**Fungal Generation of Organic Acids for Removal of Lead from
Contaminated Soils**

by

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Abstract

Four strains of fungi of the genera *Aspergillus* were evaluated for organic acid production on solid substrates (corncoobs, apple pomace, rice and hay). It was demonstrated that different organic acids were produced from the different substrates by each fungal strain.

Through a comparison of the lead extraction by the filtrates of four fungal strains generated on different substrates, it was demonstrated that the fungal filtrates generated during *A. foetidus* NRRL 337 fermentation of rice performed the best with respect to lead extraction. These filtrates had the highest concentrations of citric acid as compared to the other filtrates, therefore it was concluded that citric acid was the strongest metal chelator among the fungal organic acids produced.

The soil column studies demonstrated that the lead extraction efficiency up to 34% could be achieved. It was concluded that higher metal extraction efficiency could be attained by better control of the process variables.

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List of Abbreviations

ATCC – American Type Culture Collection

DAD – Diode Array Detection

DcyTA – 1,2-Diaminocyclohexane- *N,N,N',N'*-tetraacetic Acid

DI water – De-Ionized Water

DOC – Degree of Complexation

DTPA – Diethylenetriaminepentaacetic Acid

EDTA – Ethylenediaminetetraacetic Acid

EGTA – Ethylene Glycol-(-aminoethyl ether)-*N,N,N',N'*-tetraacetic Acid

FAAS – Flame Atomic Absorption Spectrometry

GC vial – Gas Chromatograph vial

HPLC – High Performance Liquid Chromatography

Na₂EDTA – Disodium Salt of Ethylenediaminetetraacetic Acid

Na₂S₂O₅ – Sodium Metabisulfite

NTA – Nitrilotriacetic Acid

PDA – Potato Dextrose Agar

PZSE – The Point of Zero Salt Effect

QA/QC – Quality Assurance/Quality Control

SDS – Sodium Dodecyl Sulfate

SLF – Submerged Liquid Fermentation

SS – Stabilization/Solidification

SSF – Solid State Fermentation

1. INTRODUCTION

1.1 Background

Industrial or commercial sites on which production remains were stored or buried incorrectly often have soil that is contaminated by toxic heavy metals such as lead, mercury, arsenic, chromium, cadmium and copper (Abumaizar and Smith, 1999; Yarlagadda et al., 1995). Soil contamination often results in limited exploitation of the sites, and a complete prohibition on cultivation or other potential use of the area. In comparison to organic contaminants, heavy metals can not be destroyed by biodegradation, chemical oxidation, or incineration, and they can be an environmental concern by remaining on site for a long time unless they are removed from the ecosystem (Kim et al., 2003; Hong et al., 1999).

The current technologies employed for remediation of soil contaminated with heavy metals are stabilization/solidification (SS) methods and excavation of the contaminated material with subsequent disposal at a permitted land disposal facility. Though these approaches represent a rapid method of dealing with a contaminated site, they are becoming more and more costly because of limited landfill space and processing cost. Thus, removal of heavy metals from soils using mineral processing and/or chemical extraction techniques such as soil washing and in situ soil flushing are soil decontamination options that present numerous environmental benefits (Tampouris et al., 2001). These technologies employ washing solutions that contain acids, bases, chelating agents, or other additives (Griffiths, 1995).

One of the ways of mobilizing metal in soils is through formation of soluble complexes. The addition of complexing ligands (chelating agents) converts solid-bound

heavy metal ions into soluble metal complexes (Peters and Shem, 1995). In practice, acid extraction and extraction with chelating agents are the two most common removal methods (Rampley and Ogden, 1998), however the high cost of chelating reagents such as EDTA has limited their use in remediation of metal contaminated sites. Weak organic acids also have the capacity of washing heavy metals from contaminated soils without seriously deteriorating the soil properties (Wasay et al., 1998b).

It is known that certain strains of fungi are capable of producing organic acids. In fact, the entire worldwide demand for citric acid is met by fungal fermentation. Organic acids such as oxalic, gluconic, malic, succinic, tartaric and ascorbic acids can be synthesized at the same time with citric acid by various species of the *Aspergillus* genera. These fungal generated organic acids may be effective metal chelators due to the electron-donor groups in their organic structures.

Submerged liquid fermentation (SLF) is the most popular method for commercial production of organic acids, however solid state fermentation (SSF) is an adequate method of fungal fermentation that is still employed. The potential to use naturally occurring substrates, which include waste products from agriculture, food industry and biotechnological processes, is one of the main advantages of SSF (Burgstaller and Schinner, 1993; Raimbault, 1998). In general, SSF is a cheaper process than SLF for the production of organic acids, therefore, if SSF variables (culture conditions) can be well controlled, this technology may be a more competitive process than is commonly thought.

Relatively few studies have examined fungal leaching of heavy metals, and only a few papers have been published where fungal leaching was carried out on a greater scale than

shake flasks (Burgstaller and Schinner, 1993). Nevertheless, the few investigations in this field have demonstrated that fungal generated organic acids such as citric acid were capable of leaching heavy metals in a similar range with commercially available organic acids.

1.2 Objectives and Scope of Study

The main objectives of this study were (1) to investigate fungal growth and organic acid production under solid-state fermentation conditions, and (2) to test the effectiveness of fungal generated organic acids for leaching heavy metals from a real field soil. The effect of different culture conditions on fungal production of organic acids from solid substrate was investigated during preliminary testing. The fungal strain *Aspergillus niger* NRRL 2001 and corncobs as a low-cost substrate were selected for this part of the study.

A series of bench scale experiments were conducted in order to evaluate organic acid production by different fungal species grown on various solid substrates. In this portion of the study four strains of fungus (*A. niger* NRRL 2001, *A. niger* ATCC 64065, *A. foetidus* NRRL 337 and *A. aculeatus* NRRL 358) and four solid substrates (corncobs, rice, apple pomace and hay) were evaluated for their potential for acid formation.

The effectiveness of fungal generated organic acids for leaching heavy metals from a real field soil was tested in batch metal extraction experiments, where contaminated soil samples were mixed with fungal organic acid-containing filtrates, and the soil slurries were allowed to contact for a period of time to allow metal extraction.

From the evaluation experiments, *A. foetidus* NRRL 337 and boiled white rice were selected for soil column studies as this fungal strain produced the highest concentrations

of citric acid from this substrate among all the strains tested. Citric acid was demonstrated in the batch metal extraction experiments as the strongest chelating agent in comparison with the other organic acids generated by fungi. The purpose of the soil column tests was to further evaluate the performance of the organic acid-containing flushing filtrates in soil columns that simulated in situ washing or a soil pile-leaching process. The impact of process variables on the leaching of lead in the soil columns was investigated in detail in this portion of the study.

2. LITERATURE REVIEW

2.1 Remediation of Heavy Metal-Contaminated Soils

2.1.1 Heavy Metals in Soils

The earth's crust is dominated by the elements such as Si, Al, Fe, Ca, Na, K, Mg, Ti, and P, and the remainder of the elements are present in trace quantities (Alloway, 1995). Many of these trace metals are very important for plants, animals and microorganisms; but, practically all of them possess significant toxicities when present at excessive concentrations (Hayes and Traina, 1998). Although natural constituents in all soils, the trace metal concentrations have been significantly increased by anthropogenic inputs. Industrial or commercial sites on which production remains were stored or buried incorrectly are a common reason of toxic heavy metal contamination of soil and subsequent potential fouling of ground water. Soil contamination often results in limited exploitation of the sites, and a complete prohibition on cultivation or other potential use of the area.

The most regularly found metal contaminants are lead, mercury, arsenic, chromium, cadmium and copper (Yarlagadda et al., 1995). It has been estimated that over 20 million cubic yards of soil at current National Priority List sites in the United States are contaminated with metals (Griffiths, 1995). The widespread nature of lead presents a more serious environmental and health hazard than other heavy metals (Ma et al., 1995).

Lead is known to adsorb strongly to soil surfaces by forming inner sphere complexes with mineral surface oxides that are present in soils (VanBenschoten et al., 1997). Lead also can be retained in soil by mechanisms other than sorption (e.g. solid-state diffusion and precipitation reactions) especially when lead exists as PbCO_3 , PbSO_4 , or as an

organic lead form (Cline and Reed, 1995). The transfer of lead and other heavy metals in aquatic media, their uptake by plants and their introduction into the food chain cause a potential risk to human health (Cid et al., 2002).

Anthropogenic sources of heavy metal pollution and lead pollution in particular have been classified by originating activity: agricultural, industrial and urban activities (Ma et al., 1995). Sewage sludge application to farmland introduces lead pollution to farmlands (Alloway and Jackson, 1991). Soil contamination by lead, as a result of industrial processes such as mining, refining and manufacturing, can be a pollution problem long after the site has ceased operating. Lead battery reclamation is one of the major sources of lead contamination of soil as well urban activities which include use and disposal of lead-based paints (Kim et al., 2003). Lead contamination may also result from the bullets and pellets from firearms at rifle range (Kim et al., 2003).

In comparison to organic contaminants, heavy metals can not be destroyed by biodegradation, chemical oxidation, or incineration. Metal contaminants can remain on site and be an environmental concern for a long time unless they are removed from the ecosystem (Kim et al., 2003).

2.1.2 Soil Remediation

Several management options for contaminated soils are possible (Abumaizar and Smith, 1999):

- leave the contaminants as is and limit the use of the site in question;
- immobilize the contaminants at the site and impose continuous monitoring;
- remove the polluted soil and store it in a special disposal site; and

- decontaminate the soil on- or off-site.

The current baseline technology for remediation of soil contaminated with heavy metals is excavation of the contaminated material and subsequent disposal at a permitted land disposal facility (Peters, 1999). This approach represents a rapid method of dealing with a contaminated site and is attractive due to relatively low landfill disposal costs. However, with this approach contaminants are only moved to a different location, and the long-term integrity is not well understood, hence, this approach is not ideal (Wood, 1997). Also recent increases in the cost of hazardous material disposal in landfills may result in some decline of this approach.

Stabilization/solidification (SS) remediation techniques have been widely applied to make metals in soils unavailable (Tampouris et al., 2001). These methods accomplish this by solidifying contaminated material, converting contaminants into a less mobile chemical form and/or binding them within an insoluble matrix, and presenting a minimal surface area to leaching agents (Wood, 1997). Materials such as Portland cement, fly ash, lime, and silicates have been used as the binder. The main disadvantages which limit the application of this technique are (1) the volume of the stabilized product can be significantly greater than the original contaminated material due to the quantities of stabilization materials (Wood, 1997), and (2) the technique is becoming more and more costly because of limited landfill space and processing costs (Peters, 1999). In addition, neither SS methods nor dig-and haul technology result in the removal and/or concentration of the heavy metals from soils, or can be practically implemented using in situ strategies (Peters, 1999).

When site reutilization is an important issue, decontamination can be an economically competitive alternative. Removal of heavy metals from soils using mineral processing and/or chemical extraction techniques are soil decontamination options that present numerous environmental benefits (Tampouris et al., 2001). The permanent removal of toxic elements from the soil matrix can be ensured by applying these techniques, which allow recycling of clean soil and provide improved future land-use options (Peters, 1999). As mentioned previously the soil can be decontaminated on- or off-site. Generally speaking, in situ technologies are more economical than ex situ because excavation is not required. However, there are concerns with in situ technologies that not all the mobilized contaminants will be captured by the recovery system, thereby leading to an increased public health risk (Peters, 1999). Therefore, to encourage the application of these innovative processes, it is important to improve their performance and reduce their cost. Soil decontamination technologies such as soil washing and in situ soil flushing are discussed in more detail in the following sections.

Soil washing

Soil washing is an ex situ, water-based decontamination process that utilizes physical and chemical separation and extraction for removing organic, inorganic, and radioactive contaminants from soil (Mann et al., 1993). This remediation technique is usually employed as a pretreatment process by reducing the volume of feedstock for other remediation processes. The contaminated soil is excavated, pretreated to remove oversized material, and then washed with water and other cleaning agents to separate and segregate the contaminants (Mann et al., 1993). The process recovers a clean soil

fraction and segregates the contaminants in a residual soil portion, facilitating the application of other remediation processes.

Figure 2-1 illustrates a generic soil washing process, which consists of the following modules (Kuhlman and Greenfield, 1999):

1. soil preparation module for removal and cleaning of oversize material and debris;
2. soil washing module to remove the contaminants;
3. wastewater treatment module in which the dispersed soil and contaminants are removed from the water;
4. residual management module;
5. volatile emissions control module;
6. wash water storage module of recycled water that can be heated with the addition of fresh makeup water.

The efficiency of a soil washing system is typically determined by the volume reduction achieved (Mann, 1999):

$$\text{Volume reduction (\%)} = 1 - [(feed\ soil\ (tons) - clean\ products\ (tons)) / feed\ soils\ (tons)] \times 100 \quad (2-1)$$

Soil washing systems are assembled from standard mining and material handling equipment (Mann, 1999). The overall soil washing process can be either simple or complicated, but it must be well designed to combine high productivity with low processing and capital costs (Kuhlman and Greenfield, 1999).

Figure 2-1: Flowchart for generic soil washing process (Kuhlman and Greenfield, 1999).

Three specific fractions of soil are important for the soil washing process (Mann, 1999):

- the *oversize* fraction consisting of materials larger than 5 mm;
- the *sand* fraction consisting of materials less than 5 mm and larger than 63 μm (0.063 mm);
- the *fines* (silts and clays) consisting of materials smaller than 63 μm .

Usually contaminants are concentrated in the *fines* while lower concentrations of the key contaminants are present in the *sand* and the *oversize* fractions (Mann, 1999). The finest fractions possess the highest surface area per unit volume that favors specific adsorption phenomena (Peters, 1999). The soil washing concept of reducing soil contamination through particle size separation is based on the tendency of most organic and inorganic contaminants to bind (chemically or physically) to the *fines* (clays and silts) (Mann et al., 1993). Heavy metal contaminants that concentrate in fines include chromium, lead and uranium, while strontium, barium, and cesium are almost uniformly distributed through the soil size fractions (Griffiths, 1995). The soil washing process is most effective when applied to soils containing large proportions of sand and gravel, whereas the fine particles such as silt and clay cannot be efficiently treated as they are difficult to remove from the washing fluid (Mann et al., 1993, Avogadro and Ragaini, 1994).

The technology often employs washing solutions that contain acids, bases, chelating agents, alcohols, or other additives (Griffiths, 1995). When the soil contains widely ranging contaminant concentrations, the selection of suitable reagents is necessary. Sequential washing steps may be required in order to achieve high removal efficiencies though residual solvents and surfactants can be difficult to remove from the soil (Peters,

1999). In order to determine whether soil washing can be applied to effectively remove the contaminants, bench- and pilot-scale tests should be conducted on representative contaminated soil samples (Mann et al., 1993).

Soil Flushing

Soil flushing is a soil decontamination process which can be accomplished through in situ soil washing by applying a washing solution to the unexcavated contaminated zone through sprinkling, surface flooding, subsurface leach fields, or subsurface injection in order to extract pollutants from the soil (Abumaizar and Smith, 1999, Mann et al., 1993). The fluids can be drawn from the groundwater in the immediate area. Once the contaminants have been flushed to a certain location, the contaminated fluids must be removed either from a perched condition or from a groundwater system (Mann et al., 1993). Contaminant migration into the groundwater is prevented by using proper control measures that are specific to each location. In situ soil flushing is one of the alternative treatment methods to reduce the quantity and the impact of soil pollutants (Abumaizar and Smith, 1999). The process accelerates the in situ mobilization of contaminants from soil for recovery and treatment (Mann et al., 1993).

The efficiency of soil flushing depends on the distribution of the contamination over the various phases in the soil system. The distribution (speciation) depends on the properties of the contaminants and the soil, and on the source of the contamination. In other words, to realize convective transport, the following conditions must be met (Otten et al., 1997):

- the soil must be sufficiently permeable;

- the contaminants must be made soluble;
- the contaminants must be available for transport.

Permeability of soil

Table 2-1 demonstrates the relationship between the permeability of different types of soil and the degree to which they are suitable for advective transport. As can be seen from Table 2-1, different soil fractions have a different permeability (k).

Table 2-1: The suitability of soil types for contaminant removal by convective transport (Otten et al., 1997).

Soil type	Suitability for convective transport	k-value (m/day) for water
gravel	good	> 100
very coarse sand		10 – 100
moderately coarse sand		5 – 10
fine sand	moderate	0.1 - 5
silty sand	bad	< 0.1
clay		
peat		

Soil regularly has layers of different permeability, and a very heterogeneous soil negatively affects the effectiveness of advective transport (Mann et al., 1993). In these conditions the flushing solution will mostly flow through the most permeable layers with hardly any flow in the less permeable layers (Otten et al., 1997).

Solubility of contaminants

In many cases, (ground)water can be sufficient for flushing water-soluble contaminants (Mann et al., 1993). However, in some situations, the solubility of a contaminant can be enhanced artificially by adding particular flushing agents (Otten et al., 1997). Flushing solutions may include dilute acids and bases, reducing agents, complexing and chelating agents, solvents, or surfactants. For example, surfactants increase the solubility of the contamination by forming clusters of molecules, so-called

micelles (Otten et al., 1997). Chelating agents (e.g. EDTA) form soluble complexes with heavy metals (Hong et al., 1999). Heavy metals such as cadmium, zinc and copper dissolve more easily in an acid environment as the acid addition alters soil properties due to mineral dissolution and results in an increase in contaminant mobility (Otten et al., 1997, Reed et al., 1996).

Availability of contaminants

Just like clay minerals, organic substances (e.g. humus) have a negative charge that attracts positively charged cations such as lead, copper, zinc and cadmium. High organic carbon content in the soil leads to strong adsorption and therefore low availability of the metal contaminants (Table 2-2). When the contaminants are present in micropores, or as pure product in soil, low availability is also expected (Otten et al., 1997).

Table 2-2: Relation between organic carbon content of soil, and the availability of contaminants (Otten et al., 1997).

Organic substance content	% organic substance	Availability for convective transport
low	0 – 1 %	no problems caused by adsorption
moderate	1 – 5 %	possibly limited availability
high	> 5 %	availability probably too low

Soil flushing has a positive prospect for sites where the depth to contamination, contaminant distribution, permeability, and permeability heterogeneities are compatible with the process (Mann et al., 1993). One major drawback of soil flushing is the large quantities of contaminated elutriate, which is the mixture of water, surfactants, and contaminants that is recovered in the soil flushing process, and therefore often on-site

elutriate treatment must be set up. In addition, the groundwater flow pattern must be well defined to guarantee complete recovery of the elutriate (Mann et al., 1993).

2.1.3 Chelant Extraction

Many different mechanisms influence the partitioning of metals between the soil and solution phases, thereby affecting the leachability of metals from contaminated soils. These mechanisms include dissolution and precipitation, sorption and exchange, complexation, and biological fixation (Peters and Shem, 1995). Four ways of mobilizing metal in soils have been identified (Pickering, 1986):

- changes in the acidity;
- changes in solution ionic strength;
- changes in the oxidation-reduction potential;
- formation of complexes.

In the latter technique, the addition of complexing ligands converts solid-bound heavy metal ions into soluble metal complexes (Peters and Shem, 1995). The efficiency of these ligands for metal release depends on the strength of bonding to the soil surface, the stability and adsorbability of the complexes formed, and the suspension pH. In practice, acid extraction and extraction with chelating agents are the two most common removal methods (Rampley and Ogden, 1998).

There are several major concerns in the selection of complexing agents for soil remediation (Elliott and Peters, 1993):

1. Reagents should be able to form stable complexes over a wide range of pH;
2. The formed metal complexes should be nonadsorbable on soil surfaces;

3. Biodegradability of the complexing agents and metal complexes should be low;
4. The chelating agent should have a low toxicity and potential for environmental harm;
5. The agents should be cost-effective.

Chemistry of metal extraction using chelating agents

Effective chelating agents have multiple coordination sites that are available for complexation with a metal (Peters, 1999). The chelating agents are usually multi-protic acids (H_nL) capable of undergoing acid-base equilibrium reactions in the aqueous phase:

and consequently,

where L represents a ligand anion, H is a hydrogen ion, n is the number of hydrogen atoms, and m is the number of ligand atoms.

When a strong complex with the metal may be formed by each conjugate acid/base of the chelating agent, various complexes may form as per (Peters, 1999):

$$k = [M_x(H_{n-m}L)_y^{2x-my}] / [xM^{2+}] [yH_{n-m}L^{m-}] \quad (2-5)$$

where M represents a metal cation and k is the formation constant. The value of the formation constant is a measure of the strength of the association between the metal and the ligand.

The total concentration of complexes is the sum of all of the individual complex types as per:

$$ML_{tot} = \sum M_x(H_n - mL)_y^{2x - my} \quad (2-6)$$

The total metal solubility is computed by:

$$M_{tot} = M_{aq} + ML_{tot} \quad (2-7)$$

where M_{aq} represents an aqueous metal.

On the basis of these equilibrium calculations, the complexation power of chelating agents toward heavy metals can be evaluated (Peters, 1999). The strong chelators demonstrate a total solubility M_{tot} , which is much higher than the M_{aq} (without chelators). In addition, for target heavy metal extraction, the chelating agents should match well with the criteria such as (Peters, 1999):

- ligands should possess higher metal complexing abilities toward heavy and transition metals than toward hard sphere cations (e.g. Ca, Mg);
- ligands with sulfur and nitrogen as donor atoms are generally preferred as they generally form more stable complexes with metals of interest (e.g. Zn, Cd, Pb, Cu, Ni), whereas ligands containing oxygen as the donor atom prefer hard sphere cations.

Previous studies involving extraction of heavy metals from contaminated soils using chelating agents

For the past 20 years, numbers of studies have been conducted in order to evaluate the extraction of heavy metals from contaminated soils using various chelating agents. The environmental research on heavy metal chelation has focused on the following areas (Hong and Pintauro, 1996a as referenced by Peters, 1999):

- the effects of chelators on the release of heavy metals from soil, sediment, and solid waste into the adjacent water phase;
- chelators as scavenging agents for removal of heavy metals from sludge at wastewater treatment plants;

The most studied chelating agent is ethylenediaminetetraacetic acid (EDTA). EDTA and several other aminocarboxylic acids form stable complexes with numerous metal ions (Peters and Shem, 1995). Table 2-3 summarizes the properties of three chelating agents that have been widely used as soil extractants (either for analytical or remediation purposes).

Table 2-3: Properties of chelating agents (Peters and Shem, 1995).

Chelating agent	Molecular weight (g/mol)	Acidity constants					Metal chelate					
		pK ₁	pK ₂	pK ₃	pK ₄	pK ₅	log (formation constant)					
							Cd	Zn	Cu	Pb	Mn ²⁺	Fe ³⁺
nitrilotri-acetic acid (NTA)	191	1.89	2.49	9.73	--	--	10.5	11.2	13.7	11.8	8.1	17.0
ethylene-diaminetetra-acetic acid (EDTA)	292	2.08	3.01	6.4	10.44	--	17.5	17.2	19.7	17.7	14.5	26.5
diethylene-triamine-pentaacetic acid (DTPA)	93	2.08	2.81	4.49	8.73	10.6	20.1	19.7	22.6	21.0	16.7	29.2

As has been mentioned previously, the chelating agents are multi-protic acids capable of undergoing acid-base equilibrium reactions in the aqueous phase. K_a is the acidity or dissociation constant that indicates the extent of dissociation of hydrogen ions from an acid. pK_a does not depend on the pH of the solution. The formation constant of a chelating agent is a measure of the strength of the association between the metal and the

ligand as mentioned earlier. Many reported formation constants for soluble metal complexes are available in the literature (Evans, 1989).

It has been pointed out previously that different factors affect the extraction of metals from contaminated soils. The factors such as soil type, pH of the extraction media, and selectivity of different chelating agents towards metals have been the main focus of the chelant extraction studies.

pH of the extraction media

The desorption/complexation/dissolution behavior of cadmium from kaolin using four chelators such as NTA, EDTA, ethylene glycol-(-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 1,2-diaminocyclohexane- *N,N,N',N'*-tetraacetic acid (DcyTA) and the pH-dependence of cadmium adsorption/desorption were investigated (Hong and Pintauro, 1996a as referenced by Peters, 1999). The ability of the four chelators to dissolve cadmium over the pH range of 2.5 to 12.0 varied considerably. For NTA, incomplete Cd desorption/dissolution was observed when the pH ranged from 4.0-7.5 and 9.0-12.0. Approximately 45% of the kaolin-bound cadmium was detected in the solution at pH's of 6 and 12. With EDTA, 85% of the cadmium was dissolved at pH values in the range of 5 to 6, and all of the cadmium dissolved when the pH of the kaolin suspension was greater than 8. Complete dissolution was observed over the entire pH range for DcyTA, and for EGTA, complete cadmium dissolution was observed at all pH values with the exception of pH around 4, where approximately 2% of the cadmium remained undissolved. The authors concluded that the capacity of the four chelators for removing Cd was in the following order: DcyTA > EGTA > EDTA > NTA.

Pichtel and Pichtel (1997) as referenced by Peters (1999) investigated the ability of EDTA and NTA to solubilize Cr and Pb from a real field soil over a wide pH range (2 to 11). The extraction of Cr and Pb by these two chelating agents was compared with the extraction by anionic surfactant (sodium dodecyl sulfate (SDS)) and hydrochloric acid (HCl). The extent of Cr and Pb solubilization was strongly influenced by both solution pH and the chelant-metal chemistry. A 0.1 M EDTA solution removed 100% of Pb at a pH of 4.3, while 54% of Cr and 96% of Pb were recovered at a pH of 12 with this chelant solution. The NTA was less effective with 33-48% removal of Cr (pH 8.9-11.0), and a maximum of 38% lead removal was achieved at pH 4.5. SDS was not effective at removing Pb and Cr, even at molar ratios of surfactant: metal greater than 1:1. Soil washing by HCl (concentrations ranging from 2% to 8%) removed 100% of the Cr and Pb. However 49-51% of the matrix solids were also dissolved. A 1% acid solution was much less effective in metal removal (15.7% and 3.8% removal of Cr and Pb, respectively).

As can be seen from these studies, the chelating agents performed differently with respect to metal extraction at different pH values of the soil-chelant solutions. This demonstrates the important effect of this parameter on metal extraction by chelating agents. Also it can be noted that though a strong acid such as HCl was competitive with EDTA for the metal extraction, HCl was more toxic on the soil matrix than EDTA.

Metal selectivity

The competitive desorption/dissolution of kaolin-bound heavy metal mixtures by NTA, EDTA, EGTA and DcyTA was studied by Hong and Pintauro (1996b). When 50 μM of kaolin-adsorbed Cd^{2+} , Pb^{2+} , and Cu^{2+} were treated with the chelators (concentration of

150 μM), none of the chelating agents removed all of the adsorbed metals. These concentrations were chosen by the authors in order to ligand-bind all the adsorbed metals assuming one metal ion combined with one chelant molecule. The selectivity (order) of metal removal differed for each chelant type. With NTA, Cu was dissolved to a greater extent than Cd and Pb. EDTA and DcyTA demonstrated the same order for metal removal as NTA with higher Cd extraction followed by Pb and then Cu when the chelant concentration was less than 150 μM . For EGTA, the chelant exhibited a stronger chelating affinity for Cd and Cu as compared to Pb. Thus this study demonstrated that every chelating agent possesses different chelating affinity for different metals. Table 2-4 presents the selectivity of metal removal by the four chelants when the chelant concentration was insufficient to combine all the metals adsorbed on the kaolin.

Table 2-4: Selectivity of metal removal by different chelating agents (Hong and Pintauro, 1996b).

chelating agent	metal selectivity
EGTA	Cd > Cu > Pb
EDTA	Cd > Pb > Cu
DcyTA	Cd > Pb > Cu
NTA	Cu > Cd > Pb

Soil type

Several lead-contaminated soils from Superfund sites, one rifle range soil, and one artificially lead-contaminated soil were used and variables such as soil : solution ratio, and EDTA : Pb stoichiometric molar ratios were studied by Kim et al. (2003). Table 2-5 presents the soil properties and major cation concentrations for the soil samples. One of the main objectives of this study was to investigate the effects of the major cations present in soils (e.g. Fe, Al, Ca, Zn, Cu, Mn, and Mg) on the extraction efficiency of Pb by EDTA.

In batch experiments, it was demonstrated that a washing solution : soil ratio as low as 3 : 1 had similar lead extraction results with that of a washing solution : soil ratio of 10 : 1. The authors concluded that the extraction efficiencies were dependant on the molar concentration of EDTA present in the washing solutions. Experimental results demonstrated that if a sufficiently large amount of EDTA was applied (EDTA : Pb ratio greater than 10), most of the lead was extracted from all tested soils. For a stoichiometric ratio of unity approximately 75% of Pb was extracted from the artificially contaminated soil, 55% of Pb was extracted from Soil C (battery recycling), 40% from Soil D (rifle range), 10% from Soil A (lead smelter), and none of the Pb was extracted from Soil B (lead mine). All the soils except for Soil B (pH=2.7) had a soil pH higher than 8. In addition, Soil B had a high amorphous iron content (31700 mg/kg) and calcium content (103900 mg/kg) as compared to the other soils.

It was concluded that the differences in extraction efficiencies may be a result of the major cations present in soils which may compete with lead for active sites on EDTA. For example, for a pH less than 6, dissolved ferric ions appeared to compete with lead for EDTA ligand sites. In addition, copper and zinc may potentially compete with lead when pH value less than 6. Also the type of lead species (e.g. for some of the soil tested, lead may have been occluded in the iron oxides) present in the soil may be one of the factors affecting lead extraction efficiency.

Table 2-5: Soil properties and major cations concentrations (mg/kg) for the soil samples (Kim et al., 2003).

Sample	Pb	Fe	Mn	Al	Ca	Mg	Cu	Zn	Specific surface area (m ² /g)	CEC (meq/100g)	%Organic carbon	soil pH
Artificial	2413	---	---	---	---	---	---	---	9.7	11.6	0.75	8.16
Soil A	4180	544	384	25940	19580	6780	129	1860	37.3	17.8	2.26	8.55
Soil B	1247	31720	91	707	103900	115	557	1078	4.4	4.3	0.10	2.68
Soil C	13260	316	2820	14330	12410	5270	30	86	15.4	17.5	2.52	8.13
Soil D	6238	328	21	2440	7450	436	279	70	0.7	5.8	0.18	8.47

Different extraction mechanisms

Some of the chelant extraction studies have focused on enhancing the metal extraction by combining two different extraction mechanisms. For example, the feasibility of heavy metal extraction by chelating and reducing agents was evaluated in laboratory-scale batch and column experiments by Abumaizar and Smith (1999). Samples of silty sand spiked with Cd, Cr, Pb, and Zn were subjected to chelant extraction using a solution containing either the disodium salt of EDTA (Na_2EDTA), sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) (low-priced reducing reagent), or a solution containing a mixture of the two reagents. Preliminary soil washing with deionized water (DI water) revealed that approximately 70% of the Cd was weakly bound and readily mobilized in aqueous solution at neutral pH, while 25%-30% of Zn, 20%-25% of Cr, and around 10% of Pb were water extractable. The extraction of the metals with a 0.1 M $\text{Na}_2\text{S}_2\text{O}_5$ solution resulted in very slight improvement in Cr removal over DI water flushing, and moderate enhancement in Pb and Cd extraction (10%-15%), while Zn removal was doubled. The best results for $\text{Na}_2\text{S}_2\text{O}_5$ were achieved for a soil to solution ratio of 1:25. In most cases, the extraction with 0.1 M Na_2EDTA indicated higher removal efficiencies than those of $\text{Na}_2\text{S}_2\text{O}_5$. Complete removal of Cd and Pb, 40.7% removal of Cr, and 81.8% removal of Zn were observed for a 1:12.5 soil to solution ratio. The effect of combining the two mechanisms of reduction and chelation was demonstrated with a set of batch experiments. The removal of heavy metals with 0.01 M Na_2EDTA was enhanced considerably by inclusion of 0.1 M $\text{Na}_2\text{S}_2\text{O}_5$. These results suggest that a mixture of the two reagents may provide an economically optimum solution for certain polluted soils.

Reusability of chelating agents

A degree of complexation (DOC) parameter can be defined as the ratio of the total metal solubility with chelation to the aqueous solubility without chelation (i.e. $M_{\text{tot}}/M_{\text{aq}}$). The DOC ratio is a function of pH (Hong et al., 1995 as referenced by Peters, 1999). For strong chelating agents, DOCs have been found to remain high over a broad pH range (e.g. 1 to 14), whereas for moderate strength chelating agents, the DOCs were typically found to be high at low or moderate pH value, but low at high pH (Hong et al., 1995 as referenced by Peters, 1999). This means that a chelator of moderate strength can enhance metal solubilization by complexation at lower pH values, but release the complexed metal as precipitates at a higher pH value. This feature can be used to recover extracted heavy metals and the chelator for further reuse.

The extraction, recovery, and biostability of EDTA as a potential remediation agent were investigated by Hong et al. (1999). Metal extraction and recovery from a sandy loam soil containing 15500 mg Pb/kg soil and a loamy sand with 2370 mg Pb/kg soil were examined. The effects of EDTA concentrations from 3 to 50 mM, soil content from 5% to 40%, washing cycles (1 through 6), precipitant concentration and pH (6.5-11) were examined. The experimental studies demonstrated that after one cycle of washing up to 91, 60, and 56% of Pb, Zn, and Cu, respectively, were extracted from the contaminated soil. The chelator was capable of being reused several times with some loss of extraction activity. EDTA was relatively biostable and was partially degraded by acclimated culture. At the actual site, the soil microorganisms were not acclimated to EDTA and have little potential to biodegrade it (Hong et al., 1999). Therefore, the authors

concluded that EDTA is a strong, reusable, and relatively biostable chelating agent, which is very valuable for remediation of heavy metal contaminated soils.

The reuse of chelating agents such as aminocarboxylic acids is a very important issue because of their strong chelation nature. The high cost of chelating reagents such as EDTA has precluded their use in remediation of metal contaminated sites. Methods for reuse must be developed in order to make chelant extraction economically viable. In addition, Abumaizar and Khan (1996) reported, as referenced by Abumaizar and Smith (1999), that the permeability of EDTA was considerably reduced when contacted with certain types of soil, indicating that in-situ washing with this chelating agent may require extended clean-up time.

2.1.4 Organic Acids as Metal Chelators

It has been known for a long time that weak organic acids have the capacity of washing heavy metals from contaminated soils without seriously deteriorating the soil properties (Wasay et al., 1998b). In addition, they do not linger in the soil after treatment like other chemical agents do. Though strong chelating agents and acids such as EDTA, HCl and H₂SO₄ effectively increase the solubility of heavy metals, they may destroy the basic soil structure leaving it unsuitable for revegetation (Peters, 1999). Furthermore, soil treatment with these acids is costly, and there are problems with disposal of the end product. Organic acids are much less toxic, and therefore much easier to dispose of. Veeken and Hamelers (1999) stated that organic acids could be an attractive extracting agent as the extraction of metals by organic acids can be performed at mildly acidic conditions (pH 3-5). Low molecular weight organic acids are released by

microorganisms in soil and in the phytosphere of plants in natural ecosystems (Zhou et al., 2003), so these acids are biologically degradable.

As has been discussed in the previous section, the successful chelant extraction of heavy metals from soil depends on various factors. From the literature review on metal leaching by organic acids, it has been observed that the main focus of these studies is to compare the chelating strength of different low molecular weight organic acids (e.g. citric, tartaric, formic and oxalic acids) towards various metal cations. The effect of soil-organic acid solution pH and soil type on metal extraction by organic acids have also been investigated in these studies.

Wasay et al. (1998a) studied several weak organic acids and their salts for leaching heavy metals from a naturally contaminated clay loam. Leaching was tested at various pH values, concentrations of organic acids, and reaction times. It was found that for citrate extraction of Cr, Hg, Pb, and Mn, the optimum pH ranged from 5 to 7. For tartarate extraction a pH range from 2 to 3 was optimal for Cr, Hg, and Mn and pH's between 2 to 7 were optimal for Pb. More than 85% of Pb was removed after 24 hours with citrate and tartarate at concentrations of 0.12 M and 0.2 M, respectively. At these acid concentrations, 86 to 99% of Hg was removed by tartarate and citrate, and up to 60% of Cr was removed.

The same authors (Wasay et al., 1998b) also investigated remediation of a loam and a sandy clay loam that were naturally polluted by heavy metals for over three years. The chelating characteristics of various weak organic acids and/or their salts were compared against two strong synthetic chelating agents (EDTA and DTPA). Among the 7 weak organic acids and/or their salts, citrate, tartarate and oxalate were found to be the most

effective in removing Cd, Cu, Pb, and Zn from the two soils in double extractions, over a wide range of pH values. Citrate removed 80 to 99.9% of all four metals within 24 hours at pH values from 2.3 to 7.5, and tartarate removed 84 to 99.9% at pH values from 2.1 to 6.7. Oxalate alone was not efficient in leaching metals particularly Pb, but in combination with ammonium citrate (1:1 ratio) removed 82, 70, 99.9, and 99.9% of Cd, Pb, Cu, and Zn, respectively at pH values from 2.6 to 5.8. Wasay et al. (1998b) concluded that in comparison with EDTA and DTPA, citrate and tartarate were as effective in leaching the metals as the chelating agents, plus they removed 80% less macronutrients from the soils.

Wasay et al. (2001) continued to investigate the same salts of weak organic acids (i.e. citrate and tartarate) for leaching heavy metals from artificially polluted soils in laboratory columns. Three soil types (clay loam, loam, and sandy clay loam) were contaminated with different heavy metals in a laboratory. Three soil columns were packed with each soil type, and connected to a 1.0 L reservoir holding a solution of the salts of organic acids (NH_4 base), a mixture of two salts of organic acids, or one of the two chelating agents (EDTA and DTPA) at optimum pH. The optimum pH value for the citrate extraction was 5.0 in this study, while tartarate extraction was conducted at pH values ranging from 3.6 to 4.5. A 0.16 M solution of ammonium citrate and 0.3 M ammonium tartarate were applied for the soil columns with clay loam and loam, and 0.08 M ammonium citrate and 0.3 M ammonium tartarate were used in the soil column with sandy clay loam. A large-scale experiment was conducted in a 7.7 kg tub using the sandy clay loam contaminated with Cd, Cu, Pb, and Zn, to simulate in situ conditions. The washing solution of 0.16 M ammonium citrate was applied at a pH of 5.0 to the soil

surface and allowed to soak to the soil for 12 hours. The procedure was repeated 6 times.

Table 2-6 presents the metal extraction results.

Table 2-6: Metal extraction (%) by citrate and tartarate from three soil types (Wasay et al., 2001).

Soil type	citrate				tartarate			
after 20 pore volumes	Cr	Mn	Hg	Pb	Cr	Mn	Hg	Pb
Clay loam	43%	37%	92%	75%	45%	41%	91%	75%
after 20 pore volumes	Cd		Pb		Cd		Pb	
Loam	98%		89%		91%		89%	
after 15 pore volumes	Cd	Cu	Pb	Zn	Cd	Cu	Pb	Zn
Sandy clay loam	91%	84%	70%	81%	83%	73%	89%	79%

As can be seen from Table 2-6, the ammonium citrate and tartarate demonstrated similar metal extractions when they were applied to the clay loam and loam soils. For the clay loam, the highest extraction was observed for Hg (92% and 91% for citrate and tartarate, respectively) followed by Pb (75%). For the loam, both salts achieved high extraction of Cd and Pb (Table 2-6). For the sandy clay loam, the highest extraction (91%) was achieved for Cd by the citrate, and with tartarate the highest extraction was 89% of Pb.

When compared to EDTA and DTPA, Wasay et al. (2001) observed that for the clay loam, these two chelating agents were only effective in removing Pb, but were much less effective (30%) in comparison with the citrate and tartarate in removing Cr, Mn, and Hg after 20 pore volumes. For the loam and sandy clay loam, EDTA and DTPA removed 93 to 97% of the metals after 20 pore volumes. During the large scale experiment, 81, 82, 73 and 90% of Cd, Cu, Pb, and Zn were extracted respectively.

Wasay et al. (2001) again stressed the point that though EDTA and DTPA effectively removed all heavy metals from the loam and sandy clay loam soils, they also extracted large quantities of soil nutrients and polluted the soils by being nonbiodegradable and

adsorbed on the soil particles. Whereas, with salts of citrate and tartarate, soil-clean-up criteria were reached after 10 to 20 pore volumes and within 10 to 15 hr of flushing, while only small amounts of macro-nutrients were leached out.

The effect of citric acid on the adsorption of lead and cadmium by red soil was investigated by Chen et al. (2003). Fifteen milliliters of 0.003 M KCl solution containing 0, 1 or 3 mmol/l of citric acid and 500 $\mu\text{mol/l}$ of either PbCl_2 or CdCl_2 was added to 3 g of red soil, and then the suspensions were shaken for 17 hours, centrifuged and filtered. Pb and Cd concentrations in the supernatant were analyzed by atomic absorption spectrophotometry. These experiments demonstrated that the addition of citric acid decreased the amount of lead and cadmium absorbed by the soil. Chen et al. (2003) observed that with increasing citric acid concentrations the amount of lead and cadmium adsorbed by the soil decreased in the following matter: Pb adsorption was decreased by 1.9%, 4.4% and Cd adsorption by 25.3%, 43.9%, when 1 and 3 mmol/l of citric acid were applied, respectively. It was clear from these results that the effect of citric acid on the adsorption of cadmium was greater than that of lead. The effect of the solution pH on the adsorption of Pb and Cd by the soil was also investigated in this study, and the following conclusions were presented:

1. The decrease in the adsorption of Pb and Cd was mainly due to the decrease of pH in the presence of citric acid.
2. When the citric acid decreased the pH to below the point of zero salt effect (PZSE), the negatively charged Me-L complex was apparently adsorbed onto the soil surface.

Based on the reviewed studies of metal extraction by organic acids, the following observations can be made:

1. Metal extraction by low molecular weight organic acids is comparable with the extraction by common chelating agents such as EDTA. In addition organic acids are biodegradable and do not affect soil properties as much as EDTA.
2. Citric and tartaric acids were the most frequently applied acids as both acids have demonstrated high chelating capacities for heavy metals such as Cd, Cu, Pb and Zn. Oxalic acid was not efficient in leaching metals, but in combination with citric acid, significant metal extraction was demonstrated.
3. Different organic acids had different optimal pH values for metal extraction, which also varied based on the metal type. It also has been observed that in the case of metal extraction by organic acids at low pH values, the metal leaching is mostly attributed to protonation reactions rather than complexation reactions.
4. The soil type and metal speciation have been demonstrated as important parameters that affect the metal extraction by organic acids. For example, when Wasay et al. (2001) studied the extraction of Cd and Pb from the loam soil by the salts of citric and tartaric acids, at 7 pore volumes and 3.5 hr, 83 and 62% of Cd and Pb were removed, respectively. The authors stated that this fraction of heavy metals was mainly released from the easily exchangeable form of metals. After 7 pore volumes, the metals were released more slowly as they were more strongly bound to oxides and organic matter. In addition, Wasay et al. (2001) pointed out that Pb was mainly bound by oxides and organic matter, while Cd was primarily present in an exchangeable form.

2.2 Fungal Fermentation

2.2.1 Organic Acid Production by Fungi

It has been known for decades that certain strains of fungi are capable of producing organic acids. The entire worldwide demand for citric acid is met by fungal fermentation. *Aspergillus niger* remains the organism of choice for commercial production of citric acid (Ikram-ul-Haq et al., 2002). The various species of the *Aspergillus* genera, which have been found to accumulate citric acid in their culture media, include strains of *Aspergillus wentii*, *A. carbonarius*, *A. aculeatus*, and *A. awamori* (Grewal and Kalra, 1995).

Organic acids such as oxalic, gluconic, malic, succinic, tartaric and ascorbic acids have been synthesized at the same time with citric acid by fungal fermentation. The fungus *A. niger* is known as the best oxalic acid production species (Strasser et al., 1994). In the production of citric acid with *A. niger*, oxalic acid appeared as an undesired by-product. Industrial processes have been developed for the production of gluconic acid from glucose syrups using *Aspergillus niger* (Moresi and Parente, 1999). Bercovitz et al. (1990) studied organic acid production by thirteen strains of nine *Aspergillus* species in a liquid medium. Their study demonstrated that strains of *A. aculeatus* and *A. foetidus* are capable of producing malic and citric acids while the main acid produced by *A. wentii* was gluconic acid. The production of citric acid by *A. foetidus* was also shown by Kristiansen and Sinclair (1979) and Chen (1994). Growth of *A. niger* 280 in a molasses medium that was enriched with 0.6% ammonium nitrate resulted primarily in the production of oxalic acid followed by citric acid, malic acid, and succinic acid (Industrial Biotechnology Division, 2003). *A. niger* cultivated in a Czapek Dox medium generated

tartaric and citric acids (Golab and Orlowska, 1988 as referenced by Burgstaller and Schinner, 1993). Thus, it has been demonstrated that the production of various organic acids by species of the *Aspergillus* genera has been widely investigated for a long period of time. These fungal generated organic acids may be effective metal chelators due to the electron-donor group organic structures presented in Figure 2-2.

Figure 2-2: Organic structures of fungal generated acids.

Fungal fermentation of citric acid

Among the various organic acids produced by fungi, citric acid has been by far the most important in economic terms (Zidwick, 1992), with an estimated world annual production of 400,000 tons (Roehr et al., 1992 as referenced by Gadd, 1999), and therefore, it is one of the most important fermentation products currently manufactured (Harvey and McNeil, 1994). The primary use of citric acid is in the food industry, while citric acid has been demonstrated by many investigators to complex heavy metals due to

the electron-donor groups in its structure. It was the work of Currie (1917) that formed the basis of commercial citric acid production employing *A. niger* in a surface fermentation process (Zidwick, 1992). An improved process employing submerged fermentation was developed in the USA in the 1950's (Grewal and Kalra, 1995). The three main citric acid fermentation processes currently employed these days are: (1) liquid surface process, (2) Koji process, and (3) submerged liquid fermentation (Harvey and McNeil, 1994).

Liquid Surface Process

The liquid surface process was the original large scale technology for microbial citric acid production (Grewal and Kalra, 1995). Even though more sophisticated fermentation methods such as submerged fermentation have been developed, the surface process is still used due to its simple operation and installation. The mycelium is grown as a surface mat in shallow pans, made of either stainless steel or very high quality aluminum, in a disinfected room (Harvey and McNeil, 1994). The carbohydrate sources that are employed as substrates include refined or crude sucrose, high test cane syrup or beet molasses which is currently the preferred substrate (Grewal and Kalra, 1995). Molasses is diluted to a sugar content of 15%, brought to pH 5-7 and additional nutrients are added before medium sterilization. The appropriate strain of *Aspergillus* is selected in order to minimize the production of by-products such as oxalate (Harvey and McNeil, 1994). The incubation period is 6-12 days at 30 °C and the relative humidity is maintained between 40-60% (Harvey and McNeil, 1994; Grewal and Kalra, 1995). Kubicek and Roehr (1986) stated, as referenced by Grewal and Kalra (1995), that during fermentation considerable heat is generated necessitating high aeration rates. At the end of the

process, a dense mycelial mat can be easily separated from the fermented liquor that contains citric acid, which is then sent for further processing (Harvey and McNeil, 1994). Fermentation yields are in the range of 70-75%.

Zidwick (1992) pointed out that surface fermentation requires acres of surface area and manual labor for mixing and aeration of the fungus and substrate. In addition, control of contaminating metal ions is almost impossible. The main advantage of this process in comparison with submerged fermentation is the low energy costs.

Koji Process

The Koji process that was developed in Japan accounts for approximately one fifth of the annual citric acid production in this country (Harvey and McNeil, 1994; Grewal and Kalra, 1995). This process is the solid state equivalent of surface fermentation. Wheat bran, rice or sweet potato waste is moistened with water, and then steamed for sterilization (Harvey and McNeil, 1994; Grewal and Kalra, 1995). During the sterilization process, the substrate acquires sufficient moisture to give a final moisture content of 70-80% (Harvey and McNeil, 1994). The sterilized “mash” with a pH of approximately 5.5 is then inoculated with spores of *A. niger* and fermented for a period of 5-8 days at a temperature of around 28 °C (Harvey and McNeil, 1994, Grewal and Kalra, 1995). At the end of the fermentation, citrate is extracted from the collected mash using water. Grewal and Kalra (1995) noted that the main problem of the Koji process is the presence of trace elements which can not be removed by standard methods.

Submerged Liquid Fermentation

Submerged liquid fermentation is the most popular method for commercial production of citric acid due to its reduced space and labour requirements, and high production rate. Either stirred tank reactors or tower fermentors that are made of acid resistant materials are employed in this process (Grewal and Kalra, 1995). The medium preparation involves dilution of a suitable substrate, pretreatment, addition of nutrients and sterilization. Liquid fermentation substrates are usually beet or cane molasses, glucose syrup, or starch hydrolysates (Harvey and McNeil, 1994). The main pretreatment of the medium is to reduce the heavy-metal concentrations in the molasses. The inoculum is introduced by adding either a suspension of spores or precultivated mycelia (Grewal and Kalra, 1995). Two phases, including a rapid growth phase and a production phase, are monitored during the fungal growth. During the rapid growth phase little or no citric acid is excreted, while the production phase is characterized by intensive uptake of substrate for citric acid formation (Harvey and McNeil, 1994). The fermentation time is 2-5 days at temperatures between 25 and 30°C. Temperature and pH are very easily and accurately controlled in the process. With initial pH values of molasses between 5 and 7, the pH of the medium will decrease to around pH 2 during the fermentation.

Grewal and Kalra (1995) noted that submerged liquid fermentation can be performed by batch, fed batch and continuous modes, but generally the batch mode is employed. Continuous cultivation has not been applied commercially.

Due to a continued increase in demand for citrate and its derivatives, manufacturers are constantly trying to improve the fermentation process (Harvey and McNeil, 1994). Fungal strain improvement for increased productivity, decreased fermentation time, and

enhanced tolerance of the fungus to heavy metals are recent developments in this research area. Harvey and McNeil (1994) also pointed out that an improved understanding of the genetics of this group of fungi will promote production processes in time.

Fungal fermentation of solid substrates has been employed in the present research. The review of currently employed fungal fermentation processes has provided the essential understanding of fungal fermentation techniques. The Koji process has been taken as a model for solid state fermentation implemented in this research. Even though the submerged liquid fermentation is the most popular method for commercial production of organic acids, the solid state fermentation is an adequate method of fungal fermentation that has been employed in the past.

2.2.2 Culture Conditions

Many papers have reported attempts to control and enhance fungal citric acid production (Zidwick, 1992). Considerable variations in culture conditions have been reported for citric acid production by *Aspergillus* species. A medium that contains major nutrients like carbon, nitrogen and phosphorous is required for high citric acid production (Grewal and Kalra, 1995). Also, the fermentation process has been found to be influenced by the trace metal content of the medium, pH, temperature, aeration and other factors that are discussed in the following paragraphs.

Carbon Source

The type of sugar and its concentrations have been demonstrated to influence the yield of organic acids. The influence of various carbon sources on the production of citrate by *A. niger* was studied by Xu et al. (1989). *A. niger* was found to assimilate almost all carbon sources of this study, however, despite good assimilation, only growth on maltose, sucrose, glucose, mannose, fructose and sorbitol resulted in high production of citric acid. Xu et al. (1989) noted that maltose and sucrose, two disaccharides, were better carbon sources than the corresponding monomers of glucose and fructose. Hossain et al. (1984), as referenced by Grewal and Kalra (1995), reported that sucrose was the most favorable sugar followed by glucose and fructose. When El-Holi and Al-Delaimy (2003) investigated citric acid production by *A. niger* in a liquid surface fermentation of cheese whey that was fortified with either sucrose, glucose, fructose and galactose, the best citric acid production was achieved on the whey-sucrose medium. The whey-fructose, -galactose and -glucose media resulted in decreasing yields of citric acid. Maddox et al. (1985) investigated production of citric acid from the sugars present in wood hemicelluloses by *A. niger*. The study demonstrated that *A. niger* readily assimilated glucose, xylose, mannose and arabinose, however the highest yield of citric acid was from glucose. When two sugars were employed together, glucose was utilized by *A. niger* at a faster rate than mannose, xylose and arabinose. There was however no increase in citric acid production (Maddox et al., 1985). Hence, it can be concluded that *A. niger* is able to assimilate a large variety of carbon sources during its growth phase, but only few carbon sources (i.e. sucrose, glucose, maltose and fructose) result in high production of citric acid.

Shu and Johnson (1948) reported, as referenced by Grewal and Kalra (1995), that the maximum rate of production of citric acid was achieved at sugar concentrations of 14-22%. When using raw molasses as substrate, it is usually diluted to sugar concentrations of 12-15% for optimal yield of citric acid (Ikram-ul-Haq et al., 2002, Sikander et al., 2002, Garg and Sharma, 1991). Optimal yields of citric acid were observed at sugar concentrations of 10% (w/v) for maltose, sucrose, mannose and fructose, with the exception of glucose (7.5%) (Xu et al., 1989). It was reported that no citric acid was produced on media containing less than 2.5% sugar (Xu et al., 1989). In the El-Holi and Al-Delaimy (2003) study, optimal yields of citric acid were observed when sucrose, glucose, fructose and galactose sugar solutions of 15% (w/v) were added to whey.

Two-stage fermentation may be applied in order to utilize poor carbon source wastes. The mycelium of *A. niger* obtained from surface cultures in cotton waste medium yielded more citric acid when transferred to 14% sucrose-containing medium than when this medium was directly inoculated by the fungus (Kiel et al., 1981). The two-stage fermentation approach will be considered in a portion of this research.

Nitrogen and Phosphorous Source

The nitrogen requirements for citric acid production by fungus are typically met by the addition of ammonium sulfate and ammonium nitrate (Zidwick, 1992; Sikander et al., 2002; Dawson et al., 1989; and Nowakowska-Waszczyk and Maslinski, 1988 as referenced by Chen, 1994). The concentrations of ammonium salts have generally been in the range of 0.2-0.25% (w/v) (Gupta and Sharma, 1995, Pera and Callieri, 1997, Gupta and Sharma, 2002). Grewal and Kalra (1995) noted that the type and concentration of

nitrogen source influences the fungal growth and organic acid production. For example, ammonium sulfate was reported to prolong vegetative growth, while addition of ammonium nitrate resulted in a shorter period of vegetative growth (Grewal and Kalra, 1995). The addition of ammonium carbonate affected the fungal hyphal morphology during submerged fermentation; *A. niger* grew as a compact aggregate with short bulbous mycelium (Zidwick, 1992).

The presence of phosphate in the medium also has been demonstrated to affect the yield of citric acid (Grewal and Kalra, 1995). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate (concentration of 0.1%) have been reported to be the most suitable phosphorous sources (Gupta and Sharma, 1995; Pera and Callieri, 1997; Gupta and Sharma, 2002; Schrickx et al., 1995).

Conflicting results have been presented for the influence of nitrogen and phosphate limitation on citric acid production by *Aspergillus*. As referenced by Chen (1994), Kristiansen and Sinclair (1978) found that the exhaustion of nitrogen was an essential condition for citric acid production in batch fermentation. When the same authors (Kristiansen and Sinclair, 1979) investigated the production of citric acid by *Aspergillus foetidus* in single-stage continuous culture with ammonium nitrate as a nitrogen source, it was found that under nitrogen-limiting conditions the fungus produced citric acid rapidly. However, it has been reported that the amount of citric acid produced by a mutant strain of *A. niger* was 3-fold higher when 0.6 % ammonium nitrate was used in the molasses medium as compared to 0.1% ammonium nitrate (Industrial Biotechnology Division, 2003). In addition, the fermentation time was reduced to 5 days. However, it has been noted that with the higher nitrogen concentration, oxalic acid was synthesized in addition

to citric, malic and succinic acids (Industrial Biotechnology Division, 2003). This corresponds with the results of Gupta et al. (1976) as referenced by Grewal and Kalra (1995) that found that ammonium-nitrogen concentrations higher than 0.25% lead to the accumulation of oxalic acid.

Dawson et al. (1989) investigated the effect of phosphate-limited growth of *A. niger* on citric acid production in comparison with nitrogen-limited growth. Fed-batch fermentation performed under dual phosphate/nitrogen limitation demonstrated very similar citric acid production results to those from a culture limited by nitrogen alone. Dawson et al. (1989) concluded that there was no need to maintain an excess of phosphate, and the fermentation process was more cost-effective when operated under dual limitation conditions. Chen (1994) demonstrated in their study that accumulation of citric acid by *A. foetidus* grown in sucrose medium occurred when growth was limited by the supply of either nitrogen or phosphorus.

It can be seen from these studies that ammonium sulfate and nitrate have been the common additives to the fermentation medium during fungal citric acid production. As these nitrogen sources have been reported to enhance citric acid production, the addition of ammonium sulfate to the flushing solution has been applied in the present research.

Fermentation pH

During citric acid fermentation it was found that the maintenance of low pH is necessary for maximum acid production, whereas the initial pH that was required for acid production depended on the carbon source used (Grewal and Kalra, 1995). Grewal and Kalra (1995) stated that maintenance of the pH below 2.0 was generally required for

optimum production of citric acid. Ikram-ul-Haq et al. (2002) studied the effect of different initial pH values (5.0-7.0) on citric acid fermentation of cane-molasses by submerged fermentation. The maximum yield of citric acid was achieved with an initial pH of the medium of 6.0.

Strasser et al. (1994) compared the cultivation of *A. niger* on sucrose medium with three different carbon waste substrates (green syrup, lactose permeate and molasses). The initial pH of all media was adjusted to 6.0 in shake-flask experiments, while a modified sucrose medium with an initial pH of 4.5 was used for a fed-batch run. It was demonstrated in this study that maintenance of a pH of 6.0 through the fermentation led to accumulation of oxalic and gluconic acid from the sucrose, and oxalic and lactic acid from the lactose medium. In the green syrup and molasses media (pH 6.0), lactic and gluconic acid were primarily produced along with low amounts of oxalic acid. When sucrose was used with an initial pH of 4.5, *A. niger* produced gluconic acid, and very small yields of oxalic and citric acids. However, after the pH was adjusted to 6.0 and kept constant at this value, oxalic acid production was stimulated (Strasser et al., 1994). On the other hand, Kubicek et al. (1988) reported that oxalic acid biosynthesis by *A. niger* is favored in alkaline medium (8.0-9.0), and therefore there is a problem with the use of molasses due to its high buffering capacity that tends to maintain the pH in the range of 5.0-6.0 and results in oxalic acid production. Mischak et al. (1985) induced gluconic acid production by a citric acid producing *A. niger* (48 h, pH 1.8-2.0) in a sucrose medium by shifting the pH of the medium to 6.5.

The fermentation pH is one of the most important parameters that strongly affect the production of organic acids by fungi. As has been just discussed, the maintenance of low

pH is necessary for sufficient citric acid production, while pH values higher than 4 shift production from citric acid to acids such as oxalic and gluconic acid.

Trace Elements

The effects of certain metal ions on citric acid production has received much attention (Zidwick, 1992). Metals such as manganese, zinc, iron, copper and magnesium have been found to influence the production of citric acid by *A. niger* (Shankaranand and Lonsane, 1994; Gupta and Sharma, 1995; Gupta and Sharma, 2002). When Mn, Fe and Zn were present at growth-limiting concentrations, citric acid was accumulated in large quantities (Clark et al., 1966 as referenced by Gadd, 1999). On the other hand, Clark et al. (1966) reported that the presence of Mn at concentrations as low as 2 µg/l can reduce acid production by 20%. Because of such effects, industrial carbon sources (i.e. molasses) have been pretreated with cation-exchange materials or ferrocyanide prior to fermentation in order to remove some of the inhibitory metals (Zidwick, 1992; Ikram-ul-Haq et al., 2002; Sikander et al., 2002) or antagonistic substances, such as copper, lower alcohols or lipids (Kubicek, 1998 as referenced by Gadd, 1999). Due to the inhibitory effect of Mn on citric acid production, the Mn^{2+} requirement could partially be replaced by Mg^{2+} , Fe^{2+} or Zn^{2+} (Netik et al., 1997 as referenced by Gadd, 1999). Shankaranand and Lonsane (1994) reported that the addition of a mixture of Fe, Cu and Zn to the coffee husk medium increased citric acid production by 17%.

The development of high citric acid yielding mutant strains that are resistant to Mn^{2+} has been a subject of study (Gupta and Sharma, 2002, Gupta and Sharma, 1995). Gupta and Sharma (1995) induced a new mutant strain, *A. niger GS-III* from *A. niger KCU 520*

that was resistant to manganese ion concentrations up to 1.5 ppm in a sugarcane molasses. The citric acid yield in the presence of manganese ions was at least 3-fold higher with the mutant strain than with the parental strain.

Lower Alcohols

Methanol is one of the most well-known additives in commercial citric acid fermentation (Zidwick, 1992). Though the optimum amount of methanol added in the fermentation depends upon the strain used and the composition of the medium; in general the optimum range has been 1-3% (v/w) (El-Holi and Al-Delaimy, 2003, Kiel et al., 1981, Pazouki et al., 2000). Grewal and Kalra (1995) noted that methanol is not assimilated by *A. niger* and the role of methanol in stimulating the production of citric acid is not clear. Kiel et al. (1981) stated that slightly toxic concentrations of methanol greatly increase the tolerance levels of Mn, Fe and Zn by *A. niger*. Maddox et al. (1986) suggested an increase in permeability of the cell to citrate by methanol.

Conclusion

The culture conditions such as incubation temperature, air supply, agitation intensity, and incubation time also directly influence the production of citric acid. Culture conditions for citric acid production by fungi differ from strain to strain and depend on the type of fermentation process. The optimization of culture conditions is the key for high and consistent yields of fungal metabolites like organic acids.

2.2.3 Solid-State Fermentation

Solid-state fermentation (SSF) is the growth of microorganisms on moist solid substrates in the absence of free-flowing water (Perez-Guerra et al., 2004, Nagel, 2004).

In recent years, interest in SSF has intensified and led to a wide range of applications at lab scale. Comparative studies between submerged liquid fermentation (SLF) and SSF demonstrated several advantages of SSF over conventional SLF. Table 2-7 presents advantages and disadvantages of SSF as compared to SLF (Raimbault, 1998).

Table 2-7: Comparison between liquid and solid substrate fermentations (Raimbault, 1998).

Factor	Submerged Liquid Fermentation	Solid Substrate Fermentation
Substrates	Soluble substrates (sugars)	Polymer insoluble substrates: starch, cellulose, pectins, lignin
Aseptic conditions	Heat sterilization and aseptic control	Vapor treatment, non sterile conditions
Water	High volumes of water consumed and effluents discarded	Limited consumption of water. No effluent
Metabolic heating	Easy control of temperature	Low heat transfer capacity
Aeration	Limitation of soluble oxygen. High level of air required	Easy aeration and high surface exchange air/substrate
pH control	Easy pH control	Buffered solid substrates
Mechanical agitation	Good homogeneization	Static conditions preferred
Scale up	Industrial equipment available	Need for engineering & new design equipment
Inoculation	Easy inoculation , continuous process	Spore inoculation, batch

Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low growth rate fungi
Energetic consideration	High energy consumption	Low energy consumption
Volume of equipment	High volumes and high cost technology	Low volumes & low costs of equipments
Effluent & pollution	High volumes of polluting effluents	No effluents, less pollution

The use of naturally occurring substrates, which are usually composite and heterogeneous waste products from agriculture, food industry and biotechnological processes, is one of the main advantages of SSF (Burgstaller and Schinner, 1993; Raimbault, 1998). The structural macromolecule matrix of a solid substrate may simply act as an inert support (e.g. sugarcane bagasse, rice hulls (Garg and Sharma, 1991; Ooijkaas et al., 2000)) within which the carbon source (e.g. sugars) is adsorbed (Raimbault, 1998). However generally, the macromolecular matrix embodies the substrate providing the carbon and energy source (Raimbault, 1998). Preparation and pre-treatment of solid substrates are essential in order to convert substrates into a form suitable for fermentation. Raimbault (1998) listed the following preparation steps:

- size reduction by grinding, rasping or chopping;
- physical, chemical or enzymatic hydrolysis of polymers in order to increase substrate availability;
- supplementation with nutrients (phosphorus, nitrogen) and setting the pH and moisture content using a mineral solution;
- cooking or vapour treatment for macromolecular structure pre-degradation and elimination of major contaminants.

Perez-Guerra et al. (2004) stated that agitation of solid substrates may be very difficult, therefore static conditions are preferred. However, in order to avoid compaction, which causes non uniform growth, gradients of temperature, pH and moisture (Raimbault, 1998), Gervais and Bensoussan (1994) suggested three alternatives for improving the mixing of solid substrates: (1) sequential mixing; (2) the use of mobile microorganisms; (3) forced aeration through the solid substrate. The filamentous fungi are the most suitable microorganisms for SSF due to their physiological, enzymological and biochemical properties (Perez-Guerra et al., 2004). The hyphal mode of fungal growth allows fungi to penetrate into the solid substrate and occupy its parts with high levels of nutritive components (Gervais and Bensoussan, 1994). Forced aeration allows (1) the control of water content; (2) the removal of volatile compounds and CO₂ together with heat produced during the fermentation; and (3) supply of oxygen for aerobic metabolism (Gervais and Bensoussan, 1994).

The absence of free water in SSF gives the following benefits in comparison with SLF:

- reduced fermentor volumes;
- absence of large quantities of liquid waste to dispose off;
- ease of purification of products, thus lowering downstream costs.

In addition, the absence of free water reduces the contamination by microorganisms other than molds since most bacteria require a liquid environment. This results in less stringent conditions for SSF and may require no sterilization or aseptic procedures during operation thereby reducing the capital cost of the fermentation process.

Fungal citric acid production by SSF

Although up to 20% of citric acid production in Japan is by solid-state fermentation (Gervais and Bensoussan, 1994), most recent studies on fungal citric acid production from solid substrates have been carried out at bench scale. A variety of solid substrates have been investigated for citric acid production by genus *Aspergillus* by SSF (Table 2-8).

Table 2-8: Solid substrates used for citric acid production.

Solid Substrate	Initial moisture content (%)	Fungal strain	Ferm. period (days)	Yield of citric acid (g/kg dry weight)	Reference
Pineapple waste	65	<i>A. foetidus</i>	4	110, 161*	Tran and Mitchel, 1995
Apple waste	65	<i>A. foetidus</i>	4	47	Tran and Mitchel, 1995
Wheat bran	65	<i>A. foetidus</i>	4	8	Tran and Mitchel, 1995
Rice bran	50	<i>A. foetidus</i>	4	28	Tran and Mitchel, 1995
Coffee husk	50	<i>A. niger</i>	3	150	Shankaranand and Lonsane, 1994
Apple pomace	73	<i>A. niger</i>	5	324*	Hang and Woodams, 1984
Kiwi peel	--	<i>A. niger</i>	--	234*	Hang et al., 1987 as referenced by Hang and Woodams, 1998
Grape pomace	--	<i>A. niger</i>	--	267*	Hang and Woodams, 1986 as referenced by Hang and Woodams, 1998
Sweet potato	70	<i>A. niger</i>	4	140	Leangon et al., 2000
Corncoobs	25	<i>A. niger</i>	3	250*	Hang and Woodams, 1998
Corncoobs	--	<i>A. niger</i>	7	243	Olama and El Sabaeny, 1995

* in the presence of methanol

Substrate moisture is of central importance in SSF (Gervais and Bensoussan, 1994). Low moisture content can result in a reduction of nutrient solubility of the substrate making nutrients less available for fungal uptake. However, higher levels of moisture content can decrease the air filled porosity of the system, which can create oxygen transfer limitation, and increase the risk of bacterial contamination (Perez-Guerra et al., 2004). The optimal initial value of moisture content has been found to depend on both the microorganism and the solid matrix used (Table 2-8). During fermentation, the moisture content of the substrate will change due to evaporation and microbial activity hence water addition is necessary at some point of fermentation. Perez-Guerra et al. (2004) suggested the use of humidifiers or a water saturated air flow. Tran and Mitchel (1995) monitored the production of citric acid from pineapple waste over a range of moisture contents 55-85% during a 6-day-fermentation period. The highest citric acid production was achieved after 3 days with an initial moisture content of 70%. Tran and Mitchel (1995) also pointed out that the presence of methanol during fungal fermentation affects the fungal response to environmental conditions such as moisture content. For example, the optimum moisture content for citric acid production from apple pomace was 45% in the absence of methanol, but 65% in the presence of 3% methanol (Hang and Woodams, 1987 as referenced by Tran and Mitchel, 1995).

Methanol has been widely used at low concentrations (2-4% (v/w)) in SSF (Tran and Mitchel, 1995; Hang and Woodams, 1998; Hang and Woodams, 1984). Hang and Woodams (1998) studied corncobs as a substrate for citric acid production by four cultures of *A. niger*. *A. niger* NRRL 2001 produced the highest amount of citric acid in the presence of 3% methanol. The yield of citric acid was over 50% based on the amount

of sugar consumed. The same authors (Hang and Woodams, 1984) investigated citric acid production by different strains of *A. niger* from apple pomace in the presence and absence of methanol. *A. niger* NRRL 567 produced the greatest amount of citric acid from this substrate in the presence of 4% methanol.

The control of pH is not usually necessary in SSF (Tran and Mitchel, 1995, Hang and Woodams, 1998, Hang and Woodams, 1984, Leangon et al., 2000) due to the buffering effect of the solid substrates. When this variable must be controlled, buffering solutions are added in the liquid phase. Olama and El Sabaeny (1995) reported that when a buffered medium was used during fermentation of corncobs, the maximum yield of citric acid was observed at pH 4.05. Shankaranand and Lonsane (1994) also buffered their solid substrate (coffee husk) with 0.075 M NaOH to establish a pH of 4.5 at the beginning of fermentation to make the pH suitable for citric acid production. Pretreatment of chopped corncobs with 0.1 N NaOH and enzymatic treatment with Rapidase Pomaliq doubled the production of citric acid by *A. niger* (Hang and Woodams, 2001).

Fermentation temperature was found to have a profound influence on fungal production of citric acid from corncobs (Hang and Woodams, 1998). After 72 hours of incubation, the fungus produced the highest amount of citric acid (243 g/kg dry weight of corncobs) at 30 °C as compared with 23 °C and 37 °C, where yields of citric acid were only 86 and 167 g/kg dry weight of corncobs, respectively. A fermentation temperature of 30 ± 1 °C has been most frequently employed for SSF. All the fermentation experiments summarized in Table 2-8 were carried out at 30 °C.

A review of the literature has demonstrated that the culture conditions such as substrate moisture content, pH and fermentation temperature can drastically affect the yield of organic acids in SSF. In general, SSF is a cheaper process than SLF for the production of organic acids, therefore, if SSF variables (culture conditions) can be well controlled, this technology may be a more competitive process than is commonly thought.

2.2.4 Leaching of heavy metals by fungal generated organic acids

In the terrestrial environment, fungi play fundamental roles in carbon, nitrogen, and other biogeochemical cycles as decomposers, plant pathogens and symbionts (Wainwright, 1988 as referenced by Gadd, 1999). There are three major groups of fungi; moulds (filamentous), yeasts (unicellular) and mushrooms (fruiting bodies) and they inhabit many environments. Metals, both essential and inessential, and their derivatives can interact with fungi in various ways depending on the metal species, fungus species, and environment (Wainwright and Gadd, 1997 as referenced by Gadd, 1999). Certain mechanisms may mobilize metals into forms available for leaching from the system, e.g. complexation with fungal metabolites as organic acids. In biotechnology, fungi have mostly been associated with the production of antibiotics, enzymes and organic acids. Therefore, the ability of a number of fungi to solubilize metals from solid materials may open up completely new prospects for the application of fungi.

A goal of future environmental biotechnology is to establish metal-winning processes based on closed cycle concepts, which are ecologically sustainable (Burgstaller and Schinner, 1993). Chemolithoautotrophic microorganisms of the genus *Thiobacillus* have

been mainly used in biohydrometallurgy (Burgstaller and Schimmer, 1993). The lack of enthusiasm for fungal applications in this field has been attributed to:

- fungi require substantial quantities of organic carbon for growth and for the production of leaching agents;
- the handling of fungi is not well-known by many biohydrometallurgists;
- the ratio of generation of leaching material is considerably slower with fungi in comparison with autotrophic bacteria.

However, the following aspects of fungal applications may be advantageous (Burgstaller and Schimmer, 1993):

- many metal containing materials such as contaminated soils, sewage sludge, coal wastes, mining wastes, increase the pH of the medium making it unsuitable for the commercially important acidophilic *Thiobacillus* species;
- the ability of fungal leaching agents (organic acids) to form complexes with the metal ions thereby increasing their solubility in neutral environments and reducing their toxicity;
- fungi grow on cheap organic wastes.

For these reasons, fungal applications can be suitable in special niches where chemical and bacterial leaching have environmental, economic or operational disadvantages.

Chemistry of metal interactions with fungal generated organic acids

Bosshard et al. (1996) underlined the following mechanisms that fungal bioleaching is based on:

- acidolysis which consists of the solubilization of the material due to acidification (the most important mechanism);
- complexolysis that involves complexation of metals by the excreted organic acids or amino acids;
- redoxolysis that involves the mediation of reduction of metal (i.e. ferric iron) by organic acids (i.e. oxalic acid);
- bioaccumulation in which the mycelium acts as a “sink” for the metal ions.

The production of metal-complexing organic acids provides essential metals and anions as nutrients for fungi, other microbes and plants through the solubilization of salts and minerals (Gadd, 1999). The properties of such acids also determine metal speciation and mobility in the environment and hence can affect the transfer between terrestrial and aquatic habitats, biodeterioration and weathering. Gadd (1999) pointed out that even though a lot of information about fungal generated organic acids exists because of the commercial importance of citric acid, the significance of citric and oxalic acids in influencing metal speciation and mobility should not be neglected.

Organic acids can form coordination compounds or complexes with metals, which may be non-ionic, anionic or cationic. The charge on the complex will depend on the sum of the charges of the central metal atom or ion and surrounding ions and molecules (Gadd, 1999). Organic acid complexation of metal ions will depend on the comparative concentrations of the organic acid anions and metals in solution, the pH and the stability

constants of the various complexes (Denevre et al., 1996 as referenced by Gadd, 1999).

An example of the chemistry of fungal generated organic acids and metals is demonstrated for oxalic and citric acids in Figures 2-3 and 2-4.

As referenced by Dixon-Hardy et al. (1998), it was reported by Strasser et al. (1994) that fungal generated oxalic acid acts as a leaching agent for a variety of metals that form soluble oxalates, e.g. Fe, Al and Li. However, most metal oxalates are poorly soluble, and precipitate as crystalline or amorphous solids. The oxalate ion, $\text{C}_2\text{O}_4^{2-}$ (Figure 2-3B), is a bidentate ligand that forms a five-membered chelate when binding to a metal (Figure 2-3C) (Gadd, 1999). Three oxalate molecules can be bound to a metal that usually forms octahedral six-coordinate complexes (e.g. Al^{3+} , Cr^{3+} , Fe^{3+}) (Figure 2-3E), and metals that form square planar four-coordinate complexes (e.g. Cu^{2+} , Zn^{2+}) can bind two oxalate molecules (Figure 2-3D). Gadd (1999) reported that among divalent metal oxalates, the most soluble is magnesium oxalate and the least soluble are calcium and lead oxalates.

Figure 2-3: Metal complex formation by oxalic acid. (A) oxalic acid; (B) oxalate; (C) bidentate metal complex formation; (D) complex anion formation with metals which form square planar four-coordinate complexes; (E) complex anion formation with metals which form octahedral six-coordinate complexes (Gadd, 1999).

Citric acid forms mononuclear, binuclear or polynuclear complexes with metals (Gadd, 1999). Metals such as Ca^{2+} , Fe^{3+} and Ni^{2+} form bidentate, mononuclear complexes with two carboxylic acid groups (Figure 2-4B); whereas Cu^{2+} , Fe^{2+} , Cd^{2+} and Pb^{2+} are bound to citric acid with two carboxylic acid groups and the hydroxyl group forming tridentate mononuclear complexes (Figure 2-4C).

Figure 2-4: Metal complex formation by citric acid. (A) citric acid; (B) bidentate complex, e.g. (Ca cit)⁻, (Ni cit)⁻, (Fe(OH)₂ cit)²⁻; (C) tridentate complex, e.g. (Cd cit)⁻, (Fe cit)⁻, (FeOH cit)²⁻, (Pb cit)⁻, (Cu cit)⁻ (Gadd, 1999).

As has been mentioned previously, organic acid complexation of metal ions depends on various parameters such as the comparative concentrations of the organic acid anions and metals in solution, the pH and the stability constants of the metal-organic acid complexes. Zhou et al. (2003) reported that citric acid dissociates in solution with pK_a values of 3.14, 4.77 and 6.39, and Figure 2-5 demonstrates the speciation of citric acid as a function of pH. It can be seen from Figure 2-5 that H_3Cit and H_2Cit^- exist mostly at $pH < 5.5$, and with the increase of pH, Cit^{3-} becomes more important, while $HCit^{2-}$ peaks around a pH of 5.5.

Figure 2-5: Speciation of citric acid vs. pH (Zhou et al., 2003).

Zhou et al. (2003) demonstrated the complexation of Cd by different species of citric acid:

$$[\text{CdH}_2\text{Cit}^+] / [\text{Cd}^{2+}] = 10^{0.97} [\text{H}_2\text{Cit}^-] \quad (2-8)$$

$$[\text{CdHCit}] / [\text{Cd}^{2+}] = 10^{2.2} [\text{HCit}^{2-}] \quad (2-9)$$

$$[\text{CdCit}^-] / [\text{Cd}^{2+}] = 10^{3.15} [\text{Cit}^{3-}] \quad (2-10)$$

Log stability constants of Cd with H_2Cit^- , HCit^{2-} and Cit^{3-} were reported to be 0.97, 2.2 and 3.15, respectively. Based on the above equations and the dissociation constants of citric acid, Zhou et al. (2003) calculated the relative quantities of $[\text{CdH}_2\text{Cit}^+] / [\text{Cd-complex}_{\text{Total}}]$, $[\text{CdHCit}] / [\text{Cd-complex}_{\text{Total}}]$ and $[\text{CdCit}^-] / [\text{Cd-complex}_{\text{Total}}]$ as a function of pH (Figure 2-6).

Figure 2-6: Plot of $[CdH_2Cit^+]/[Cd-complex_{Total}]$, $[CdHCit]/[Cd-complex_{Total}]$ and $[CdCit^-]/[Cd-complex_{Total}]$ vs. pH (Zhou et al., 2003).

It can be seen from Figure 2-6 that $CdHCit$ existed as the main complex at $pH < 5$ and $CdCit^-$ was as the major Cd complex at $pH > 6$. In the pH range of 5-6, both $CdHCit$ and $CdCit^-$ contributed to the solution. CdH_2Cit^+ as can be seen from Figure 2-6 did not appear noticeably. Zhou et al. (2003) demonstrated in their study the correlation between organic acid complexation of metal ions and parameters such as pH and stability constants. The authors reported that when the desorption of cadmium ions from a tested soil in the presence of citric acid was studied, the concentration of cadmium ions in aqueous solution decreased slowly with increasing pH. Zhou et al. (2003) speculated that this was due to the high formation (stability) constant of Cd and Cit^{3-} .

Previous studies involving metal extraction by fungus

Relatively few studies have examined fungal leaching of heavy metals, and only a few papers have been published where fungal leaching was carried out on a greater scale than shake flasks (Burgstaller and Schinner, 1993). Burgstaller and Schinner (1993) noted that in order for a fungus to be used in leaching processes it must excrete substantial amounts of organic acids, and it must be resistant to heavy metals. Some fungal strains are not resistant to heavy metals, but can excrete large amount of organic acids. Such strains can be employed in two-stage processes. Burgstaller and Schinner (1993) stated that there are two steps involved in developing highly efficient leaching processes:

1. a fungal strain that is suitable for metal leaching processes must be chosen, and then its capacity to solubilize the specific insoluble metal compound is tested;
2. the mechanisms of the excretion of metabolites is studied and subsequently optimized (e.g. even recently, a complete understanding of the mechanisms of overproduction of citrate by fungi has not been achieved (Finkelstein, 1992)).

Most active leaching fungi that have been used in the past belong to the genera *Penicillium* and *Aspergillus*, and the most important species were *P. simplicissimum* and *A. niger* (Burgstaller and Schinner, 1993).

The investigation of metal leaching from municipal waste incineration fly ash by *Aspergillus niger* in one and two-stage processes was conducted by Bosshard et al. (1996):

In the one-stage process, *A. niger* was incubated in the presence of fly ash.

In the two-stage process, *A. niger* was incubated in a sucrose medium without ash addition at first. When the substrate was consumed, the suspension was filtered and the filtrate was used for the fly ash leaching.

A. niger was incubated in a sucrose medium with fly ash concentrations varying from 0 to 10% (w/v) at a temperature of 30 °C. It was found that *A. niger* produced gluconate in the one-stage process, while citrate was produced in the two-stage. The authors speculated that the citrate production was due to the absence of manganese (among other elements such as Zn, Co, Fe, and Ca) in the medium without fly ash. Bosshard et al. (1996) pointed out that low pH values have been reported to stimulate citric acid production, while higher pH in the presence of fly ash likely stimulated oxalic and gluconic acid production. The leaching efficiency of gluconate in the one-stage process was higher for Al, Fe, Mn, and Ni (average 4.2%), and lower for Cd, Cu, and Zn (average 20.2%) in comparison with the leaching efficiency of citrate in the two-stage process. It was observed that 81% of Cd, 66% of Zn, 57% of Cu, 52% of Pb, 32% of Mn, 27% of Al, and less than 10% of Cr, Fe, and Ni were extracted by the citrate in the two-stage process after one day of operation. The authors concluded that with the exception of Pb the leaching efficiency in the two-stage process was established quickly. Thus this study demonstrated that two-stage processes are quite applicable where fungal leaching of metals involved as fungal excretion of metabolites (i.e. organic acids) is very sensitive towards different culture conditions as demonstrated in Section 2.2.2.

Metal complexation by fungal generated organic acids was compared with commercially available organic acids such as citric, oxalic and gluconic acid by Sayer and Gadd (2001). *A. niger* was grown in an aerated liquid glucose culture medium

(control) and in the same medium which was modified with either $\text{Co}_3(\text{PO}_4)_2$ or $\text{Zn}_3(\text{PO}_4)_2$. In the control filtrate, citric acid at a concentration of 2.8 mM was the only acid detected after ten incubation days, whereas both citric and oxalic acid were detected in the $\text{Zn}_3(\text{PO}_4)_2$ -amended filtrate. The highest concentration of citric acid in the $\text{Zn}_3(\text{PO}_4)_2$ -amended filtrate was 7.0 mM after 25 days, and the concentration then decreased to 1.8 mM by the 49th day. The oxalic acid concentration remained at about 0.1 mM throughout. In the $\text{Co}_3(\text{PO}_4)_2$ -amended filtrate, gluconic acid with a concentration of 55.8 mM was the main acid produced after 49 incubation days. Only 6 mM of citric acid and 0.05 mM of oxalic acid were detected in the filtrate at the same time.

The control filtrate bound 77% of Zn^{2+} , and all the Co^{2+} present after 49 days. The formation of insoluble zinc oxalate was observed in the $\text{Zn}_3(\text{PO}_4)_2$ -amended cultured filtrate, but formation of cobalt oxalate was not detected in the medium amended with $\text{Co}_3(\text{PO}_4)_2$. The $\text{Co}_3(\text{PO}_4)_2$ -amended filtrate with large amounts of gluconic acid did not bind Zn^{2+} . The $\text{Zn}_3(\text{PO}_4)_2$ -amended culture filtrate was able to bind 100% of the Co^{2+} after 5 days.

The study demonstrated that the culture filtrates were as efficient, if not better, at binding the metal ions as the individually-supplied commercial organic acids. The commercial citric acid bound 78% of the Zn and 63.8% of the Co, while oxalic acid bound 41% of the Zn and 26% of the Co. Commercially-available gluconic acid did not demonstrate any apparent complexation of Co^{2+} and Zn^{2+} . Bosshard et al. (1996) also compared metal complexation by commercially available citric acid with that by fungal generated citric acid. The study demonstrated that metal leaching with commercially

available citric acid of equal molarity was only slightly higher (at the average 4.5%) than fungal filtrate leaching.

This review has revealed that there is a lack of studies that evaluate metal leaching by fungal generated organic acids. Nevertheless, the few investigations in this field have demonstrated that fungal generated organic acids such as citric acid were capable of leaching heavy metals in a similar range with commercially available organic acids.

3. EXPERIMENTAL AND ANALYTICAL METHODS

3.1 Experimental Plan

This work was divided into the following four components:

- Preliminary testing
- Evaluation of fungal species and solid substrates
- Batch metal extraction
- Soil column testing

This section outlines the experimental plan for each of the four components. The experimental apparatus that was employed and the associated methods are described in subsequent sections.

3.1.1 Preliminary testing

Preliminary testing of fungal growth and organic acid production under solid-state fermentation conditions was conducted. Based on the research conducted by Hang and Woodams (1998), the fungal strain *Aspergillus niger* NRRL 2001 and corncobs as a low-cost substrate were selected for this part of the research. DI water was employed as a flushing solution.

The effect of culture conditions on fungal production of organic acids from corncobs was investigated. The following culture conditions were examined:

- addition of an alternate carbon source
- addition of a nitrogen source
- initial pH adjustment of the corncobs with acid
- pre-treatment of corncobs with NaOH

- flushing frequency

Table 3-1 presents an overview of the experiments conducted in order to investigate fungal growth and organic acid production under the various environmental conditions.

Table 3-1: Summary of the preliminary testing experiments.

Experiment #	Additional carbon	Additional nitrogen	Initial pH of corncobs	Pre-treatment	Flushing frequency
1			4.1		daily
2	0.1 % molasses ^a		4.1		daily
3			3.0		daily
4	0.1 % molasses		3.0		daily
5		10 mg N/100 ml of flushing solution	4.1		daily
6	0.1 % molasses	10 mg N/100 ml of flushing solution	4.1		daily
7			4.1		every 3 rd day
8	0.1 % molasses		4.1		every 3 rd day
9			5.0	0.1 N NaOH	daily
^b Control_1			4.1		daily
Control_2	0.1 % molasses		4.1		daily
10			4.5		daily
11			2.0		daily
Control_3			4.5		daily
Control_4			2.0		daily

Note: ^a 0.1 % molasses as a flushing solution

^b corncobs were not inoculated by fungus (control)

3.1.2 Evaluation of fungal species and solid substrates

The following strains of genera *Aspergillus* were employed in this study for evaluation of fungal potential to produce organic acids from different solid substrates:

- *Aspergillus niger* NRRL 2001
- *Aspergillus niger* ATCC 64065
- *Aspergillus foetidus* NRRL 337
- *Aspergillus aculeatus* NRRL 358

The substrates indicated below were evaluated based on their nutritional suitability for organic acid production by fungi:

- corncobs
- apple pomace
- rice
- hay

Each fungal strain was grown on these substrates for an incubation period of 8 days. The inoculated substrates were flushed with DI water, and the flushing filtrates were analyzed for organic acid contents. Table 3-2 summarizes the experiments conducted in order to evaluate organic acid production by different fungal species grown on various solid substrates.

Table 3-2: Summary of the experiments conducted for the fungal species and solid substrate evaluation.

Experiment #	Fungal strain	Solid substrate
12 (in duplicate)	<i>A. niger</i> NRRL 2001	corncobs
13 (in duplicate)	<i>A. niger</i> NRRL 2001	apple pomace
14 (in duplicate)	<i>A. niger</i> NRRL 2001	boiled white rice
15 (in duplicate)	<i>A. niger</i> NRRL 2001	hay
16 (in duplicate)	<i>A. niger</i> ATCC 64065	corncobs
17 (in duplicate)	<i>A. niger</i> ATCC 64065	apple pomace
18 (in duplicate)	<i>A. niger</i> ATCC 64065	boiled white rice
19 (in duplicate)	<i>A. niger</i> ATCC 64065	hay
20 (in duplicate)	<i>A. foetidus</i> NRRL 337	corncobs
21 (in duplicate)	<i>A. foetidus</i> NRRL 337	apple pomace
22 (in duplicate)	<i>A. foetidus</i> NRRL 337	boiled white rice
23 (in duplicate)	<i>A. foetidus</i> NRRL 337	hay
24 (in duplicate)	<i>A. aculeatus</i> NRRL 358	corncobs
25 (in duplicate)	<i>A. aculeatus</i> NRRL 358	apple pomace
26 (in duplicate)	<i>A. aculeatus</i> NRRL 358	white rice
27	<i>A. aculeatus</i> NRRL 358	hay
28	<i>A. aculeatus</i> NRRL 358	boiled white rice

3.1.3 Batch metal extraction

The effectiveness of fungal generated organic acids for leaching heavy metals from a real field soil was tested. Batch metal extraction experiments were conducted, where contaminated soil samples were mixed with fungal organic acid-containing filtrates, and the soil slurries were allowed to contact for a period of time to allow metal extraction.

The slurries were then centrifuged and the aqueous phase was analyzed for heavy metal content. Control tests were also conducted (the soil was mixed with water) during each experimental sequence in order to observe the effects of water alone on the soil matrix.

Table 3-3 presents an overview of the flushing filtrates employed in the batch metal extraction series. Filtrates from flushing all four substrates were employed for each

series. Each series comprised of four batch metal extraction tests (in duplicate) and one control.

Table 3-3: Summary of batch metal extraction series.

Series	Fungal strain	Filtrate collection day
1	<i>A. niger</i> NRRL 2001	after 2 days of incubation
2	<i>A. niger</i> NRRL 2001	after 5 days of incubation
3	<i>A. niger</i> NRRL 2001	after 8 days of incubation
4	<i>A. niger</i> ATCC 64065	after 2 days of incubation
5	<i>A. niger</i> ATCC 64065	after 5 days of incubation
6	<i>A. niger</i> ATCC 64065	after 8 days of incubation
7	<i>A. foetidus</i> NRRL 337	after 2 days of incubation
8	<i>A. foetidus</i> NRRL 337	after 5 days of incubation
9	<i>A. foetidus</i> NRRL 337	after 8 days of incubation
10	<i>A. aculeatus</i> NRRL 358	after 2 days of incubation
11	<i>A. aculeatus</i> NRRL 358	after 5 days of incubation

3.1.4 Soil column testing

In this portion of the study, four soil columns with an inoculated substrate established at their top were prepared. Boiled white rice was chosen as a solid substrate, and *A. foetidus* NRRL 337 was employed as the fungal strain. The rice was flushed with DI water, and leachates were collected at the bottom of the soil columns. Samples of the filtrates (at the top of the soil column) were collected before contacting with the soil, and analyzed for pH and organic acid content. Leachate samples were analyzed for pH, the total dissolved lead concentration and for organic acid content.

The impact of process variables on the leaching of lead from a real field soil by fungal generated organic acids was tested in the soil column studies. Soil column testing consisted of two main parts:

1. Evaluation of the soil column depth and substrate addition;

2. Evaluation of the quantity of substrate, substrate type, substrate addition and frequency of flushing.

Table 3-4 summarizes the experiments conducted during the soil column studies, and indicates the process variables that were tested in these experiments.

Table 3-4: Summary of soil column studies.

Soil column	Column depth, mm	Soil dry mass, g	Rice type	Boiled rice mass, g	Substrate addition	Flushing frequency
Part 1						
1	60	20	A	80	after 14 incubation days, 50 g of rice A	every day
2	120	40	A	80	after 14 incubation days, 50 g of rice A	every day
3	180	60	A	80	after 14 incubation days, 50 g of rice A	every day
4	240	80	A	80	after 14 incubation days, 50 g of rice A	every day
Part 2						
5	60	20	B	200	after 35 incubation days, 200 g of rice B	every other day
6	60	20	A	200	after 35 incubation days, 200 g of rice A	every other day
7	60	20	C	200	after 35 incubation days, 200 g of rice C	every other day
8	60	20	A	400	after 35 and 79 days, 400 g of rice A	every other day

Note: Rice A and B were purchased from two different local grocery stores, while rice C was type A mixed with 1.5 g of Styrofoam.

3.2 Apparatus

3.2.1 Fungal leaching apparatus

A 2-stage funnel, with inside diameters of 114 mm and 84 mm, and a height of 112 mm, and with a perforated stainless steel disk mounted in its throat was employed to support the inoculated growth media (Figure 3-1). 12 funnels were constructed for this research. During the incubation period the funnels were fixed on a stand and covered with aluminum foil in order to minimize spreading of fungal spores. The flushing solution was collected at the funnel's bottom into a 200-ml glass Erlenmeyer flask.

Figure 3-1: Fungal leaching apparatus.

3.2.2 Evaluation of fungal species and solid substrates

In this portion of the study four strains of fungus and four solid substrates (corncobs, rice, apple pomace and hay) were evaluated for the potential of acid formation. *A. niger* NRRL 2001, *A. niger* ATCC 64065, *A. foetidus* NRRL 337 and *A. aculeatus* NRRL 358 were purchased from the American Type Culture Collection (ATCC) in a lyophilized condition. Apple pomace was obtained from a local apple farm. While white rice was purchased from a local grocery store, and hay was purchased from a local craft shop. Corncobs were obtained from the Ottawa Experimental Farm, air dried, and cut laterally into approximately 4 mm thick disks.

3.2.3 Batch metal extraction

A contaminated soil that was collected from a lead-battery contaminated site was employed in this study. Batch metal extraction experiments were conducted in 500-ml glass Erlenmeyer flasks that were covered by aluminum foil (Figure 3-2). The fungally-generated organic acid-containing filtrates were added to the soil samples in the ratio of 3 to 1 (volume (ml) to mass (g)).

Figure 3-2: Metal extraction tests.

3.2.4 Soil column testing

Soil column

Glass columns with an inside diameter of 25 mm, and a height of 300 mm were employed in this portion of the study. Glass wool with a depth of 1 cm was placed at the bottom of the glass columns as a leachate filter. The columns were loaded with contaminated soil, and fungal growth apparatus with substrate inoculated by fungus were fastened on top of the column in a manner that fungal filtrates would drain into the soil column. Flushing was performed by manually pouring water on the inoculated substrate at the top of the soil columns. The water that passed through the soil was collected as a leachate in a flask (Figure 3-3). A schematic of the soil columns is also shown in Figure 3-4.

Figure 3-3: Soil column testing.

Figure 3-4: Schematic of the soil column testing.

3.2.5 Analytical Instrumentation

Three analytical instruments were used to analyze samples that were generated in this study. Samples were analyzed for pH with an Accumet polymer body, gel-filled pH probe, attached to a Corning model 340 pH meter. Organic acids were measured with a Varian high performance liquid chromatography (HPLC) system that was equipped with a model 9100 autosampler, a model 9012 solvent delivery system and a model 9065 Polychrom diode array detection (DAD). Data acquisition and reporting were performed by Star Chromatography Software, version 4.51.

Two HPLC columns were used, one at early stages of testing and the other was installed later, hence the two names of the analytical methods (A) and (B) were assigned, respectively. The HPLC column that was used in Method (A) was a Rezex 8 μm , Org Acids, 8% H , and a Rezex, Org Acids guard column was used (Phenomenex, Torrance, California). The HPLC column used in Method (B) was an ICSep ICE-CORGEL-64H (Tarnsgenomic, San Jose, California). The guard column was a Rezex, Org Acids (Phenomenex, Torrance, California). The column heater, Thermosphere TS-130, was from Phenomenex. The HPLC experimental conditions are depicted in Table 3-5.

Table 3-5: HPLC experimental conditions used in Method (A) and Method (B).

Test parameters	Method A	Method B
Injection volume	10 μl	10 μl
Mobile phase	0.001N H_2SO_4	0.008N H_2SO_4
Wavelengths, λ_{max}	210, 263 nm	210, 263 nm
HPLC column	Rezex, Org Acids	ICSep ICE-CORGEL-64H
Guard column	Rezex, Org Acids	Rezex, Org Acids
Column heater temperature	No column heater used	40 $^{\circ}\text{C}$

The calibration of the HPLC was achieved first by analysis of each organic acid separately to establish retention time and wavelength at the peak of the absorbance signal,

$\lambda_{\max}$. A mixture of reference organic acids in solution was then prepared and injected for calibration of the HPLC. Table 3-6 shows the list of organic acids in the standard solution used for calibration of the HPLC.

Table 3-6: List of organic acids used for calibration of HPLC.

Name of organic acid	Retention time, min.	$\lambda_{\max}$, nm		ppm
		Analysis	Confirmation	
Oxalic	6.850	210		240
Citric	10.306	210		680
Tartaric	10.889	210		270
Gluconic	11.492	210		1000
L-Ascorbic	12.100	210	263	500
Malic	12.500	210		403
Succinic	16.000	210		960
Formic	17.351	210		2077

Metals were analyzed with a Perkin-Elmer, model 3100 flame atomic absorption spectrometry (FAAS) system. Hollow cathode lamps were used as the source of radiation. The sample was aspirated into an air/acetylene flame for atomization of target heavy metals. The instrument response was recorded for quantitation. The calibration was performed by analysis of at least five standard calibration solutions. The peak-height absorbance was plotted versus concentration to develop the calibration curve.

3.3 Experimental Methods

3.3.1 Preliminary testing

Fungus recovery and maintenance

A. niger NRRL 2001 was recovered from a lyophilized condition by adding approximately 0.3 ml of DI water with a Pasteur pipette to the inner vial of a batch vial. The entire inner vial contents were then drawn into the pipette, and transferred to a test tube containing 5 ml of sterile water. The fungus was left overnight for rehydration before transferring to potato-dextrose agar (PDA) plates and slants.

The fungus was maintained by monthly transfers on PDA slants that were incubated at room temperature (25 ± 1 °C), and stored at 4 °C. Potato-dextrose agar was prepared as described by ATCC (www.atcc.org).

Preparation of corncobs and flushing solutions

A 0.1 % (w/v) molasses flushing solution was prepared with molasses purchased from a local grocery store and distilled water. The molasses solution was sterilized by boiling 500 ml of the solution in a 1000-ml glass flask for 5-7 min. The flask was capped with a tin foil and foam plug in order to hinder over-boiling.

The additional nitrogen source employed in Experiments 5 and 6 was added to the flushing solutions (DI water in Exp. 5, and 0.1 % molasses in Exp. 6) in the form of $(\text{NH}_4)_2\text{SO}_4$. A nitrogen concentration of 100 mg/L in the flushing solution was employed.

For pH adjustment, the corncobs (prior sterilization) were acidified to pH 3.0 and 2.0 by titrating the corncob solution with 0.1 N HCl at 30-min intervals until a stable value was achieved.

Pre-treatment of corncobs was accomplished as described by Hang and Woodams (2001). In the treatment, the corncobs were blended with 0.1 N NaOH (20g/100 ml) for one hour, and then acidified to pH 5.0 by the addition of HCl prior to sterilization.

Experimental procedure for corncob fermentation by *A. niger* NRRL 2001

The following experimental procedure was employed in the preliminary experiments. In each experiment, 20 g (dry mass) of corncobs was autoclaved at 121 °C for 15 min in 100 ml of DI water. The sterilized corncobs were then inoculated with 2 ml of *A. niger* NRRL 2001 spore suspension (about $6.5\text{--}7 \times 10^6$ spores/ml) from a stock culture. The inoculated corncobs were left for an incubation period of 3 days at 25 °C. After incubation, the corncobs were flushed with 40 ml of flushing solution each day for 10 consecutive days. The filtrates were collected, and the pH of the filtrates was measured. Filtrate samples were also analyzed for organic acid content by HPLC. In experiments 10 and 11, the sterilized corncobs were inoculated with 1 ml of *A. niger* NRRL 2001 spore suspension (about 4×10^6 spores/ml), and the total incubation period was 9 days.

Spore suspensions were prepared by suspending pieces of PDA with 1-week-old cultures in 20 ml of sterilized water, and then filtering the solution through a glass wool in order to remove large mycelium fragments and clumps of agar. The approximate number of spores in the prepared spore suspension was determined by the spectrophotometric method that was specifically developed for this research (Appendix A).

3.3.2 Evaluation of fungal species and solid substrates

Preparation of fungal species and solid substrates

The recovery of fungus from a lyophilized condition and fungus maintenance for *A. foetidus* NRRL 337, *A. niger* ATCC 64065 and *A. aculeatus* NRRL 358 were carried out in the same manner as for *A. niger* NRRL 2001 described in the section 3.3.1.

The rice was prepared prior to inoculation by boiling 240 g of white rice in 750 ml of water for 20 min. The moisture content of the apple pomace and boiled white rice was calculated based on weight basis using the oven dry method (Trautmann and Richard, 2004). The initial pH of the corncobs, apple pomace, boiled rice and hay was measured by immersing the gel-filled pH probe in a solution of the substrate (in ratio 2:1(DI water, ml:substrate, g)) prior to sterilization.

A standard experimental procedure was employed to evaluate acid generation and is summarized as follows:

Duplicate samples of the solid substrate were autoclaved at 121 °C for 15 min in DI water as per:

- 30 g of corncobs (dry mass) in 150 ml;
- 15 g of apple pomace (dry mass) in 50 ml;
- 40 g of boiled white rice (dry mass) in 80 ml;
- 10 g of hay was in 200 ml.

The sterilized solid substrate samples were then inoculated with a fungal spore suspension prepared from a stock culture:

- 1ml of *A. niger* NRRL 2001 spore suspension (about $5-5.5 \times 10^6$ spores/ml);
- 2ml of *A. niger* ATCC 64065 spore suspension (about $2.5-3 \times 10^6$ spores/ml);

- 3ml of *A. foetidus* NRRL 337 spore suspension (about 1.7×10^6 spores/ml);
- 2 ml of *A. aculeatus* NRRL 358 spore suspension (about 2×10^6 spores/ml).

The inoculated substrates were incubated for 8 days at 25 °C. The substrates were flushed with 100 ml of DI water, and filtrates were generated on the 1st, 2nd, 5th, and 8th days. The pH of the filtrates was measured, and the filtrates were analyzed for organic acid content by HPLC.

Figures 3-5 and 3-6 demonstrate the growth of fungus on the different substrates in the solid-state fermentation systems.

Fig. 3-5: Fungal fermentation on corncobs and apple pomace.

Fig. 3-6: Fungal fermentation on boiled rice and hay.

3.3.3 Batch metal extraction

The soil employed in this study was heavily contaminated with lead. Soil samples were sent by SAIC Canada for ICP-OES analysis to Accutest Laboratories LTD. The initial lead concentration of 8190 $\mu\text{g/g}$ was determined. Hence the main focus of the heavy metal leaching tests by fungal generated organic acids was on lead extraction.

The contaminated soil was air dried, and the fraction that passed through a 2-mm sieve was employed in this study. The organic acid containing filtrates were collected after the 2nd, 5th and 8th days of fermentation. In parallel to the extraction tests, the initial pH of the filtrates was measured and samples of the filtrates were analyzed for organic acid contents by HPLC.

The organic acid-containing filtrates were added to the contaminated soil samples in ratio of 3 (volume) to 1 (mass) in duplicate flasks. The pH of the soil solutions was measured immediately. The soil slurries were contacted for 24 hr at room temperature (25 ± 1 °C) to allow desorption of heavy metals from the soil to the aqueous phase. Aliquots were withdrawn from the soil-filtrate mixtures, filtered through glass wool, and then centrifuged to fully separate the liquid from soil. The resulting aqueous solutions were analyzed by FAAS for heavy metal content (i.e., Pb, Mn, Cu, Zn), and by HPLC for organic acid content.

In preliminary metal extraction tests, the effect of contact time on metal extraction was investigated. Soil slurries were contacted for 3 days, and samples of the soil solutions were withdrawn for FAAS analysis each day.

3.3.4 Soil column testing

Preparation of rice

Rice A was cooked by boiling the raw rice in water (1 part of rice to 2 parts of water) for 10 min. Rice B was boiled in water (1 part of rice to 1.5 part of water) for 8 min. Rice C was prepared by mixing 200 g of cooked Rice A with 1.5 g of Styrofoam. A moisture content of the two types of boiled white rice was calculated using the oven dry method (Trautmann and Richard, 2004) and the initial pH of the rice was measured by immersing the pH probe in the substrate solution.

Evaluation of the process variables in the soil column studies

In Part 1 of the soil column studies, a mass of 80 g (wet mass) (for each column) of boiled Rice A with a moisture content of 65% was placed in the support apparatus at the top of the columns, and inoculated with 1 ml of the fungal spore suspension (about 2×10^6 spores/ml). The columns were loaded with 20, 40, 60, and 80 g of the contaminated air-dried soil. The inoculated rice was left without flushing for four incubation days, and then was flushed daily with 50 ml of DI water. After 13 fermentation days, an additional mass of 50 g of boiled Rice A was added to each column and mixed with the old rice layer with the fungus growing on it, and then left for four days without flushing. On the 18th incubation day, flushing was continued until the end of this run (28 fermentation days in total). During the run, samples of the filtrates (at the top of the soil column) were collected before contacting with the soil, and analyzed for pH and organic acid content. The leachates from the bottom of the columns were also collected. The leachate quantity was recorded, and leachate pH was measured. The leachate samples were analyzed by FAAS for the lead concentration and by HPLC for organic acid content.

In Part 2 different quantities and types of rice were placed in the support apparatus at the top of the columns including:

- 200 g of boiled Rice B with a moisture content of 55% that was inoculated with 3 ml of the fungal spore suspension (about 15×10^6 spores/ml) (column 5);
- 200 g of boiled Rice A with a moisture content of 65% that was inoculated with 3 ml of the fungal spore suspension (about 15×10^6 spores/ml) (column 6);
- 200 g of boiled Rice C with a moisture content of 65% that was inoculated with 3 ml of the fungal spore suspension (about 15×10^6 spores/ml) (column 7);
- 400 g of boiled Rice A with a moisture content of 65% that was inoculated with 3 ml of the fungal spore suspension (about 15×10^6 spores/ml) (column 8).

An additional 100 ml of DI water was added to the rice after inoculation of columns 5, 6 and 7, and 200 ml was added to column 8 as the rice appeared dry. All four columns were loaded with 20 g of the contaminated air-dried soil. After three days of incubation, flushing of all columns with 100 ml of DI water was started every other day. The pH of the filtrate samples was measured, and they were analyzed for organic acid content by HPLC. The leachates from the bottom of each column were also collected. The leachate quantity was recorded, and the leachate pH was measured. The leachate samples were analyzed by FAAS for the dissolved lead concentration and by HPLC for organic acid content. Manual mixing of the rice in columns was conducted for the first 35 incubation days in order to enhance aeration.

After 35 incubation days, an additional rice layer was added on the top of the old layer without mixing. A mass of 100 g of boiled Rice B was added to column 5, 100 g of boiled Rice A was added to column 6, 100 g of boiled Rice C was added to column 7,

and 200 g of boiled Rice A was added to column 8. The new rice layer was inoculated with 2 ml of a freshly prepared spore suspension (about 7.5×10^6 spores/ml) in every column, and after four incubation days, the columns flushing with DI water was resumed.

Columns 5, 6 and 7 experimental runs were stopped after 61 days, while column 8 run was continued. After 79 incubation days, a new rice layer (400 g of boiled Rice A) was added (no mixing) to column 8, and inoculated with 2 ml of the fungal spore suspension (about 14×10^6 spores/ml). Column 8 was flushed until the day 101.

With an increase in experimental time of the soil column tests, the soil inside the columns became more saturated, which resulted in different soil column permeability. As the flushing filtrates started passing through the columns with much slower rate than at the beginning of the soil column tests, the flushing frequency changed to irregular, and was based on the soil permeability in each soil column. The leachates of different quantities were still collected and analyzed for lead concentrations every other day. The lead extraction was calculated based on the measured volumes of the collected leachates. All soil column studies were conducted at the room temperature of 19 ± 1 °C.

3.4 Analytical Methods

pH

To determine pH, the electrode of the pH meter was immersed in a 200 ml flask containing flushing filtrate. Soil slurry pH measurement was accomplished in the same manner. The pH was calibrated prior to each batch of sample measurements.

Organic Acids

Measurement of organic acids was performed using the HPLC-DAD. A small aliquot (about 1 ml) of the liquid sample was transferred with a Pasteur pipette into a GC vial that was capped and loaded into the autosampler for analysis. All data acquisition, reporting and communication with the HPLC-DAD modules was accomplished using the Star Chromatography software. The identification and quantitation of the target organic acids were achieved by comparison of analyte signals with those of the reference calibration solution. As a QA/QC measure, a blank, which consisted of reagents, and a control, which was a reference solution of organic acids, were analyzed with each batch of samples. When deemed necessary, a correction of retention time shift was performed and the sample was re-integrated to reflect the changes made. This process was done very carefully based on column performance history for separation of closely eluted analytes. Reporting under different analysis wavelengths was used for confirmation purposes, specifically L-ascorbic acid that absorbed well at two different wavelengths, 210 and 263 nm.

Metals

The dissolved metal concentration was determined by aspirating the sample into the FAAS. Prior to injection the sample was centrifuged to ensure that suspended soil particles were removed. For quantitation and identification of the target heavy metal, the peak height absorbance of the sample was compared with that of the reference calibration solution. Those concentrations falling outside the linear dynamic range of the calibration curve were diluted appropriately and re-analyzed.

4. RESULTS AND DISCUSSION

4.1 Preliminary testing

This section addresses the results from a series of preliminary experiments that involved fermentation of corncobs by *A. niger* NRRL 2001. The impact of different culture conditions on the fermentation process was examined. The addition of an alternative carbon source was examined in Experiments 2, 4, 6 and 8, where the corncobs inoculated by *A. niger* were flushed with 0.1 % molasses. The addition of a nitrogen source was examined in Experiments 5 and 6 by adding $(\text{NH}_4)_2\text{SO}_4$ to flushing solutions. The effect of initial corncob pH was studied in Experiments 3, 4 and 11, when the corncobs (prior to sterilization) were acidified to pH 3.0 and 2.0 by 0.1 N HCl. In Experiment 9, the corncobs were pre-treated with NaOH, and the pH of the corncobs prior to inoculation was 5. Flushing frequency was examined in Experiments 7 and 8, where the flushing of corncobs was conducted every 3rd day, while the rest of the experiments carried out daily flushing. Only Experiments 10 and 11 were conducted in duplicate in the preliminary testing.

4.1.1 Fungal growth

In this section visual observations of fungal mycelium and spore development were used in order to characterize the stage of growth of the fungus. As all 9 sets of corncobs (Exp. 1 to 9) were inoculated on a Friday, the fungal growth was not monitored over the weekend. On Monday all corncob sets were entirely covered by white mycelium and showed many brown spores on the mycelium surface. The difference in the fungal growth on raw and pre-treated corncobs could not be visually noticed. An increase in the

fungal sporulation was observed with each subsequent incubation day. Flushing with different flushing solutions (i.e., 0.1 % molasses, 0.1 % molasses and water enriched with a nitrogen source) did not radically affect fungal growth. When a reduced flushing frequency was employed (every third day), only a slight decrease in the *A. niger* fungal growth was noticed. In general, it was concluded that the mycelium growth continued only for the first couple of days then fungal sporulation took over the entire fermentation process. According to Kiel et al. (1981), sporulation is regarded as detrimental to citric acid production and this was proved by HPLC analysis of the filtrate samples.

Fig. 4-1: *A. niger* NRRL 2001 on corncobs.

In successive attempts to influence initial fungal growth and metabolite development, additional experimental runs (Exp. 10 and 11) were conducted, where the fungal fermentation of acidified corncobs (pH=2) in Experiment 11 was compared with that of untreated corncobs in Experiment 10. The concentration of the spore suspension used for inoculation was decreased to one third of the original concentration in order to provide the fungus with sufficient amounts of carbohydrates (corncobs) as the fermentation

funnel could be loaded just with a certain amount of corncobs. After 2 incubation days visible signs of white mycelium appeared on untreated corncobs, whereas on acidified corncobs, no signs of fungal growth were observed. As the incubation period increased, the acidified corncobs were covered by brown spores of *A. niger*. Superior fungal growth was observed throughout the control runs (Controls 3 and 4). Control 3 using sterilized, untreated corncobs that were flushed daily with DI water, became contaminated by *A. niger* brown spores believed to be from the laboratory ambient air almost instantly. Control 4 using acidified corncobs had no visible signs of fungal growth throughout an entire 8 day incubation period. It was concluded that the adjustment of the initial corncob pH to 2 resulted in a weaker fungal growth on these corncobs compared to the untreated ones.

4.1.2 pH of filtrates

Figures 4-2 and 4-3 present the patterns in pH that were observed in the filtrates generated in this portion of the study. It can be seen from these figures that the pH of the corncob filtrates rapidly decreased over the first four incubation days and then stabilized at similar levels (pH 2.6 to 2.8). As can be seen from Figure 4-2, the lowest pH values were in Experiment 9 (corncobs pre-treated with NaOH), while the highest pH values were in Experiment 5 (nitrogen addition). The pH of filtrates in Experiments 1 and 3 were very similar demonstrating that the adjustment of the initial corncob pH to 3 in Experiment 3 did not substantially affect the fermentation process. When the corncobs were flushed with the molasses solution (Figure 4-3), the highest pH values were observed in Experiment 6 (nitrogen addition).

The decrease in pH of the flushing filtrates was indicative of organic acid production.

In general it can be concluded that the differences in the culture conditions had no significant effect on the pH.

Fig. 4-2: pH of filtrates vs. time with DI water flushing.

Fig. 4-3: pH of filtrates vs. time with molasses flushing.

4.1.3 Organic acid production

In Experiments 1 to 9 only citric, oxalic, and L-ascorbic acids were monitored in the filtrates (Analytical method A). Citric acid was studied as this acid has been demonstrated to possess a high metal leaching ability due to its four electron-donors. Oxalic acid forms insoluble calcium and aluminum oxalates causing a breakdown of soil particles and releasing entrapped heavy metals. Ascorbic acid was an acid of interest because its chemical structure also possesses several electron-donor groups. In this phase of the study the HPLC analysis detected only oxalic and ascorbic acids in the filtrates. Citric acid could not be detected in the filtrates either due to its low concentration or due to difficulties with Analytical method A. Figure 4-4 demonstrates cumulative organic

acid production under the different environmental conditions examined in this phase of the study.

Fig. 4-4: Cumulative organic acid production by *A. niger* NRRL 2001.

Insignificant quantities of citric acid were generated under any of the experimental conditions evaluated in this portion of the study. The impact of environmental conditions on the generation of ascorbic and oxalic acid production can be seen in Figure 4-4. The most ascorbic acid production was achieved in Experiment 3 using acidified corncobs (pH 3.0) that were flushed with DI water, while 0.1 N NaOH pre-treated corncobs (pH 5) (Exp. 9) resulted in the generation of the highest amount of oxalic acid. Ascorbic acid was not generated in this case suggesting that initial substrate pH had an influence on fungal metabolism.

When Experiments 10 and 11 were carried out, Analytical method B was employed. In this method the HPLC was calibrated with more organic acids including tartaric, malic, succinic, formic and gluconic acids. These organic acids have been shown to be synthesized by different *A. niger* cultures (Bercovitz et al., 1990; NIBGE, 2003), and may also leach heavy metals from soils due to the electron-donor groups present in their molecules. As Experiments 10 and 11 were conducted in duplicate, Table 4-1 presents the variability index for the cumulative organic acid production. The variability index (variability of two data relevant to their mean) was defined as a measure of the variability of duplicates as an alternative to coefficient of variance since the calculation of standard deviation did not have a significant statistical value in this case:

$$V_I = |d_1 - d_2| / \bar{I} \quad (4-1)$$

where d_1 , d_2 represent duplicate values and $\bar{I}$ is their mean.

Table 4-1: Variability index for cumulative organic acid production in Experiments 10 and 11.

Experiment	Fungal Generated Organic Acid				
	oxalic	citric	malic	ascorbic	succinic
10	0.21	0.15	0.10	0.52	0.39
11	0.23	0.06	0.69	0.65	2.00

As can be seen from Table 4-1, the highest variability index in Experiment 10 (untreated corncobs) was for succinic acid (0.39), and the lowest variability index was for malic acid (0.1). In Experiment 11 (acidified corncobs), the data demonstrated higher variability than in Experiment 10 for organic acids such as succinic, malic and ascorbic acids. The variability index of 2 for succinic acid in Experiment 11 indicates high variability for succinic acid production as no succinic acid was detected in the filtrates of one of the duplicate runs. The citric acid production data in Experiment 11 had the

lowest variability as can be seen from Table 4-1. The mean cumulative organic acid production from Experiments 10 and 11 are depicted in Figures 4-5 and 4-6.

Fig. 4-5: Cumulative organic acid production in Experiment 10.

Fig. 4-6: Cumulative organic acid production in Experiment 11.

The results presented in Figures 4-5 and 4-6 demonstrate that oxalic, citric, malic, ascorbic and succinic acids were synthesized by the fungus. Oxalic, citric and ascorbic acids were the main acids produced with oxalic acid as the predominant acid generated. Yields of 20.9 g/kg of oxalic acid, 2.5 g/kg of citric acid and 2.0 g/kg of ascorbic acid were generated in Experiment 10, while 7.9 g/kg, 0.6 g/kg and 1.8 g/kg of oxalic, citric and ascorbic acids, respectively, were generated in Experiment 11. Thus it was concluded that generation of organic acids on untreated corncobs (Exp. 10) was higher than on acidified corncobs (Exp. 11).

The pattern of organic acid production by *A. niger* NRRL 2001 on corncobs can be generalized in the following manner (Tables B-12 and B-13):

- At the beginning of the fungal fermentation cycle (2 incubation days), the amount of citric acid produced was highest in comparison with the other organic acids generated; oxalic acid production was relatively low.
- With an increase in incubation period (3 - 4 days), oxalic acid production increased significantly, whereas citric acid excretion reached its peak.
- After 7 - 8 days of incubation, oxalic acid production was still increasing, while citric acid production was declining.

The results suggest that the excessive oxalic acid excretion suppressed the production of the other organic acids.

4.2 Evaluation of fungal species and solid substrates

In this section of the study, an evaluation of the suitability of different solid substrates for organic acid fermentation by four fungal species was performed. The assessment was based on observations of fungal growth on the substrates, pH values of filtrates, and the types and quantities of organic acids produced by the fungi.

4.2.1 Fungal growth

The visual observations of the growth of the fungal strains on different solid-state substrates through eight incubation days are summarized in Table 4-2.

Table 4-2: Fungal growth on solid substrates.

Substrate	<i>A. niger</i> NRRL 2001	<i>A. niger</i> ATCC 64065	<i>A. foetidus</i> NRRL 337	<i>A. aculeatus</i> NRRL 358
corncoobs	Visible signs of mycelium growth with small brown spores on the surface appeared after 3 incubation days. On the 5 th day corncoobs were completely covered by brown spores. The mycelium could not be seen any more.	Only after 5 incubation days, small spots of white mycelium could be observed with no visible fungal spores. On 7 th incubation day, the mycelium covered lightly with grey fungal spores.	Mycelium growth could be seen after 2 incubation days. After 5 days, there were no visible signs of fungal mycelium, corncoobs were covered entirely by black spores. Increase in fungal sporulation was observed throughout the entire fermentation period.	Sustained mycelium growth. After 5 incubation days, substantial quantities of white fuzzy mycelium could be observed, with small purple spores on the surface.
apple pomace	Fungal growth was very similar to the growth observed on corncoobs. Lots of brown spores covered the substrate at the end of fermentation period.	Almost from the first incubation days, apple pomace was contaminated by <i>A. niger</i> NRRL 2001 spores. On 6 th incubation day, the substrate was completely covered by them. No fungal growth of <i>A.</i>	Analogous fungal growth as on corncoobs. Little mycelium growth (only during the first few days), then sporulation took over the entire process.	Sporulation was not as high as seen in the other strains. The fungal growth pattern was similar to the growth on corncoobs.

		<i>niger</i> ATCC 64065 was observed through the entire fermentation.		
rice	White mycelium completely covered the entire rice surface before sporulation occurred (after 5-6 incubation days). Sporulation started from the substrate edges, and moved gradually toward the center.	Mycelium development was very high. Rice was covered completely by white mycelium after 4 incubation days. Whereas, sporulation was very poor. At the end of incubation, only several small grey spores could be seen on the mycelium.	Dark yellow mycelium fully covered boiled rice in 3-4 incubation days and then sporulation (black fungal spores) started throughout the rice surface.	A lot of white mycelium developed on this substrate throughout the fermentation period. Fungal sporulation was not as high as <i>A. niger</i> and <i>A. foetidus</i> sporulation.
hay	Fungal growth was very difficult to observe. After 4 days of incubation, small brown spores could be seen on the hay without mycelium formation.	Similar fungal growth with other strains. No fungal mycelium developed through the incubation. Small fungal spores on hay straws could be barely seen.	The same fungal growth as <i>A. niger</i> NRRL 2001 on hay was observed. Sporulation was very low compared to the sporulation on corncobs, apple pomace, and rice.	Visible signs of fungal sporulation appeared after 5 incubation days: not many purple spores on hay straws.

It can be seen from Table 4-2 that except for *A. aculeatus* NRRL 358, corncobs, which are considerably rich in cellulose and hemicelluloses, were not a good carbon source for mycelium growth. Intensive sporulation was observed during the incubation. Apple pomace was not the most desirable substrate either as in general a lot of fungal spores were developed on its surface, but with a low mycelium formation. Boiled white rice favored mycelium formation for all fungal strains. The spore development was also very high on rice for *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 demonstrating that this solid substrate was especially suitable for growth of these two strains, and starch was a sufficient carbon source in this case. Sporulation of *A. aculeatus* NRRL 358 and *A. niger*

ATCC 64065 was minimal on rice. The weakest growth of all fungal strains was on hay which was likely due to the nutritional deficiency and low moisture content of hay.

Among the fungal species, *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 demonstrated the strongest growth on the tested solid substrates with very similar growth general characteristics (Table 4-2). *A. aculeatus* NRRL 358 growth was quite different as the mycelium formation was predominant on the substrates, with the exception of hay, while the spore development was low. *A. niger* ATCC 64065 was the weakest strain tested. On apple pomace the growth of this strain was dominated completely by *A. niger* NRRL 2001 growth from ambient air.

4.2.2 Filtrate pH

The initial pH values of the corncobs, apple pomace, boiled white rice and hay were about 4.5, 3.8, 6.8 and 7.0, respectively. Figures 4-7, 4-8, 4-9, and 4-10 depict the mean pH values of the filtrates collected during solid-state fermentation with the four fungal species on the four substrates. Table 4-3 presents the variability index for the pH values of filtrates collected during *A. niger* NRRL 2001 fermentation of the solid substrates.

Table 4-3: Variability index for filtrate pH with *A. niger* NRRL 2001.

incubation day	Solid Substrate			
	corncobs	apple pomace	boiled rice	hay
1	0.05	0.00	0.07	0.09
2	0.02	0.01	0.00	0.00
5	0.02	0.00	0.07	0.10
8	0.02	0.03	0.03	0.00

As can be seen from Table 4-3, there was low variability in the filtrate pH data during *A. niger* NRRL 2001 fermentation. The pH values of the hay filtrates collected on the 1st and 5th days demonstrated the highest variability.

Fig. 4-7: pH of filtrates with *A. niger* NRRL 2001.

Fig. 4-8: pH of filtrates with *A. foetidus* NRRL 337.

Table 4-4 presents the variability index for the pH values of filtrates collected during *A. foetidus* NRRL 337 fermentation of the solid substrates.

Table 4-4: Variability index for filtrate pH with *A. foetidus* NRRL 337.

incubation day	Solid Substrate			
	corncoobs	apple pomace	boiled rice	hay
1	0.01	0.02	0.04	0.00
2	0.03	0.01	0.04	0.05
5	0.01	0.02	0.03	0.06
8	0.03	0.03	0.01	0.02

As can be seen from Table 4-4, the pH values of *A. foetidus* NRRL 337 filtrates had very low variability with the highest variability index of 0.06 and 0.05 for the hay filtrates of days 2 and 5, respectively.

Fig. 4-9: pH of filtrates with *A. aculeatus* NRRL 358.

Table 4-5 presents the variability index for the pH values of filtrates collected during *A. aculeatus* NRRL 358 fermentation of the solid substrates. The variability index was not

presented for the hay and boiled rice filtrates as the *A. aculeatus* NRRL 358 fermentation of these substrates was not conducted in duplicate (Table 3-2). It can be seen from Table 4-5 that the highest variability was in the rice filtrate pH values collected on the 8th day with a variability index of 0.18.

Table 4-5: Variability index for filtrate pH with *A. aculeatus* NRRL 358.

incubation day	Solid Substrate		
	corncobs	apple pomace	rice
1	0.02	0.01	0.06
2	0.05	0.02	0.03
5	0.03	0.01	0.05
8	0.03	0.04	0.18

Fig. 4-10: pH of filtrates with *A. niger* ATCC 64065.

Table 4-6 presents the variability index for the pH values of filtrates collected during *A. niger* ATCC 64065 fermentation of the solid substrates. As can be seen from Table 4-6,

the lowest variability was demonstrated in the pH of apple pomace filtrates, while the hay filtrates of days 2 and 5 had the highest variability in their pH values.

Table 4-6: Variability index for filtrate pH with *A. niger* ATCC 64065.

incubation day	Solid Substrate			
	corncoobs	apple pomace	boiled rice	hay
1	0.02	0.01	0.02	0.00
2	0.02	0.00	0.02	0.11
5	0.02	0.02	0.07	0.09
8	0.02	0.00	0.22	0.04

As can be seen from Figures 4-7 to 4-10, the pH patterns of the filtrates from *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 were similar, while *A. aculeatus* NRRL 358 and *A. niger* ATCC 64065 filtrates demonstrated different patterns in pH values.

In general a decrease in the filtrate pH occurred with *A. niger* NRRL 2001 fermentation of all substrates (Exp. 12–15). Though the initial pH of these solid substrates was very different, after about 5 fermentation days the pH of all filtrates was in a very similar range with the exception of the rice filtrates. At the end of the incubation the non-rice pH values reached approximately the same values (2.5-2.8). While the mean final pH value of the rice filtrates was 3.4.

The pH of the filtrates collected during fermentation by *A. foetidus* NRRL 337 (Exp. 20-23) decreased in a similar manner as the pH of the *A. niger* NRRL 2001 filtrates. However, some differences in pH responses were observed. For instance, *A. niger* NRRL 2001 had a more rapid decrease in the filtrate pH with hay and corncoobs fermentation during the first four incubation days. On the 5th fermentation day, the filtrates from *A. niger* NRRL 2001 fermentation of corncoobs and hay had pH values of about 2.7 and 3.2, respectively, whereas *A. foetidus* NRRL 337 yielded pH values of 3.3 for corncoobs filtrate, and 3.6 for hay filtrate. At the end of the incubation, *A. foetidus* NRRL 337

filtrates from corncobs, apple pomace and hay had higher pH values (3.1, 3.0 and 3.2, respectively) than that of *A. niger* NRRL 2001 filtrates. The final pH of the rice filtrates was lower (2.9) for *A. foetidus* NRRL 337 in comparison with *A. niger* NRRL 2001 rice filtrates.

A. niger ATCC 64065 fermentation of corncobs resulted in similar filtrate pH values as *A. niger* NRRL 2001 and *A. foetidus* NRRL 337. The pH of the corncobs filtrates from *A. niger* ATCC 64065 decreased from approximately 4.3 to 2.7 during the incubation period of eight days. The apple pomace pH values of this strain could not be compared with other strains as contamination by *A. niger* NRRL 2001 occurred during fermentation. The pH of the rice and hay filtrates (Exp. 18 and 19) decreased in a similar manner. After five incubation days, the pH of the filtrate was 5.4 for rice incubation, and 5.0 for hay incubation, while at the end of incubation the pH reached about 3.8 for the rice filtrate, and 4.2 for the hay filtrate. This differed from *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 fermentation, where the final pH values for all substrates were between 2.4 and 3.4.

In the case of *A. aculeatus* NRRL 358 fermentation (Figure 4-9), the pH values of the corncob, apple pomace and hay filtrates were similar throughout the whole incubation time. When this fungus was cultivated on sterilized but not boiled rice, the filtrate pH decreased from 6.8 to about 4.8. During boiled rice fermentation (Exp. 28), the pH decreased to 5.0. The pH values from rice fermentation by *A. aculeatus* NRRL 358 were close to *A. niger* ATCC 64065 rice fermentation, but with a less rapid decrease during five incubation days.

It was observed that the growth of the four fungal strains on the solid substrates resulted in different pH values of the filtrates. The pH patterns of corncob and apple

pomace filtrates were quite similar among the fungal strains, while that of rice and hay filtrates differed among the strains. The pH values of rice and hay filtrates were similar for *A. niger* NRRL 2001 and *A. foetidus* NRRL 337, whereas *A. aculeatus* NRRL 358 and *A. niger* ATCC 64065 filtrates from these substrates had much higher pH values during the fermentation process.

4.2.3 Organic acid production

Cumulative organic acid production was calculated on the basis of the acid concentrations measured in the filtrates on the 2nd, 5th and 8th incubation days and the dry weight of the substrates. Figure 4-11 presents the mean of cumulative production of organic acids (g/kg dry matter of substrate) by the four strains of fungi after 8 days of growth on corncobs. Table 4-7 presents the variability indices for the cumulative organic acid production by the four fungal strains from corncobs.

Table 4-7: Variability index for cumulative organic acid production from corncobs.

Fungal Strain	Fungal Generated Organic Acid				
	oxalic	citric	malic	formic	succinic
<i>A. niger</i> 2001	0.1	0.5	0.6	0.7	0.4
<i>A. foetidus</i>	0.1	0.0	0.4		
<i>A. aculeatus</i>	0.1	0.1	0.2		
<i>A. niger</i> 64065	0.5	0.2	0.1	0.2	

It can be seen from Table 4-7 that generally the organic acid production data had low variability in the case of corncob fermentation. *A. niger* 2001 organic acid production data had the highest variability among the fungal strains, with malic and formic acid production in particular. These two acids had variability indices of 0.6 for malic acid and 0.7 for formic acid. The rest of the fungal strains had variability indices equal or less than 0.5 for the cumulative organic acid production data.

Fig. 4-11: Cumulative organic acid production from corncobs using Analytical method B.

As can be seen from Figure 4-11, oxalic, citric, malic, succinic and formic acids were produced by *A. niger* NRRL 2001 from the corncobs. These results differed from the previous results of Experiment 10 (section 4.1.3). This can be explained by the difficulties with the analytical method employed. Overlapping signals and shifts in the retention time during HPLC analysis of the filtrate samples made separation of the organic acids difficult in some cases. In addition, at this part of the study, ascorbic acid was eliminated from HPLC calibration due to difficulties with separation of citric and ascorbic acid HPLC signals. Among the five acids produced by *A. niger* NRRL 2001, oxalic acid contributed about 58 % (mass basis) of the total organic acid production. An oxalic acid yield of 6.14 g/kg was produced by *A. niger* NRRL 2001 after eight incubation

days as compared to 1.31 g/kg of citric acid, 2.69 g/kg of malic acid, 0.23 g/kg of formic acid, and 0.28 g/kg of succinic acid.

Corncob fermentation by *A. foetidus* NRRL 337 resulted in the production of oxalic (1.38 g/kg), citric (0.82 g/kg) and malic (0.71 g/kg) acid over the 8 days. Though the oxalic acid production was still the highest among the three organic acids produced, in comparison with *A. niger* NRRL 2001 its production was much lower.

A. aculeatus NRRL 358 produced the lowest amount of citric acid (0.37 g/kg) in comparison with other strains. Malic (0.75 g/kg) and oxalic (0.48 g/kg) acids were also produced by *A. aculeatus* NRRL 358 in low quantities. This strain produced the least amount of oxalic acid from corncoobs.

A. niger ATCC 64065 had a similar pattern of organic acid production as *A. niger* NRRL 2001 with oxalic acid production the highest (5.29 g/kg) followed by malic (2.38 g/kg), citric (1.78 g/kg), and then formic (0.84 g/kg) acid. This was the highest citric acid production from corncoobs among the fungal strains examined.

Generally, it was concluded that *A. niger* ATCC 64065 had similar organic acid production behavior to *A. niger* NRRL 2001 on corncoobs. Though *A. foetidus* NRRL 337 and *A. aculeatus* NRRL 358 did not produce as much oxalic acid on corncoobs as the *A. niger* strains, their cumulative production of oxalic acid was higher than that of citric acid, which was the most desirable acid in this study.

The production of organic acids by the different fungal species from apple pomace is presented in Figure 4-12. Table 4-8 presents the variability indices for the cumulative organic acid production by four fungal strains from apple pomace.

Fig. 4-12: Cumulative organic acid production from apple pomace using Analytical method B.

Table 4-8: Variability index for cumulative organic acid production from apple pomace.

Fungal Strain	Fungal Generated Organic Acid					
	oxalic	citric	tartaric	succinic	formic	gluconic
<i>A. niger</i> 2001	0.1	1.3	0.3	0.6	0.4	
<i>A. foetidus</i>	0.7	0.5	0.6	0.2		0.5
<i>A. aculeatus</i>	0.9	0.1	0.1	0.1	2.0	2.0
<i>A. niger</i> 64065	0.2	0.6	0.8	1.3	0.9	

As can be seen from Table 4-8, the cumulative organic acid production from apple pomace had higher variability than that from corncobs (Table 4-7). The large variability for formic and gluconic acids was demonstrated during *A. aculeatus* fermentation of apple pomace. The variability index of 2.0 indicates that these acids were not detected in one of the duplicate runs. For *A. niger* NRRL 2001, the highest variability was for citric acid production (1.3), while for *A. niger* ATCC 64065, it was for the succinic acid cumulative production. The *A. foetidus* data for apple pomace fermentation had the

lowest variability among the strains as the highest variability index was 0.7 in the case of oxalic acid.

As presented in Figure 4-12, oxalic, citric, tartaric, succinic, and formic acid were produced by *A. niger* NRRL 2001 from apple pomace with total yields of 10.60 g/kg, 0.97 g/kg, 1.87 g/kg, 3.11 g/kg, and 2.14 g/kg, respectively. As seen from Figure 4-12, production of oxalic acid by *A. niger* NRRL 2001 was the highest on apple pomace among all the fungal strains used. About 5% (mass basis) of the total organic acid production was citric acid, while oxalic acid contributed 57%. Considerably higher amounts of succinic acid (3.11 g/kg) were produced from apple pomace as compared with citric and tartaric acid production.

Apple pomace fermentation by *A. foetidus* NRRL 337 resulted in the highest yield of succinic acid (6.02 g/kg) of the fungal strains employed (Figure 4-12). Oxalic acid production by *A. foetidus* NRRL 337 was just 1.82 g/kg, and hence much lower than succinic acid production. Tartaric and gluconic acid were generated in very low concentrations that were similar to the citric acid production.

A. aculeatus NRRL 358 produced oxalic (0.13 g/kg), citric (0.49 g/kg), tartaric (0.35 g/kg), succinic (3.71 g/kg), formic (0.09 g/kg) and gluconic (0.15 g/kg) acids on apple pomace with the highest succinic acid production in comparison with the rest of acids generated. *A. aculeatus* NRRL 358 produced the lowest amount of oxalic acid among the all fungal strains growing on apple pomace.

During *A. niger* ATCC 64065 fermentation of apple pomace, the main acid produced was succinic (3.38 g/kg), followed by formic acid (2.64 g/kg), which with the exception of oxalic acid formation was similar to *A. niger* NRRL 2001 fermentation of apple

pomace. However the *A. niger* ATCC 64065 apple pomace fermentation results were suspect as the apple pomace was heavily contaminated by *A. niger* NRRL 2001.

Only *A. niger* NRRL 2001 fermentation of apple pomace resulted in high production of oxalic acid, while for the rest of fungal strains, succinic acid was the predominant acid in the apple pomace filtrates. Citric acid production from apple pomace was very low for all the fungal strains.

Figure 4.13 presents the results of the boiled white rice fermentation by *A. niger* NRRL 2001, *A. foetidus* NRRL 337 and *A. niger* ATCC 64065. Table 4-9 presents the variability index for the cumulative organic acid production from boiled white rice.

Fig. 4-13: Cumulative organic acid production from boiled white rice using Analytical method B.

Table 4-9: Variability index for cumulative organic acid production from rice.

Fungal Strain	Organic Acid	
	oxalic	citric
<i>A. niger</i> 2001	0.46	0.01
<i>A. foetidus</i>	0.40	0.04
<i>A. niger</i> 64065	0.82	1.49

As can be seen from Table 4-9, the highest variability with *A. niger* ATCC 64065 was for citric acid production data, with a variability index of 1.49. This was followed by the variability index of 0.82 of oxalic acid production. The citric acid production data of *A. niger* NRRL 2001 and *A. foetidus* fermentation of the boiled rice had very low variability.

No organic acids were detected in the rice filtrates collected during *A. aculeatus* NRRL 358 fermentation, and only oxalic and citric acids were produced by the other fungal strains. *A. niger* NRRL 2001 produced 0.08 g/kg of oxalic acid, and 0.18 g/kg of citric acid. *A. foetidus* NRRL 337 had a higher yield of citric acid (2.39 g/kg) as compared to *A. niger* NRRL 2001 and *A. niger* ATCC 64065 (0.49 g/kg) citric acid production.

As the citric acid production from the boiled rice by *A. foetidus* NRRL 337 contributed about 94 % (mass basis) of the total organic acid production, it was concluded that this fungal strain favored citric acid production on boiled white rice. The two *A. niger* strains did not produce as much citric acid from this substrate, though they did not favor oxalic acid production from boiled rice.

The results from hay fermentation by the fungus are summarized in Figure 4-14, and Table 4-10 presents the variability index for the cumulative organic acid production from hay.

No organic acids were detected in the hay filtrates collected during *A. aculeatus* NRRL 358 fermentation. *A. niger* NRRL 2001 produced oxalic (9.31 g/kg) and malic (1.32 g/kg)

acids while *A. foetidus* NRRL 337 and *A. niger* ATCC 64065 generated only oxalic acid (2.11 g/kg and 1.16 g/kg, respectively).

Fig. 4-14: Cumulative organic acid production from hay using Analytical method B.

Table 4-10: Variability index for cumulative organic acid production from hay.

Fungal Strain	Organic Acid	
	oxalic	malic
<i>A. niger</i> 2001	0.08	0.08
<i>A. foetidus</i>	0.20	
<i>A. niger</i> 64065	0.01	

As can be seen from Table 4-10, the organic acid production data had low variability during hay fermentation. The highest variability index (0.2) was for the oxalic acid production by *A. foetidus*.

The cumulative organic acid production from the four different substrates by *A. niger* NRRL 2001 is presented in Figure 4-15.

Fig. 4-15: Cumulative organic acid production by *A. niger* NRRL 2001 from four different substrates.

As can be seen from Figure 4-15, *A. niger* NRRL 2001 favored production of oxalic acid (undesirable fermentation product) on all substrates except for boiled rice. This may have been due to a different carbon source such as starch, which is the main constituent of rice. As oxalic acid concentrations were much higher than the concentrations of other acids produced, this may suggest that the high level of oxalic acid generated could suppress the production of other organic acids. The apple pomace fermentation resulted in higher production of formic (2.14 g/kg) and succinic (3.11 g/kg) acids in comparison with the corncob fermentation, when only 0.23 g/kg and 0.28 g/kg of formic and succinic acids, respectively, were generated. Tartaric acid (1.87 g/kg) was only produced from the apple pomace by *A. niger* NRRL 2001, while malic acid was generated by this fungal

strain from the corncobs (2.69 g/kg) and hay (1.39 g/kg). The citric acid production was very low by this strain, with the highest production (1.31 g/kg) from the corncobs and the lowest (0.18 g/kg) from the rice. No citric acid was generated by *A. niger* NRRL 2001 from the hay.

The cumulative organic acid production from the four different substrates by *A. niger* ATCC 64065 is presented in Figure 4-16.

Fig. 4-16: Cumulative organic acid production by *A. niger* ATCC 64065 from four different substrates.

As can be seen from Figure 4-16, *A. niger* ATCC 64065 had similar organic acid production behavior with *A. niger* NRRL 2001 on corncoobs, and excreted a large amount of oxalic acid (5.29 g/kg). The rice fermentation results were also close to that of *A.*

niger NRRL 2001, though *A. niger* ATCC 64065 produced slightly higher amounts of citric (1.15 g/kg) and oxalic (0.18 g/kg) acids. On the hay, *A. niger* ATCC 64065 produced approximately 8-fold less oxalic acid as compared to *A. niger* NRRL 2001. On the apple pomace, *A. niger* ATCC 64065 produced similar amounts of citric, tartaric, formic and succinic acids with that of *A. niger* NRRL 2001, while the oxalic acid production was much lower (0.7 g/kg) in comparison with *A. niger* NRRL 2001 oxalic acid production (10.6 g/kg). Thus it was concluded that *A. niger* ATCC 64065 did not favor oxalic acid production as much as *A. niger* NRRL 2001.

Figure 4-17 presents the cumulative organic acid production from the four different substrates by *A. foetidus* NRRL 337. Though *A. foetidus* NRRL 337 did not produce as much oxalic acid as *A. niger* strains (Figure 4-17), the cumulative production of oxalic acid was higher than that of citric acid, which was the most desirable acid in this study. The exception was boiled white rice fermentation, when the highest amount of citric acid (2.39 g/kg) was produced with this fungal strain and substrate. The succinic acid production (6.02 g/kg) from apple pomace was the highest production among all the substrates for *A. foetidus*.

Fig. 4-17: Cumulative organic acid production by *A. foetidus* NRRL 337 from four different substrates.

Figure 4-18 presents the cumulative organic acid production from the four different substrates by *A. aculeatus* NRRL 358. *A. aculeatus* NRRL 358 was the weakest fungal strain for solid substrate fermentation as no organic acids were detected in the rice and hay filtrates. The highest production was succinic acid (3.71 g/kg) from the apple pomace. The production of the remaining acids from both substrates was less than 1 g/kg (Figure 4-18).

Fig. 4-18: Cumulative organic acid production by *A. aculeatus* NRRL 358 from corncobs and apple pomace.

Considering all of the fermentation parameters including fungal growth, filtrate pH and cumulative organic acid production, the following conclusions were made:

1. Mycelium formation and sporulation can be parameters directly related to organic acids production. For example, it was suggested by Kiel et al. (1981) that citric acid production is directly correlated to the amount of mycelium formed. This could be demonstrated by *A. foetidus* NRRL 337 fermentation of rice, when rich mycelium formation followed by flourishing sporulation resulted in the highest citric acid production. The development of spores could be correlated to the beginning of organic acid production during *A. foetidus* NRRL 337 fermentation of rice. The first visible signs of the fungal sporulation on rice were distinguished

after 4 days (Table 4-3), at the same time as the first detection of organic acids in the flushing filtrates (Tables C-21 and C-22).

2. The pH decrease of the flushing filtrates can be considered both as an indicator of fungal growth and organic acid production. For instance, the pH of rice filtrates started decreasing from the first incubation day during *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 fermentation, while the production of organic acids began after about 4-5 incubation days. Therefore it can be concluded that the mycelium growth caused a drop in the filtrate pH that might be due to the excretion of some metabolites, which were undetectable by the employed HPLC analytical method.

4.3 Batch metal extraction

The effectiveness of fungal generated organic acids for leaching of heavy metals, lead in particular, from a real field soil was investigated in this section. The fungal organic acid-containing filtrates collected on the 2nd, 5th and 8th incubation day were mixed with soil samples, and the soil slurries were allowed to contact for a period of time to allow metal extraction. As the fungal growth on the solid substrates was conducted in duplicate, all batch metal extraction series were in duplicate also. In preliminary metal extraction tests, the effect of contact time on metal extraction was investigated. Soil slurries were contacted for 3 days, and samples of the soil solutions were withdrawn for FAAS analysis each day. It was demonstrated that metal extraction did not increase over the contact time of 3 days (Tables D-1 to D-3), therefore all the results of the batch metal extraction tests presented below are after 24 hours of contact. In addition, the organic acid content of *A. niger* NRRL 2001 filtrates (Tables D-12 to D-19) discussed in this section differed from that of Section 4.2 as two different experimental runs (different flushing frequency) of *A. niger* NRRL 2001 solid state fermentation were conducted.

4.3.1 *A. niger* NRRL 2001

The parameters such as organic acid content of the fungal filtrates and pH values of the soil slurries are presented in parallel with the lead extraction in order to examine the effect of these two factors on lead extraction. Figure 4-19 shows the lead concentrations in the soil extracts that were generated with organic acids generated by *A. niger* NRRL 2001 from the different solid substrates.

As can be seen from Figure 4-19, the corncob and hay filtrates demonstrated very poor lead extraction capacities. In the case of the corncob filtrates, the highest concentration of lead that was extracted by the acids in the corncob filtrates was 2.1 ppm on Day 2. The corncob filtrates from Day 5 and Day 8 extracted only 1.3 and 1.6 ppm, respectively. Their duplicate filtrates extracted similar amounts of lead; 1.0 and 1.5 ppm for Day 5 and Day 8, respectively. The hay filtrates performed even lower extraction of lead. The highest concentration of lead extracted during the hay filtrate extraction tests was 1.5 ppm on Day 8. The lowest extraction by the hay filtrates was observed on Day 5, the same as in the case of corncob filtrates where Day 5 was characterized by the lowest lead extraction. The hay filtrates from Day 2 ranged from 1.3 to 1.0 ppm. It needs to be pointed out that the extraction values of the duplicate series were very close and in some cases identical during lead extraction by corncob and hay filtrates (Figure 4-19).

The apple pomace filtrates demonstrated much better extraction of lead from the contaminated soil in comparison with the corncob and hay filtrates. Figure 4-19 demonstrates that the maximum lead concentrations measured in the aqueous solution of the soil slurry for the apple pomace filtrate extraction tests were 5.7 and 7.8 ppm for Day 2. The Day 5 filtrates had the lowest extraction among the apple pomace filtrates, with values of 2.4-4.2 ppm. The filtrates from Day 8 extracted 6.7-6.8 ppm of lead. As it can be seen from Figure 4-19, the lead extraction by apple pomace filtrates had wider variations in the extracted lead concentrations compared to the extraction by corncob and hay filtrates.

As demonstrated in Figure 4-19, the best lead extraction was achieved with the rice filtrates collected on the 8th day of fermentation. Lead concentrations of 24.2-33.9 ppm

were observed for Day 8, while the filtrates of Days 2 and 5 extracted very low amounts of lead. The rice filtrates from Day 2 extracted 1.1 ppm of lead, while the filtrates from Day 5 extracted 0.6-0.8 ppm of lead.

In general the lead extraction by the filtrates collected on Day 5 performed the lowest lead extraction. Quite high repeatability of the duplicate series was demonstrated during the batch metal extraction tests, especially when low amounts of lead were extracted (Figure 4-19).

Fig. 4-19: Extraction of lead using filtrates generated by *A. niger* NRRL 2001.

Tables 4-11 and 4-12 show the pH values of the soil slurries that were measured at the beginning and 24 hr after the soil solutions preparation, respectively.

Table 4-11: pH values of the initial soil solutions with *A. niger* NRRL 2001.

	control	corn. filtrate	app.pom. filtrate	rice filtrate	hay filtrate
Day 2-1	7.1	6.4	5.6	7.1	6.8
Day 2-2	7.1	6.5	6.0	7.0	6.9
Day 5-1	7.3	5.8	5.4	6.6	5.9
Day 5-2	7.3	5.5	5.5	6.3	6.0
Day 8-1	7.3	5.7	5.0	4.9	5.8
Day 8-2	7.3	5.5	4.9	5.0	5.6

Table 4-12: pH values of the soil solutions after 24 hr with *A. niger* NRRL 2001.

	control	corn. filtrate	app.pom. filtrate	rice filtrate	hay filtrate
Day 2-1	7.0	6.7	6.3	7.2	7.0
Day 2-2	7.0	6.7	6.5	7.1	7.0
Day 5-1	7.1	6.7	6.4	6.8	6.7
Day 5-2	7.1	6.6	6.4	6.6	6.4
Day 8-1	7.3	6.8	6.4	6.4	6.9
Day 8-2	7.3	6.7	6.4	6.6	6.8

It can be seen from Table 4-11 that the soil slurries prepared with the rice and hay filtrates from Day 2 had pH values similar to the values of the control test. The corncob and apple pomace filtrate soil solutions from Day 2 had lower pH values than the control solutions. The pH of all the soil slurries decreased when the solutions were prepared with the filtrates collected after five and eight incubation days (Table 4-11). The corncob filtrate soil slurries had pH values of 5.5-5.8 for the Day 5 and Day 8 solutions. Hay filtrate soil slurries had similar pH values of 5.7-6.0. The apple pomace filtrate soil slurries had the lowest pH values for Day 8 (4.9-5.0). These were similar to the rice filtrate soil slurries with pH values of 4.9-5.0. The pH of the rice filtrate soil solutions was the highest for Day 5 slurries with values in the range 6.3-6.6.

After 24 hr of soil-filtrate contacting, the pH of the soil solutions was much more similar for all series (Table 4-12). This was likely due to the soil buffering capacity. The pH of the corncob filtrate-soil slurries for Day 2, 5, 8 were buffered to 6.6-6.7 after 24 hours though the original pH values of the soil solutions were different for the different filtrates (Table 4-11). The pH values of the apple pomace filtrate-soil solutions ranged from 6.3-6.5 after 24 hours of contact. In the case of the rice filtrate-soil solutions, it can be seen from Table 4-2 that the variation among the pH values was quite high. The highest pH values were for the Day 2 slurries (7.1-7.2). These values corresponded to the high pH values of the initial solutions (Table 4-11). The lowest pH value among the rice filtrate-soil solutions was 6.4 for Day 8, which also corresponded to the low pH value (4.9) for the same solution in Table 4-1. The pH of the hay filtrate-soil solutions presented in Table 4-12 varied in the range of 6.4-7.0. The highest pH was 7.0 for Day 2-2 and the lowest was 6.4 for Day 5-2.

No clear relation can be seen between the extracted lead concentrations (Figure 4-1) and the pH of the soil solutions (Tables 4-11 and 4-12). For example, the pH of the rice filtrate-soil solutions of Day 8 in both Tables 4-11 and 4-12 were the lowest among all rice filtrate-soil solutions and corresponded to the highest lead extraction for these days (Figure 4-19). On the other hand, the pH values of the soil solutions for all substrates on Day 5 were either lower or quite similar to that of Day 2, whereas the lead extraction was always the lowest for Day 5.

The organic acid production by the studied fungal strains from the different substrates has been discussed in detail in Section 4.2.3. This section addresses the effect of organic

acid speciation and the concentrations of lead extracted from the soil. Figures 4-20 to 4-23 present the concentrations of organic acids in the fungal filtrates that were used in the batch metal extraction tests and the lead concentrations extracted by these filtrates.

Corncobs

Figure 4-20 demonstrates the organic acid concentrations in the corncob filtrates versus the extracted lead concentrations in these filtrates. As it was demonstrated in Figure 4-19, the extraction of lead by the corncob filtrates was very low. The highest lead extraction was by the corncob filtrates during Day 2. These filtrates as it can be seen from Figure 4-20 had the highest concentrations of citric and malic acid, and very low amounts of oxalic acid in comparison with the corncob filtrates of Day 5 and 8. The latter filtrates can be characterized by high concentrations of oxalic acid (Figure 4-20). Though the filtrate of Day 2-1 had higher concentration of malic acid than the filtrate of Day 2-2, both filtrates leached out the same amount of lead (2.1 ppm). This may be explained by the similar concentrations of citric acid in both filtrates. As citric acid has been known to possess a high metal leaching ability due to its four electron-donor groups, it can be speculated that citric acid had a more profound role in lead leaching than malic acid. In the case of oxalic acid as a metal chelator, Gadd (1999) reported that most simple metal oxalates are sparingly soluble in water, with the least soluble being calcium and lead oxalates. That was apparently demonstrated in Days 5 and 8 for the lead extraction by the corncob filtrates as with high concentrations of oxalic acid almost no lead was leached out from the soil, though the pH values of the soil solutions (Table 4-11) were lower than on Day 2.

Fig. 4-20: *A. niger* NRRL 2001 generated organic acid concentrations (a) in the corn cob filtrates vs the extracted lead concentrations (b).

Apple pomace

Figure 4-21 presents the organic acid concentrations in the apple pomace filtrates and the lead concentrations extracted by these filtrates.

Fig. 4-21: *A. niger* NRRL 2001 generated organic acid concentrations (a) in the apple pomace filtrates vs the extracted lead concentrations (b).

As can be seen from Figure 4-21, the highest lead concentrations extracted by the apple pomace filtrate happened in Day 2-1 (7.8 ppm). This was associated with high concentrations of ascorbic (796 ppm) and succinic (526 ppm) acid, while less lead was extracted for Day 2-2 (5.7 ppm) as the concentrations of ascorbic (572 ppm) and succinic (362 ppm) acid were lower. On the other hand, more lead was extracted in Day 8-2 (6.8 ppm) than on Day 2-2 though, as indicated in Figure 4-21, the filtrate of Day 8-2 had much lower concentrations of ascorbic (142 ppm) and succinic (96 ppm) acid. One of the possible explanations for this behavior may be the lower pH of the soil slurry of Day 8-2

(4.93) as compared to pH 5.96 for Day 2-2 (Table 4-11). The organic acid content and concentrations were quite different between the Day 8 replicates, but the lead extraction was similar (Figure 4-21). As the concentration of ascorbic acid in Day 8-1 was approximately 4.5-fold higher than that of Day 8-2, the high amount of oxalic acid (854 ppm) in the Day 8-1 filtrate may be the reason why this filtrate extracted the same amount of lead as the Day 8-2 filtrate that had much lower amounts of organic acids (Figure 4-21). Day 5-2 filtrate leached out only 2.4 ppm of lead, while Day 5-1 filtrate extracted 4.2 ppm, though these filtrates had similar organic acid content and concentrations as presented in Figure 4-21. The pH of the soil slurries was similar in these samples and hence the difference in the lead extraction was difficult to explain. It may have been due to analytical variability.

Rice

Figure 4-22 presents the organic acid content and concentrations in the rice filtrates collected for the batch metal extraction tests and the extracted lead concentrations. As it has been previously mentioned, the best lead extraction among the *A. niger* NRRL 2001 filtrates was achieved with the Day 8-1 rice filtrate (33.9 ppm). From Figure 4-4 it can be seen that this filtrate had the highest concentration of citric acid (508 ppm) among the filtrates. The filtrate of Day 8-2 extracted less lead (24.2 ppm) and this can be explained by the lower concentration of citric acid in the filtrate (Figure 4-22). Though the concentration of citric acid in the rice filtrates of Day 8-1 and Day 8-2 was lower than the concentrations of ascorbic and succinic acid in the apple pomace filtrates (Figure 4-21), the rice filtrates leached out much more lead than any of the apple pomace filtrates.

Therefore the results strongly suggest that citric acid is the strongest metal chelator among the fungal organic acids produced. The pH of the soil slurry of the rice filtrate of Day 8-1 was the lowest in comparison with the rest of the soil solutions and this would also favor higher lead extraction.

Fig. 4-22: *A. niger* NRRL 2001 generated organic acid concentrations (a) in the rice filtrates vs the extracted lead concentrations (b).

Hay

The organic acid concentrations in the hay filtrates and the concentrations of the extracted lead extracted by these filtrates are presented in Figure 4-23. The hay filtrates demonstrated the least lead extraction, and this behavior was likely due to the organic acid content and concentrations in the filtrates (Figure 4-23).

Fig. 4-23: *A. niger* NRRL 2001 generated organic acid concentrations (a) in the hay filtrates vs the extracted lead concentrations (b).

The highest extracted lead concentrations were 1.3, 1.55 and 1.50 ppm and these were extracted in the Days 2-1, 8-1 and 8-2 filtrates. The values were higher than the extraction of lead by the control series, where DI water was used for the extraction. The

lead extracted on Day 2-1 was likely due to the presence of tartaric acid. The elevated lead concentrations in the Days 8-1 and 8-2 extracts were possibly due to the decreased pH of the soil solutions (Table 4-11) that was caused by the fairly high concentrations of oxalic acid in the filtrates.

As an overall conclusion for this section, the following can be said:

- Citric acid generated by *A. niger* NRRL 2001 was very effective in the extraction of lead from the contaminated soil.
- Other fungal generated organic acids such as malic, succinic and tartaric acid were also capable of lead extraction, though they were much weaker chelators than citric acid.
- Oxalic acid decreased the pH of the soil solutions, but could not leach out lead. This behavior was likely due to the formation of insoluble lead oxalate (Gadd, 1999).

4.3.2 *A. foetidus* NRRL 337

The lead extraction by *A. foetidus* NRRL 337 filtrates collected during the fermentation of different solid substrates is presented in Figure 4-24.

Fig. 4-24: Extraction of lead using organic acids generated by *A. foetidus* NRRL 337.

The extraction of lead by corncob filtrates was limited (Figure 4-24), with the highest concentration of 6.5 ppm observed in the Day 2-1 filtrate. The duplicate filtrate (Day 2-2) extracted only 2.5 ppm of lead. Day 5 filtrates demonstrated the lowest extraction among the corncob filtrates, leaching out only 2.1 and 1.7 ppm of lead, respectively. The Day 8 extracts contained the same concentrations of lead (3.3 ppm).

Apple pomace filtrates demonstrated better lead extraction in comparison with the corncobs filtrates. The highest extraction was achieved with the Days 2-1 and 5-1 filtrates when 7.8 and 7.7 ppm of lead were extracted respectively. The Day 8-1 extraction of lead (6.1 ppm) by the apple pomace filtrate was similar to the Day 2-1 extraction by the corncob filtrate. However, there was a lack of repeatability of duplicate series during batch metal extraction by apple pomace filtrates. The lowest extraction (2.6 ppm) was demonstrated by the filtrate of Day 5-2. Day 2-2 and Day 8-2 apple pomace filtrates leached out 4.2 and 3.3 ppm, respectively.

It can be seen from Figure 4-24 that the extraction by hay filtrates was the least. The lead concentrations extracted by the hay filtrates were similar for all days, and ranged between 1 and 2 ppm. The rice filtrate extraction in the Day 8-1 and Day 8-2 samples leached out lead concentrations of 36 and 34 ppm, respectively. These concentrations were considerably higher than those observed with the rest of the *A. foetidus* filtrates (Figure 4-24). The lowest lead extraction by the rice filtrates was with Day 2-1 and Day 2-2, when only 1.3 and 1.9 ppm were extracted respectively. Days 5-1 and 5-2 rice filtrates extracted 5.7 and 6.9 ppm, respectively.

The following subsections address the effect of organic acid speciation on lead extraction by the filtrates collected from four different substrates.

Corncoobs

The pH values of the soil slurries of the batch metal extraction tests by *A. foetidus* filtrates were not available; therefore all further discussion of lead extraction by the filtrates is based on the organic acid content of the filtrates. Figure 4-25 presents the

organic acid content of the corncob filtrates and the extracted lead concentrations in these filtrates. As it can be seen from Figure 4-25, the highest lead extraction (6.5 ppm) by the corncobs filtrates was achieved with the filtrate of Day 2-1. This filtrate had the highest concentration of malic acid (170 ppm) among all filtrates, which could have caused the higher lead extraction. This can also be supported by the Day 2-2 filtrate. Both filtrates had very low concentrations of oxalic acid (11 and 5.2 ppm) and moderate concentrations of citric acid (83.7 and 61.5 ppm) for Days 2-1 and 2-2, respectively. The main difference in the organic acid concentrations between these two filtrates was the malic acid. The Day 2-1 filtrate had 170 ppm of malic acid as compared to 108 ppm for Day 2-2 filtrate. It can be seen in Figure 4-25 that the Day 2-2 filtrate extracted almost three-fold less lead (2.5 ppm) in comparison with the Day 2-1 filtrate.

Though the Day 5 corncob filtrates had the highest citric acid concentrations among the corncob filtrates, 120.1 and 94.3 ppm, respectively, and citric acid is considered as the strongest leaching agent in this study, the lead extraction was very low (Figure 4-25). One of the possible explanations of this was the presence of increased concentrations of oxalic acid (199 and 145 ppm) and the decreased concentrations of malic acid (51 and 23 ppm) in the filtrates of Day 5-1 and Day 5-2 as compared to the Day 2 filtrates. It was mentioned before that oxalic acid is not favorable for lead extraction as it tends to form an insoluble lead oxalate (Gadd, 1999). However, the results of the Day 8-1 and Day 8-2 extractions contradict the above assumption as more lead was extracted (3.3 ppm) by these filtrates as compared to the Day 5 filtrates, even though the oxalic acid concentrations were higher (220 and 247 ppm) for the Day 8 filtrates. The citric and

malic acid concentrations of the Day 8 filtrates were either similar or lower than those of the Day 5 filtrates.

Fig. 4-25: *A. foetidus* NRRL 337 generated organic acid concentrations (a) in the corncob filtrates vs the extracted lead concentrations (b).

Apple pomace

The organic acid content of the apple pomace filtrate of *A. foetidus* NRRL 337 fermentation and the corresponding concentrations of extracted lead are presented in Figure 4-26.

Fig. 4-26: *A. foetidus* NRRL 337 generated organic acid concentrations (a) in the apple pomace filtrates vs the extracted lead concentrations (b).

As it was observed in Figure 4-24, the lead extraction by the apple pomace filtrates was higher than the corncobs filtrate for Day 2-1, Day 5-1 and Day 8-1, whereas the extraction by samples from Day 2-2, Day 5-2 and Day 8-2 were similar to the corncob filtrates. The Day 2-1 apple pomace filtrate extracted 7.8 ppm of lead, while the Day 2-2 filtrate extracted only 4.2 ppm, though the organic acid content of these two filtrates was very similar. The organic acids present in these filtrates were citric, tartaric and succinic acid, where succinic acid was in predominant concentrations of 568 and 503 ppm for Day 2-1 and Day 2-2, respectively. The citric acid concentrations of these filtrates were the

highest of the apple pomace filtrates, with 62 ppm for Day 2-1 filtrate and 58 ppm for Day 2-2. Tartaric acid was present in quite low concentrations of 25 and 20 ppm for Days 2-1 and 2-2, respectively.

The difference between the Day 5-1 (7.7 ppm) and Day 5-2 extractions (2.6 ppm) was likely due to the higher concentration of citric (42 ppm), tartaric (65 ppm) and succinic (283 ppm) acids in the Day 5-1 filtrate. Citric acid was not detected in the Day 5-2 filtrate, and tartaric and succinic acids were in concentrations of only 17 and 193 ppm, respectively. However, the lead extraction with the Day 2-1 and Day 5-1 filtrates was similar, while the concentrations of citric and succinic acids of the Day 2-1 filtrate were higher than those of the Day 5-1 filtrate.

The lead extractions by the Day 8 samples with apple pomace filtrate were different, with values of 6.1 ppm and 3.3 ppm, respectively. The concentrations of organic acids such as citric, tartaric and succinic acid in these filtrates were similar as it can be observed from Figure 4-26. However, the apple pomace filtrate of Day 8-1 had a higher concentration of oxalic acid (234 ppm vs 116 ppm) as compared to Day 8-2. Oxalic acid is considered as not very favorable for the lead extraction as has been mentioned before. The data suggest that the lead extraction by the apple pomace filtrates was influenced by factors other than the organic acid content of the filtrates. Factors such as the pH of the soil slurries and fungal metabolites in the filtrates, which were not analyzed in this study, could have affected the lead extraction.

Rice

Figure 4-27 presents the organic acid concentrations of the rice filtrates vs the extracted lead concentrations in these filtrates. As depicted in Figure 4-9, only oxalic and citric acid were detected in the rice filtrates. Citric acid was the main acid in the rice filtrates as oxalic acid was present in low concentrations. The rice filtrates of Day 8-1 and Day 8-2 extracted 36 and 34 ppm of lead, respectively, which were the highest extractions among all filtrates as indicated in Figure 4-24. These two rice filtrates also had the highest concentrations of citric acid among the *A. foetidus* NRRL 337 filtrates, with 582 ppm for the Day 8-1 filtrate and 550 ppm for the Day 8-2 filtrate.

Though citric acid appeared to have played the main role in lead extraction by the rice filtrates, some additional factors may have affected the extraction. This speculation is based on comparing the lead extraction by the rice filtrates of Day 5-1 and Day 5-2. These filtrates had relatively high citric acid concentrations (339.3 and 427.4 ppm, respectively), but the lead concentrations were very low in comparison with Day 8-1 and Day 8-2. The Day 5-1 filtrate extracted only 5.7 ppm of lead, and the Day 5-2 filtrate extracted 6.9 ppm (Figure 4-27). It can be seen from Figure 4-27 that only a small amount (16.6 ppm) of citric acid was detected in the Day 2-1 filtrate, and no acids were detected in the Day 2-2 filtrate.

Fig. 4-27: *A. foetidus* NRRL 337 generated organic acid concentrations (a) in the rice filtrates vs the extracted lead concentrations (b).

Hay

Figure 4-28 presents the organic acid concentrations detected in the hay filtrates and the lead extraction by these filtrates. Only oxalic acid was identified in the hay filtrates, and the extraction by the hay filtrates was the lowest of all the substrates. The highest lead extraction by the hay filtrates was in Day 5-2 and Day 8-1 (1.6 and 2 ppm), respectively. These two filtrates had the highest oxalic acid concentrations (Figure 4-28), however it is difficult to conclude if oxalic acid affected the lead extraction. This was

because the extracted concentrations were small and not very different from those extracted by the Day 2-1 and Day 2-2 filtrates, which did not have detectable oxalic acid as it can be seen from Figure 4-28.

Fig. 4-28: *A. foetidus* NRRL 337 generated organic acid concentrations (a) in the hay filtrates vs the extracted lead concentrations (b).

The following conclusions were drawn for this section:

- Batch metal extraction tests by apple pomace filtrates demonstrated a lack of repeatability of the duplicates though the organic acid content of these duplicates

was similar. This suggests that some unidentified factors were influencing the lead extraction in addition to the effect of the organic acids.

- Citric acid demonstrated a high metal leaching capacity in the lead extraction by the rice filtrates.
- Fungal generated organic acids such as malic and succinic acid also highly affected the lead leaching in the case of corncob and apple pomace filtrate extraction.
- Oxalic acid was considered as the weakest chelator, which did not yield significant lead extraction in the case of the hay filtrates. Oxalic acid appeared to reduce lead extraction in the corncob filtrates.

4.3.3 *A. niger* ATCC 64065

This section presents the results of lead extraction by the fungal filtrates generated by *A. niger* ATCC 64065. Figure 4-29 presents the extraction of lead by different *A. niger* ATCC 64065 filtrates.

Fig. 4-29: Extraction of lead using organic acids generated by *A. niger* ATCC 64065.

As it can be seen from Figure 4-29, the lead extraction by *A. niger* ATCC 64065 filtrates was very poor, with the exception of the apple pomace filtrates from Day 2, as the extracted amounts did not exceed 1 ppm. With the apple pomace filtrates from Day 2, 6.5 and 4.6 ppm of lead were extracted, respectively. The apple pomace filtrates of Day

5-1 and Day 5-2 extracted only 0.7 and 0.8 ppm, respectively, and negligible concentrations of lead were extracted by the apple pomace filtrates from Day 8-1.

The highest extraction among the corncob filtrates was 0.9 ppm with the filtrates from Day 2 followed by the Day 5-1 filtrate extraction of 0.8 ppm. The lowest extraction was 0.4 ppm by the corncob filtrates of Day 8. The rice filtrates from Days 8 extracted similar amounts of lead (0.9 ppm) as the corncob filtrates of Day 2. This was the highest extraction for the rice filtrates as no lead was leached from the Day 2 rice filtrates, and 0.45 and 0.13 ppm of lead were detected in the rice filtrates of Day 5-1 and Day 5-2. Hay filtrates demonstrated the least extraction among all *A. niger* ATCC 64065 filtrates as the highest amounts extracted were 0.5, 0.4 and 0.3 ppm by the hay filtrates from Day 8-1, 8-2 and 5-1, respectively.

The pH values of the soil slurries during the batch metal extraction tests were recorded in order to analyze whether the pH of the soil solutions influenced the lead extraction. Tables 4-13 and 4-14 present the pH values of the soil slurries during the extraction tests by *A. niger* ATCC 64065 filtrates that were measured immediately after mixing and 24 hr after the soil solution preparation, respectively.

Table 4-13: pH values of the initial soil solutions with *A. niger* ATCC 64065.

	<i>control</i>	<i>corn. filtrate</i>	<i>app.pom. filtrate</i>	<i>rice filtrate</i>	<i>hay filtrate</i>
Day 2-1	7.1	6.8	6.3	7.9	7.5
Day 2-2	7.1	6.6	6.6	7.9	7.6
Day 5-1	7.3	6.3	6.6	7.3	7.5
Day 5-2	7.3	6.4	6.4	7.5	7.5
Day 8-1	7.3	6.3	N/A	6.8	7.5
Day 8-2	7.3	6.6	N/A	7.2	7.5

Table 4-14: pH values of the soil solutions after 24 hr with *A. niger* ATCC 64065.

	control	corn. filtrate	app.pom. filtrate	rice filtrate	hay filtrate
Day 2-1	7.0	6.3	6.0	7.3	7.4
Day 2-2	7.0	6.3	6.0	7.3	7.4
Day 5-1	7.1	6.7	6.6	6.9	7.1
Day 5-2	7.1	6.9	6.6	7.1	7.2
Day 8-1	7.23	7.0	N/A	6.8	7.3
Day 8-2	7.23	7.0	N/A	7.0	7.3

As can be seen from Table 4-13, the soil solutions of the corncob filtrates had pH values in the range of 6.3-6.8 when the pH was measured immediately after the solution preparation. The lowest pH values were 6.3-6.6 and 6.3-6.4 for the soil slurries from Day 8 and Day 5, respectively. The soil slurries from corncob filtrates on Day 2 had pH values of 6.8 and 6.6, respectively, when measured immediately after solution preparation. The initial apple pomace filtrate soil solution pH values were in the range of 6.3-6.6. For the rice and hay filtrate soil solutions, the initial pH values were higher than those of the corncob and apple pomace filtrate soil solutions (Table 4-13). For the rice filtrate soil solutions, the initial pH was in the range of 6.8-7.9, with the highest pH of 7.9 for Day 2-2, and the lowest pH of 6.8 for Day 8-1. The pH of the hay filtrate soil slurries were in the range of 7.5-7.6 initially.

It can be seen from Table 4-14 that the pH of the soil slurries of corncob filtrates from Days 2 decreased only slightly after 24 hours, with both filtrates having a value of 6.3. The pH of the corncob filtrate soil solutions for Day 5-1, Day 5-2, Day 8-1 and Day 8-2 increased after 24 hour of soil-filtrate interaction to 6.7, 6.9, 7.0 and 7.0, respectively (Table 4-14). After 24 hours, the pH of the apple pomace filtrate soil slurries of Day 2 decreased to 6.0, while that of Day 5-2 increased from 6.4 to 6.6. The pH of all rice filtrate soil solutions decreased slightly, with the highest pH of 7.3 for Day 2-2, and the lowest pH of 6.8 for Day 8-1 (Table 4-14). The pH values of the hay filtrate soil

solutions changed less during 24 hours. As can be seen from Table 4-13, the pH of these soil slurries were in the range of 7.5-7.6 initially and were in the range of 7.1-7.4 after 24 hours.

As presented in Figure 4-29, the corncob filtrates of Day 2 extracted the highest amounts of lead as compared to the rest of the corncob filtrates. The initial pH of the corncob filtrate soil solutions from Day 2 (Table 4-13) were higher than those after 24 hours (Table 4-14), therefore it would appear that the pH of the soil slurries after 24 hours of interaction (Table 4-14) demonstrated better correlation between the pH of the soil solutions and lead extraction. This also was demonstrated by the pH of the corncob filtrate for Day 8-1. Though the pH of this solution was the lowest (6.3) immediately after the solution preparation, 24 hours later it became the highest (7.0) among all the corncob filtrate soil solutions, and the lead extraction by this filtrate was the lowest. Regarding the lead extraction by the apple pomace filtrates, it was previously demonstrated in Figure 4-29 that the Day 2 apple pomace filtrates extracted the highest amounts of lead among all *A. niger* ATCC 64065 filtrates. Table 4-14 demonstrates that the pH of the apple pomace filtrate soil solutions of Day 2 were the lowest, which suggests a relationship between the pH after 24 hours of interaction and lead extraction. The pH of the rice and hay filtrate soil slurries did not appear to be low enough to result in any substantial lead extraction as the lead extraction by the rice and hay filtrates was very low (Figure 4-29).

Corncoobs

The following part of this section discusses the effect of the organic acid content of the fungal filtrates on the lead extraction. Figure 4-30 presents the organic acid content of the corncob filtrates generated during *A. niger* ATCC 64065 fermentation vs. the extracted lead concentrations in these filtrates.

Fig. 4-30: *A. niger* ATCC 64065 generated organic acid concentrations (a) in the corncob filtrates vs. the extracted lead concentrations (b).

As it can be seen from Figure 4-30, oxalic, citric, malic and formic acids were observed in the filtrates generated by *A. niger* ATCC 64065. The lead extraction by the *A. niger* ATCC 64065 corncob filtrates was very poor, with the highest amount extracted by the

Day 2 filtrates (0.9 ppm). The citric acid concentrations were relatively high in the Day 2-1 and Day 2-2 filtrates (282 and 308 ppm) and these concentrations were higher than the citric acid concentrations of *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 corncob filtrates for the same days (Figures 4-20 and 4-25), however the lead extraction was much lower by *A. niger* ATCC 64065 corncob filtrates. This demonstrated again that the organic acid content of the filtrates was not only the factor, which influenced lead extraction. It may be suggested that *A. niger* ATCC 64065 generated some unidentified metabolites from corncobs on Day 2 that negatively affected the lead extraction from the soil.

The corncob filtrates of Day 5 had high amounts of oxalic acid (687 and 465 ppm), which could be a reason for the low lead extraction by these samples (Figure 4-30). These two filtrates had substantial amounts of citric acid, 248 ppm for Day 5-1 filtrate, and 158 ppm for Day 5-2 filtrate. The Day 5-1 filtrate also had 188 ppm of malic acid, while no malic acid was detected in the Day 5-2 filtrate, which possibly caused the difference in the lead extraction between these two filtrates (Figure 4-30). Oxalic acid was the main acid in the Day 8 corncob filtrates (1045 and 976 ppm), which likely caused the least lead extraction among the corncob filtrates.

Apple pomace

Figure 4-31 demonstrates the organic acid concentrations of the apple pomace filtrates and the lead concentrations extracted by these filtrates. Apple pomace filtrates were not available on Day 8 as the apple pomace was contaminated by *A. niger* NRRL 2001 spores from the ambient air after six fermentation days.

Fig. 4-31: *A. niger* ATCC 64065 generated organic acid concentrations (a) in the apple pomace filtrates vs. the extracted lead concentrations (b).

As it can be seen from Figure 4-31, the main organic acids in the apple pomace filtrates of Day 2-1 and Day 2-2 samples were citric, succinic and formic acids. Succinic and formic acid were in the predominant concentrations in these filtrates (284 and 367 ppm in Day 2-1 filtrate, and 524 and 290 ppm in Day 2-2 filtrate). The citric acid concentrations were also substantial in Day 2 filtrates (141 and 119 ppm). The lead extraction by these apple pomace filtrates was the highest among all *A. niger* ATCC 64065 filtrates, with 6.5 and 4.6 ppm extracted by the Day 2-1 and Day 2-2 filtrates, respectively.

The organic acid content of the Day 5-1 and Day 5-2 apple pomace filtrates were similar to that of the Day 2-1 and Day 2-2 apple pomace filtrates. Oxalic acid was the only acid that was not present in the Day 2 filtrates, whereas Day 5 apple pomace filtrates had 112 and 90 ppm of oxalic acid, respectively. As it can be seen from Figure 4-31, the citric, succinic and formic acid concentrations of Day 5 filtrates were lower than those of the Day 2 filtrates. Thus it would appear that the lower organic acid concentrations and the presence of oxalic acid in the Day 5 apple pomace filtrates was the reason for the reduced lead extraction.

Rice

Organic acid content of the rice filtrates and the extracted lead concentrations in these filtrates are demonstrated in Figure 4-32. Organic acids were not detected in the rice filtrates from Day 2, and lead was not significantly extracted by these filtrates (Figure 4-32). The rice filtrate of Day 5-1, which had some traces of oxalic and citric acid, extracted 0.5 ppm of lead. The Day 5-2 filtrate, with negligible acids in it extracted 0.1 ppm of lead. Despite the presence of 339 ppm of citric acid in the Day 8-1 rice filtrate, this filtrate extracted only 0.9 ppm, and the same amount was extracted by Day 8-2 filtrate with only 50 ppm of citric acid. In general it can be observed that the lead extraction by *A. niger* ATCC 64065 rice filtrates was negligible as compared to the extraction by the *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 rice filtrates (Figures 4-19 and 4-24).

Fig. 4-32: *A. niger* ATCC 64065 generated organic acid concentrations (a) in the rice filtrates vs. the extracted lead concentrations (b).

Hay

Figure 4-33 presents the organic acid content of the hay filtrates and the lead concentrations extracted by these filtrates. As it can be seen from Figure 4-33, oxalic acid was only the acid detected in the hay filtrates, and the lead extraction was very low. Negligible oxalic acid was detected in the Day 2 filtrates, however the amount of lead extracted was similar to that extracted by the Day 5 filtrates that had 38 and 39 ppm of oxalic acid in them. It can be concluded that small amounts of oxalic acid do not affect

lead extraction. The Day 8 filtrates extracted slightly higher amounts of lead (0.5 and 0.4 ppm, respectively). However it was not clear if the oxalic acid concentrations in these filtrates (77 ppm) affected lead extraction as the extracted lead concentrations were very low.

Fig. 4-33: *A. niger* ATCC 64065 generated organic acid concentrations (a) in the hay filtrates vs. the extracted lead concentrations (b).

The main conclusion that can be made for this section is that during *A. niger* ATCC 64065 fermentation of the solid substrates some unidentified metabolites were excreted.

These metabolites that were present in the fungal filtrates affected the lead leaching from the soil. Therefore, even though the organic acid concentrations of *A. niger* ATCC 64065 filtrates were similar or higher than those observed in the *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 filtrates, the lead extraction was much lower in the case of *A. niger* ATCC 64065 filtrates. For example, the lead extraction by *A. niger* ATCC 64065 rice filtrate of Day 8-1 (Figure 4-32) was only 0.9 ppm despite 339 ppm of citric acid being present in this filtrate.

4.3.4 *A. aculeatus* NRRL 358

As has been discussed in section 4.2.1, the growth of *A. aculeatus* NRRL 358 on the solid substrates was distinguished by the formation of substantial quantities of fungal mycelium with very poor sporulation. The fungal growth on all tested substrates was stopped after six incubation days as the substrates became contaminated from the ambient air by *A. niger* NRRL 2001, *A. foetidus* NRRL 337 and some unknown microorganisms. The contamination of the substrates demonstrated that *A. aculeatus* NRRL 358 was not a suitable fungal strain for the fermentation of these solid substrates. *A. aculeatus* NRRL 358 filtrates of Days 2, 5 and their duplicates were collected, and the batch metal extraction tests were performed using these filtrates.

Figure 4-34 presents the lead extraction by *A. aculeatus* NRRL 358 filtrates that were generated on different solid substrates. It is evident from Figure 4-34 that the filtrates from the *A. aculeatus* NRRL 358 fermentation had very low capabilities for lead extraction. The concentration of lead that was extracted by the apple pomace filtrate from Day 5-1 was 3.7 ppm, and this was the best extraction performed by the *A. aculeatus* NRRL 358 filtrates. The apple pomace filtrates of Days 2-1, 2-2 and 5-2 extracted 2.4, 2.7 and 2.7 ppm of lead, respectively. The corncob filtrates from Day 2-1 and Day 2-2 extracted 1.3 and 1.8 ppm, respectively. The extraction with Day 5-1 and Day 5-2 corncob filtrates were similar to the control extraction, with only 0.6 and 0.2 ppm detected in these filtrates. The rice and hay filtrates did not extract any lead as it can be seen from Figure 4-34.

Fig. 4-34: Extraction of lead using organic acids generated by *A. aculeatus* NRRL 358.

The pH values of the soil solutions for the batch metal extraction tests were not available for this section. Hence the following conclusions about the lead extraction are based only on the organic acid content of *A. aculeatus* NRRL 358 filtrates. Organic acids were not detected in the rice and hay filtrates, which agrees with the extraction data of Figure 4-34 as no lead either was extracted by these filtrates. Figures 4-35 and 4-36 present the organic acid content of corncob and apple pomace filtrates, respectively and the lead concentrations extracted by these filtrates.

Corncobs

As it can be seen from Figure 4-35, citric and malic acid were detected in the corncob filtrates of Day 2-1 and Day 2-2 in substantial quantities. The Day 2-1 filtrate contained 58 and 129 ppm of citric and malic acid, respectively, while Day 2-2 corncob filtrate contained 106 and 262 ppm of citric and malic acid, respectively. However the lead extraction by these filtrates was very low; 1.3 and 1.8 ppm of lead were extracted by the Day 2 filtrates. The Day 5 corncob filtrates contained 76 and 74 ppm of oxalic acid, respectively, and smaller amounts of citric and malic acids. These filtrates did not leach out significant quantities of lead.

Apple pomace

Figure 4-36 demonstrates that the main organic acid in the Day 2 apple pomace filtrates was succinic acid. The Day 2-1 filtrate had 412 ppm of succinic acid, while Day 2-2 contained 511 ppm. Small amounts of citric and tartaric acid were also present in these filtrates (Figure 4-36). Despite high concentrations of succinic acid, the Day 2-1 and 2-2 filtrates extracted only 2.4 and 2.7 ppm of lead, respectively. The Day 5-1 and Day 5-2 apple pomace filtrates had much smaller concentrations of succinic acid (129 and 61 ppm, respectively) and traces of oxalic, citric and tartaric acid. The Day 5-1 apple pomace filtrate extracted the highest concentration of lead among the *A. aculeatus* NRRL 358 filtrates. This could not be explained based on the organic acid content of the filtrates.

Fig. 4-35: *A. aculeatus* NRRL 358 generated organic acid concentrations (a) in the corncob filtrates vs the extracted lead concentrations (b).

Fig. 4-36: *A. aculeatus* NRRL 358 generated organic acid concentrations (a) in the apple pomace filtrates vs the extracted lead concentrations (b).

It can be concluded that the lead extraction by *A. aculeatus* NRRL 358 filtrates was very poor, and did not correlate with the organic acid concentrations in the filtrates as some acids were present in quite high concentrations. This was similar to the extraction by *A. niger* ATCC 64065 filtrates (Section 4.3.3), where it has been suggested that there may be some unidentified metabolites of the fungal fermentation present in the filtrates that were repressing the lead extraction from the soil.

4.3.5 Selection of the best strain and substrate

The lead extraction by the filtrates of different fungal strains generated on each solid substrate was compared in this section in order to select the best fungal strain and the most suitable substrate for the further soil column studies. Figure 4-37 presents the lead extraction by the corncob filtrates of the four different fungal strains.

Fig. 4-37: Lead extraction by the corncob filtrates of different fungal strains.

When the lead extraction by the filtrates collected during corncob fermentation by four fungal species is compared it can be seen that the best extraction was performed by *A. foetidus* NRRL 337 filtrate for Day 2-1. This filtrate extracted 6.5 ppm of lead. *A. foetidus* NRRL 337 filtrates from Days 8-1 and 8-2 also extracted high concentrations of

lead (3.3 ppm) when compared to the extraction by the other fungal strain filtrates generated on the corncobs. *A. niger* NRRL 2001 filtrates as it can be seen from Figure 4-37 achieved a slightly higher extraction than *A. aculeatus* NRRL 358 and *A. niger* ATCC 64065 filtrates, which demonstrated very poor extraction capacity.

Figure 4-38 demonstrates the extraction of lead by the apple pomace filtrates with the different fungal species.

Fig. 4-38: Lead extraction by the apple pomace filtrates of different fungal strains.

Among the apple pomace filtrates, the highest extraction was achieved by *A. foetidus* NRRL 337 filtrates from Day 2-1 and Day 5-1 (7.8 and 7.7 ppm, respectively) and by *A.*

niger NRRL 2001 filtrate of Day 2-1 (7.8 ppm). The lead extraction by *A. foetidus* NRRL 337 and *A. niger* NRRL 2001 filtrates was in a similar range (Figure 4-38). *A. niger* ATTC 64065 filtrates had comparable extraction with some of *A. foetidus* NRRL 337 and *A. niger* NRRL 2001 filtrates in Days 2-1 and 2-2, with 6.5 and 4.6 ppm of lead extracted, respectively. *A. niger* ATTC 64065 filtrates from Days 5 and 8 did not leach out any lead, whereas *A. aculeatus* NRRL 358 with Days 2 and 5 extracted lead in the range of 2.4-3.7 ppm. However these amounts were low as compared with the extraction by *A. foetidus* NRRL 337 and *A. niger* NRRL 2001 filtrates.

The lead extraction by the rice filtrates from the different fungal strains is presented in Figure 4-39. As it can be clearly seen from Figure 4-21, the highest lead extraction was achieved with *A. foetidus* NRRL 337 rice filtrates for Day 8-1 and Day 8-2, when 36 and 34 ppm of lead were extracted, respectively. This was followed by *A. niger* NRRL 2001 rice filtrate extraction with Days 8-1 and 8-2, with 33.9 and 24.2 ppm, respectively. In addition, the *A. foetidus* filtrates from Day 5 extracted higher amounts of lead (5.7 and 6.9 ppm, respectively) in comparison with the rest of rice filtrates of the same day. *A. niger* ATCC 64065 and *A. aculeatus* NRRL 358 rice filtrates did not demonstrate any lead extraction capacity during the batch metal extraction tests (Figure 4-39).

Fig. 4-39: Lead extraction by the rice filtrates of different fungal strains.

Figure 4-40 presents the extraction of lead by the hay filtrates from the four fungal species. As it is demonstrated in Figure 4-40, the hay filtrates of all fungal species performed very poorly with respect to lead extraction. *A. foetidus* NRRL 337 and *A. niger* NRRL 2001 hay filtrates achieved higher extraction than *A. niger* ATCC 64065 and *A. aculeatus* NRRL 358 filtrates. *A. foetidus* NRRL 337 hay filtrate of Day 8-1 extracted the highest amount of lead, which was 2.0 ppm.

Fig. 4-40: Lead extraction by the hay filtrates of different fungal strains.

Through the general comparison of the lead extraction by the filtrates of four fungal strains generated on different solid substrates, it can be clearly seen that the fungal filtrates generated during *A. foetidis* NRRL 337 fermentation of rice performed the best with respect to lead extraction. As discussed in section 4.3.2, citric acid was the main acid in the *A. foetidis* NRRL 337 rice filtrates. Therefore, *A. foetidis* NRRL 337 and rice were selected as a fungal strain and solid substrate, respectively, for the soil column studies.

4.4 Soil column testing

After the evaluation of fungal growth and organic acids production by the different fungal strains on the solid substrates, and the batch metal extraction experiments, *A. foetidus* NRRL 337 and boiled white rice were selected for soil column studies. The purpose of the soil column tests was to further evaluate the performance of the organic acid-containing flushing filtrates in soil columns that simulated in situ washing or a soil pile-leaching process. The impact of process variables on the leaching of lead in the soil columns was investigated in detail in this portion of the study.

4.4.1 Part 1

In this part of the soil column studies, the impact of soil column depth and fresh substrate addition, on the extraction of lead by fungal generated organic acids was investigated. Visual observations of *A. foetidus* NRRL 337 growth on the rice, the organic acid content and the pH of the rice filtrates and the soil column leachates, and the lead concentrations in the leachates were monitored during this part of the soil column studies.

A mass of 80 g (wet mass) (for each column) of boiled Rice A with a moisture content of 65% was placed in the support apparatus at the top of the columns, and inoculated with 1 ml of the fungal spore suspension (about 2×10^6 spores/ml). Columns 1 to 4 were loaded with 20, 40, 60, and 80 g of the contaminated air-dried soil, respectively. The inoculated rice was flushed daily with 50 ml of DI water. After 13 fermentation days, an additional mass of 50 g of boiled Rice A was added to each column and mixed with the

old rice layer with the fungus growing on it, and then flushing was continued until the end of this run (28 fermentation days in total).

Fungal growth

The growth of *A. foetidus* NRRL 337 on boiled white rice was described in Table 4-2 (Section 4.2.1). The main difference between Exp. 22 and the growth on the top of the soil columns was that the boiled rice was not sterilized prior to inoculation in the soil column studies. Hence the moisture content of the substrate was lower (about 65%) than in Exp. 22 (about 85%) as the sterilization was performed with addition of DI water to the boiled white rice in order to avoid its burning. Also, in comparison with an eight-day incubation period in Exp. 22, in this portion of the study the fungus was fermented on the substrate for 28 days. In addition, all soil column tests were conducted at a room temperature of 19 ± 1 °C, while the room temperature during the batch tests was 25 ± 1 °C. As reported by Hang and Woodams (1998), incubation temperature was found to have a profound influence on fungal fermentation.

The following aspects of *A. foetidus* NRRL 337 growth at the top of the soil columns can be highlighted:

- in comparison with Exp. 22, where the mycelium covered the rice completely at first, and then sporulation occurred, the mycelium formation was not as flourishing for the column study. After four incubation days, several yellow mycelium spots covered by black spores were observed on the rice surface;
- with an increase in incubation time, an increase in fungal sporulation was observed with no mycelium observed;

- when the fresh rice layer was added and mixed with the old rice, it was observed that the old rice was very wet, and contained spores throughout;
- after rice addition, substantial quantities of new fungal mycelium formed on the rice surface, but sporulation was very poor on the new formed mycelium over the remainder of the experimental run (28 incubation days).

As reported by Rimbault (1998), the moisture level in SSF processes has a clear effect on microorganism growth kinetics. Based on the visual observations described above, it may be suggested that the moisture content of a solid substrate below the optimum level resulted in an increased fungal sporulation with low mycelium formation. Rimbault (1998) also noted that typically a reduction in a moisture level decreases the specific growth rate, and results in a low amount of biomass produced. The high moisture content of the rice increased the mycelium formation, but repressed the fungal sporulation.

pH

The pH of the rice filtrates was taken as a factor indicating the production of organic acids by the fungus. Figure 4-41 depicts the pH values of the rice filtrates that were measured before the filtrates entered the soil columns.

Fig. 4-41: pH of the rice filtrates in Part 1.

It can be seen from Figure 4-41 that the changes in the pH values were very similar for all four columns as the culture conditions were the same in these tests. The pH of the flushing filtrates started decreasing after 4 incubation days to the minimum pH value of 2.7-2.8 on the 6th incubation day. After the addition of the fresh rice on the 13th incubation day, the pH increased over a period of 5-6 days to about 3.5-3.8 in columns 1, 2 and 4, and to 4.4 in column 3. This was likely due to the buffering capacity of the fresh rice. The filtrate pH then slightly decreased in all the columns, and in 3 days it reached values of 3.0-3.2. After this time, a rapid increase in the pH was observed in all columns. At the end of the tests, the flushing filtrates had pH values in the range of 6.5-7.2.

Fig. 4-42: pH of the soil column leachates in Part 1.

Figure 4-42 presents the pH of the soil column leachates that were collected daily at the bottom of the soil columns. Soil depths of 60, 120, 180 and 240 mm were employed in columns 1, 2, 3 and 4, respectively. It can be seen from Fig. 4-2 that in general, there was no correlation between the column depths and pH of the leachates. Only column 4 had consistently higher pH values for the leachates as compared to the other three columns. This was observed through the whole experimental run, and may be due to the greater soil depth (240 mm) that provided increased buffering. The pH changes of the soil column leachates had relatively similar patterns for all columns. A decrease in the leachate pH was observed through the first 10 days of the tests, which corresponded to the decrease in the pH of the rice filtrates (Figure 4-41). In columns 2 and 3, the pH of the leachates decreased after 10 days to 5.0 and 4.8, respectively, while in columns 1 and 4 the pH decreased to about 5.3 after 11 days. The leachate pH started rapidly increasing after that, though the pH of the filtrates entering the columns was still decreasing (Figure

4-41). It can be speculated that though the pH of the filtrates was still low, the organic acid concentrations in the filtrates were decreasing, which resulted in an increase of the leachate pH values. On the 14th experimental day, the leachate pH values increased to 6.9, 6.1, 6.6 and 7.1 in columns 1, 2, 3 and 4, respectively. The pH values then decreased again to values of 5.3, 4.5, and 6.4 in columns 1, 2 and 4, respectively, on the 18th day, and on the 19th day the pH of column 3 was 5.3. This was probably due to the new rice addition on the 13th day, which likely resulted in new fungal growth and organic acid production. Thus it may be speculated that organic acid concentrations were increasing for a while in the flushing filtrates after day 14. After the 18th day, the pH values increased to final values of 6.6, 6.7, 5.8 and 7.8 in columns 1 to 4, respectively, which likely related to the increase in pH values of the rice filtrates (Figure 4-41).

Lead extraction

The soil column leachates were collected on a regular basis, and analyzed by FAAS for the lead concentration. Figure 4-43 presents the lead concentrations that were measured in the leachates collected during the 28-day soil column leaching tests.

As can be seen from Figure 4-43, the columns had similar trends with respect to lead leaching, with two peaks of lead extraction on the 11th day for all columns and the 18th day. The second peak was higher for all the columns except for column 3. Among the columns, column 2 had the highest peaks, where 17.4 mg/L of lead was detected in the column leachate on the 11th day, and 20.4 mg/L was detected on the 18th day. Column 3 had the lowest lead extraction through the entire experimental run. The highest lead concentration detected in the collected leachates from this column was 9.6 mg/L on the

11th day, whereas the second peak on the 19th day was just 5.9 mg/L as compared to the second peaks of columns 1, 2 and 4, which were 19.2, 20.4 and 15.3 mg/L, respectively. It was concluded that the lead leaching in these columns was directly correlated to the pH values of the leachates as the peaks of lead extraction (Figure 4-43) happened around the same time when the leachate pH had its lowest values (Figure 4-42).

Fig. 4-43: Leaching of lead in the soil columns in Part 1.

The following subsections address the interaction between the pH of the column leachates, the lead concentrations detected in these leachates and the organic acid content of the rice filtrates flushed into the columns for each column individually.

Column 1

Figures 4-44 presents the pH of the column leachates and amounts of lead detected in these leachates for column 1.

Fig. 4-44: Pb extraction and pH of the leachates in column 1.

A clear relationship can be observed from Figure 4-44 between the extracted lead concentrations and pH of the leachates. The highest concentrations of lead were detected in the leachates with the lowest pH, and with an increase in the leachate pH, the amounts of extracted lead decreased. As can be seen from Figure 4-44 and has been discussed earlier, the highest concentrations of lead for column 1 was detected in the leachate from the 18th experimental day (19.2 mg/L), which had the lowest pH value of 5.3. Also on days 10-12 and 19, higher concentrations of lead were leached out, with concentrations of 6.6, 10.9, 5.3 and 14.6 mg/L, respectively. The leachates from these days had lower pH values as well (Figure 4-44). Some exceptions from the general trend can be observed in Figure 4-44. For example, on the 7th day the leachate pH was 6.9 and on the 20th day it was 5.6. However more lead was extracted (4.4 mg/L) on day 7 than on day 20 (2.2 mg/L). It can be speculated that readily available lead may have already been extracted

before day 20 and therefore was not available on day 20 as much as on day 7. Therefore it can be concluded that though the pH of the leachates strongly influences the lead extraction, it is not only the parameter, which affects metal leaching from a contaminated soil.

The organic acid content of the rice filtrates and soil leachates was monitored during the soil column study in order to examine the behaviour of organic acids inside the soil columns, and the effect of organic acids on lead leaching from the columns. Figure 4-45 presents the organic acid content of the rice filtrates and soil leachates, and lead concentrations in column 1. From Figure 4-45a it can be seen that citric and gluconic acid were present in the flushing filtrates from the 4th to the 10th experimental day, and citric acid was in predominant concentrations. From day 11 to 13, only citric acid was detected in the filtrates, and no organic acids were detected in the filtrates on the following days.

Gluconic acid was the main acid observed in the soil leachates, and it was present in much higher concentrations than it was detected in the filtrates (Figure 4-45b). It has been mentioned by Zidwick (1992) that the major differences between citric and gluconic acid fermentation lie in the pH of the fermentation. When the pH values are in the range of 2.5-3.5, citric acid is produced, and for pH values greater than 5, gluconic acid is produced. In addition, citric acid production is shut down by excess manganese, while gluconic acid production is stimulated by its presence. Therefore, one may speculate that when the flushing filtrates, which likely contained fungal spores and rice starch, entered the soil column, gluconic acid production was stimulated due to the high pH (> 5) within

the column. It may also be possible that the high manganese concentration (193 $\mu\text{g/g}$) of the soil (Table F-1) supported the gluconic acid production.

Succinic acid was produced inside the column as well, and was detected in the leachates on the 10th and 18th days (131 and 145 mg/L, respectively) (Figure 4-45b). It may be suggested that the pH inside the soil column was more suitable for the production of succinic acid than the rice pH as no succinic acid could be seen in the rice filtrates.

It is interesting to note that citric acid was absent in the leachates collected on days 6 through 14, though the filtrates, which corresponded to these leachates, had quite high concentrations of citric acid (Figures 4-45a and 4-45b). It may be that citric acid was biodegraded within the column, and therefore the acid could not be detected in the leachates. On the other hand, 809 mg/L of citric acid was detected in the leachate collected on 18th day, which corresponded to the filtrate flushed to the column 4 days earlier where no citric acid was detected. One may speculate that as day 18 was a low pH time in the soil (Figure 4-44), citric acid formed in the column soil rather than gluconic acid.

No clear trend could be seen between lead extraction (Figure 4-45c) and organic acid concentrations entering the column (Figure 4-45a). The highest citric acid concentration (898 mg/L) was in the filtrate on the 6th day, while the highest lead extraction was observed on days 18 and 19 when no organic acids were detected in the corresponding filtrates. It may be suggested that perhaps the lead was leaching downwards at some rate that did not correspond to the movement of the liquid.

Fig. 4-45: Organic acid concentrations in flushing filtrates (a) and leachates (b), and Pb concentrations (c) in column 1.

Column 2

Figures 4-46 presents the pH of the column leachates and concentrations of lead detected in these leachates for column 2. The organic acid content of the rice flushing filtrates and leachates, and leachate lead concentrations for column 2 are presented in Figure 4-47.

Fig. 4-46: Pb extraction and pH of the leachates in column 2.

As demonstrated in Figure 4-43, column 2 had the highest lead concentrations observed in the columns, with peaks on days 11 and 18. It can be seen from Figure 4-46 that the leachate on day 18 had the lowest pH value of 4.5, which was also the lowest pH among all leachates collected from the four soil columns (Figure 4-42). Though the leachate pH on day 11 (5.5) was higher than that of day 10 (5.0), slightly more lead was extracted on day 11 than on day 10. The leachate pH on day 13 was slightly lower than

that of days 11 and 12, while the concentration of lead extracted was slightly smaller on the 13th day. In general it could be concluded that an increase in the leachate pH resulted in a decrease in the concentration of the extracted lead.

Figure 4-47 presents the organic acid content of the rice filtrates and soil leachates, and lead concentrations in column 2. As it can be seen in Figure 4-47, the organic acid content of the filtrates and leachates of column 2 were similar to that of column 1. Citric and gluconic acid were detected in the filtrates with citric acid in the predominant concentrations. Gluconic acid was the main acid in the soil leachates, and citric and succinic acid were also detected in the soil leachates. Little citric acid was produced by the fungus in column 2 during the period from days 4 to 10. Overall, production of citric acid in column 2 was lower than that of column 1, however the extracted lead concentrations were higher in column 2 (Figure 4-47c). A possible explanation is the higher gluconic acid concentrations in the leachates of column 2 for days 4 to 11 in comparison with column 1. The higher gluconic acid production inside the soil of column 2 could possibly have resulted in the lower pH within the column (Figure 4-42), and subsequently in higher lead extraction. In addition, it needs to be pointed out that the best lead extraction was achieved on day 18 that corresponded to the filtrate of day 14 and the leachate of day 18. Both samples had very high citric acid concentrations (1914 and 1550 mg/L in the filtrate and leachate, respectively).

Fig. 4-47: Organic acid concentrations in flushing filtrates (a) and leachates (b), and Pb concentrations (c) in column 2.

Column 3

Figure 4-48 presents the pH of the column leachates and the concentrations of lead detected in these leachates for column 3. The organic acid content of the rice filtrates, soil leachates, and lead concentrations from column 3 are presented in Figure 4-49.

Fig. 4-48: Pb extraction and pH of the leachates in column 3.

The general trends that were discussed for columns 1 and 2, also can be seen in Figure 4-48 for column 3. When the pH values of the leachates were in the range of 4.7-5.7, higher lead extraction was observed.

Figure 4-49 presents the organic acid content of the rice filtrates and soil leachates, and lead concentrations in column 3.

Fig. 4-49: Organic acid concentrations in flushing filtrates (a) and leachates (b), and Pb concentrations (c) in column 3.

The lead concentrations were the lowest in column 3 among the columns (Figure 4-43), whereas the soil leachate pH in this column was in the same range with the rest of the columns (Figure 4-42). The lead extraction in column 3 before day 15 was quite close to that of column 1 (Figure 4-43), while after day 15 the extraction of lead was much lower in comparison with the other columns. It may be explained by examining the organic acid concentrations in the filtrates and leachates of column 3 as presented in Figure 4-49. As can be seen from this figure, the organic acid content of the filtrates was low. Significant concentrations of citric and gluconic acid were only observed in the filtrates on days 5 and 14 (Figure 4-49a). The leachates contained some gluconic acid on days 5 to 10 that probably resulted in the extraction of lead.

Column 4

Figure 4-50 shows the pH of the column leachates and concentrations of lead detected in these leachates for column 4. Though the highest lead concentration in column 4 was observed on day 18 (15.3 mg/L), the leachate pH at this day was not the lowest (6.4) (Figure 4-50). Days 11 and 12, which also had high lead concentrations corresponded to the lowest pH values for this column, with values of 5.32 and 5.28, respectively.

Fig. 4-50: Pb extraction and pH of the leachates in column 4.

The organic acid content of the rice filtrates and leachates, and leachate lead concentrations measured for column 4 are demonstrated in Figure 4-51. The trends in organic acid production in column 4 (Figure 4-51) were similar to column 2 (Figure 4-47). Though the organic acid concentrations in column 4 were similar and even higher than that of column 2, the extracted lead concentrations were lower in column 4 (Figure 4-43). This can be explained as it has been mentioned before by the higher leachate pH values in this column in comparison with the others (Figure 4-42). This was possibly due to the greater column depth of column 4.

Fig. 4-51: Organic acid concentrations in flushing filtrates (a) and leachates (b), and Pb concentrations (c) in column 4.

Based on the organic acid production for all soil columns it can be concluded that the rice addition on the 13th incubation day resulted in a rapid citric acid production on the next day. This was followed by a complete cessation of organic acid production. The increased organic acid production on the 14th day may also have been due to the mixing of the substrate when the new rice was added that allowed better flushing of the organic acids from the rice.

Cumulative lead extraction

Figure 4-52 presents cumulative removal of lead from the soil columns. A mass of 4 mg of lead was removed from column 2, which demonstrated the highest extraction level in this part of the soil column studies. Column 3 had the lowest extraction (1.9 mg). In columns 1 and 4, 3.3 and 2.8 mg were extracted, respectively.

Fig. 4-52: Cumulative lead removal in soil column tests.

The extraction efficiency of lead in the columns was calculated as the ratio of the cumulative lead extracted after 28 days to the total amount of lead in the 20, 40, 60 and 80 g of soil for columns 1 to 4, respectively. The results showed that after 28 leaching days, only 1.2, 0.7, 0.2 and 0.3 % of the lead were extracted from the soil in columns 1, 2, 3 and 4, respectively.

Overall it was concluded that the pH of the leachates and the organic acid content of the filtrates and leachates had a profound effect on lead extraction from the columns. The leachate pH influenced the lead extraction to a greater extent than the organic acid content. However the pH of the leachates was directly correlated to the organic acid content of the rice filtrates flushed to the columns and the leachates. It was demonstrated in column 3, where low leachate pH did not result in substantial lead extraction due to the poor organic acid content of the filtrates and leachates.

Though all columns had the same culture conditions for fungal rice fermentation, the quantities and composition of acids produced differed from column to column demonstrating, as it has been mentioned in many literature reviews, how difficult it is to control fungal fermentation.

The effect of column depth on lead leaching was demonstrated in column 4. The cumulative organic acid production in column 4 was the highest among the columns, but due to the buffering effect of the increased quantity of soil in the deepest column depth, the pH of the leachates increased. This column performed more poorly with respect to lead extraction than columns 1 and 2 (Figure 4-52).

The impact of different organic acids on lead leaching was demonstrated in columns 1, 2 and 4, when the highest extracted lead concentrations were detected in the soil leachates with high citric acid concentrations. The leachates of column 3 had mainly gluconic acid and this column had the lowest lead extraction.

The acid transformation in the soil was clearly demonstrated in this part of the soil column studies. The main acid of the rice filtrates was citric acid, whereas gluconic acid was in predominant concentrations in the soil leachates. It would appear that either citric acid degraded to gluconic acid inside the soil or gluconic acid was generated in soil as the soil pH favored gluconic acid production.

4.4.2 Part 2

The impact of quantity of substrate, substrate type, substrate addition and frequency of flushing on the leaching of lead from a real field soil by fungal generated organic acids was tested in Part 2 of the soil column studies. Four soil columns with different quantities and types of rice in the support apparatus at the top of the columns were evaluated. Column 5 had 200 g of boiled Rice B with a moisture content of 55%. Column 6 was loaded with 200 g of boiled Rice A with a moisture content of 65%. Column 7 had 200 g of boiled Rice C (Rice A mixed with 1.5 g of Styrofoam), and column 8 was loaded with 400 g of boiled Rice A. After 35 incubation days, an additional rice layer was added on the top of the old layer without mixing. 100g of boiled Rice B was added to column 5, 100 g of boiled Rice A was added to column 6, 100 g of boiled Rice C was added to column 7, and 200 g of boiled Rice A was added to column 8. The new rice layers were inoculated with a freshly prepared spore suspension. The evaluation of columns 5, 6 and 7 was stopped after 61 experimental days, while column 8 assessment was continued. After 79 incubation days, a new rice layer (400 g of boiled Rice A) was added (no mixing) to column 8, and then it was inoculated with a freshly prepared fungal spore suspension. Column 8 was flushed until the 101st experimental day. The detailed description of the experimental procedures employed in the soil column studies is presented in sections 3.1.4 and 3.3.4 of Chapter 3 (Experimental and Analytical Methods).

Fungal growth

As all four soil columns had different culture conditions for fungal growth in Part 2, the following presents the separate assessment of fungal growth of each soil column. After the rice in column 5 was inoculated by *A. foetidus* NRRL 337 and incubated for three days, several spots of bright yellow mycelium with small black spores could be seen on the rice surface. With an increase in incubation time, the rice became completely covered by the mycelium with a very rich fungal sporulation. During the first 35 incubation days, manual mixing of the rice was conducted, and by mixing it could be seen that the rice was in very good condition with no smell indicating spoilage and with each rice grain separate. The fungal growth was observed to be throughout the entire rice depth. After the new rice layer was added and inoculated by a freshly prepared spore suspension, the rice became completely covered by yellow mycelium in several days, while the spore formation on this mycelium was quite poor in comparison with the first part of the incubation period (before the rice addition).

The fungal growth in column 6 was similar to that of column 5 for the first incubation week, with several spots of fungal mycelium developed with black spores on them. With an increase in incubation time, the mycelium did not develop as much as in column 5, while flourishing sporulation could be observed. It was noticed after several incubation days during the manual mixing of the rice in column 6 that the rice spoiled rapidly. After one week of incubation, the rice had a mucous like appearance and had a strong mouldy smell. Most probably this was the cause of the weak mycelium formation on the rice in column 6. When the rice was mixed, it could be seen that the fungus was growing only on the rice surface as the top layer of rice was less moist and had better aeration. Before

addition of the new rice layer (35 incubation days), substantial quantities of fungal mycelium and spores had formed in column 6 despite the unfavorable rice condition. When the new rice layer was added, new mycelium developed on the surface but less than in column 5, where the mycelium covered the whole surface. The sporulation was much higher in column 6 than in column 5 after the rice addition. A white fuzz with black *A. foetidus* spores developed on the rice of column 6.

In column 7 as it has been mentioned above, the same rice was used as in columns 6 and 8 however Styrofoam was added. The rice of column 7 became spoiled almost immediately after inoculation. After seven incubation days, only a few small spots of fungal mycelium with black spores were developed on the rice surface. The fungal growth was very poor in column 7. After 35 incubation days only several mycelium spots could be seen. The rice appeared more spoiled in column 7 than in column 6 which may have been due to the Styrofoam addition, though the Styrofoam was applied in order to enhance air flow through the rice surface. After addition of a new rice layer and inoculation by a freshly prepared spore suspension, the fungal growth in column 7 was as poor as before. Several spots of yellow mycelium and white fuzz with spores on it developed, while the new rice layer became spoiled again very quickly.

Column 8 had the same rice as column 6, but the amount was doubled. After three days of incubation, the rice surface had more fungal mycelium than in columns 5 and 6, but no spores could be seen on this mycelium. One can speculate that this difference in the fungal sporulation could be due to less oxygen uptake as the larger amount of rice was loaded into the same size apparatus in comparison with the other columns. However after a week of incubation the fungal growth and the rice condition in column 8 became

similar to that of column 6. After the first rice addition, a yellow mycelium developed with a number of black spores that were surrounded by a white fuzz. After the second rice addition, no fungal growth could be seen on the rice surface. The rice became contaminated by various microorganisms from the ambient air after several days.

It can be concluded from the visual observations discussed above that even minor differences in the culture conditions can affect the fungal growth. This was visibly demonstrated in column 7, when the addition of Styrofoam resulted in the fungal growth of the column becoming very different from that of columns 6 and 8, though the same rice was applied in all three columns.

pH

The pH of the rice filtrates was recorded after each flushing, and was considered as a parameter indicating the organic acid production by the fungus. Figure 4-53 presents the pH of the rice filtrates, which was measured before the filtrates entered the soil columns.

Fig. 4-53: pH of rice filtrates in Part 2.

It can be seen from Figure 4-53 that the pH of the filtrates from all the columns started decreasing after 5 incubation days and remained similar for 10 days. The pH of column 5 filtrates reached a minimum value (2.7) on the 12th incubation day, while the pH of column 6 was a minimum on the 14th day (2.7). The column 7 filtrates had the lowest pH value (2.9) on the 26th incubation day. As it can be seen from Figure 4-53, when the pH of column 8 filtrates reached a value of 3.7 on the 14th incubation day, it remained in the same range (3.5-3.9) until the 75th experimental day, when the second rice addition was applied. The pH of column 8 filtrates increased to 5.2 on the 82nd day and then decreased to 4.1 on the last experimental day. The pH changes of columns 5 and 6 were similar. After reaching their minimum (days 13 and 14), the pH values of the column 5 and 6 filtrates started climbing and reached their highest values of 7.8 and 7.6, respectively, on the 35th incubation day. As the new rice layer was added and freshly inoculated on the

35th incubation day, the new fungal growth resulted in the pH of the rice filtrates decreasing over 10 days to 2.9 on the 45th day. It then increased again, and reached 8.4 and 8.1 for columns 5 and 6, respectively, at the end of the experimental run. The pH of column 7 filtrates increased to 6.4 for the first time on the 39th day, and then it decreased to 5.0 on the 45th day, and then climbed again. At the end of the fermentation, the pH of column 7 filtrate reached 8.3.

It was concluded that columns 5-7 had similar pH fluctuation pattern for their filtrates. The rice addition on the 35th day resulted in a decrease in the pH values of the filtrates for a short period, though the pH values of the column 7 filtrates did not decrease as much as that of columns 5 and 6. Column 8 filtrates had the lowest pH values throughout the whole experimental run with a very low fluctuation in them. The first rice addition on the 35th day did not affect much the filtrate pH, while the second rice addition on the 75th day increased the pH values to about 5.2 (Figure 4-53). As column 8 was only the column with the doubled rice quantity (400 g), it can be concluded that this was the cause of the low pH values of the column 8 filtrates. It can be speculated that due to the doubled rice quantity, the fungus had more nutrients available for a longer organic acid production than in columns 5-7.

Figure 4-54 depicts the pH of the soil column leachates that were collected every other day at the bottom of the soil columns.

Fig. 4-54: pH of the soil column leachates in Part 2.

It is demonstrated in Figure 4-54 that there was a substantial fluctuation in the pH values of the leachates collected from all four columns. It was difficult to observe a trend with respect to the pH changes, but in general the pH values of the leachates decreased during the first 25 experimental days, which corresponded to the decrease in the pH of the rice filtrates. The pH of columns 5 and 6 leachates reached minimum values of 4.8 and 5.2, respectively, on the 25th day, while the pH of columns 7 and 8 leachates had minimum values of 4.1 and 3.8, respectively, on the 17th day. As columns 6 and 7 became completely plugged after 9 experimental days, these columns were loaded with a new soil on the 10th day, therefore the increase in the pH of these column leachates around 13th leaching day (Figure 4-54) can be explained by the new soil buffer.

It can be seen from Figure 4-54 that the pH values of the column 8 leachates were the lowest and much less fluctuating in comparison with the other columns. This trend

corresponded to the pH values of the column filtrates (Figure 4-53). However the pH of column 8 leachates had more substantial changes as compared to that of the filtrates. As demonstrated in Figure 4-54, after reaching its minimum on the 17th day, the pH of column 8 leachate started increasing, and reached a value of 5.1 on the 39th day. The pH then decreased to 3.4 on the 47th day. This could be explained by the rice addition on the 35th day that probably caused the production of additional organic acids. The pH values of the rice filtrates of column 8 had also a small decrease from days 35 to 45 as presented in Figure 4-53. The leachate pH then started increasing again, but the pH increase was less rapid; from day 47 to 82 the pH increased from 3.9 to 5.2. On the 88th day the pH rapidly increased to 8.3, which corresponded to the increase in the filtrate pH of day 82 (Figure 4-53). The pH of the leachates then decreased to 4.9 on the 96th day, and started climbing after that.

The pH values of the leachates from columns 5 and 6 reached a minimum on the 25th day as mentioned above and then started gradually increasing. On the 35th day, the pH of the column 5 leachate was 8.0, and the pH of column 6 leachate reached a maximum value (7.0) on the 39th day. The pH of the leachates then decreased to 5.6 (45th day) for column 5, and 5.0 (47th day) for column 6. These corresponded to the decrease in the filtrate pH from the new rice layer. The pH values then started increasing for both columns. The pH of the column 7 leachate stayed in quite a narrow range (5.0-5.8) after reaching a minimum value (4.1) on the 17th day. On the 47th day, the pH of the column 7 leachate rapidly increased to 7.9, and decreased to 5.2 on the 55th day, after which it climbed to a value of 8.0. These pH fluctuations did not match with the changes in the

filtrate pH as the pH values of column 7 filtrates gradually increased after the 45th experimental day (Figure 4-53).

It can be seen from Figures 4-53 and 4-54 that in general the pH pattern of the soil leachates corresponded to that of the filtrates as the column 8 leachates had the lowest pH values, while the leachates of columns 5 and 6 had the highest pH values during the experimental run.

Lead extraction

The soil column leachates were analyzed by FAAS for the lead concentration, and Figure 4-55 presents the lead concentrations that were measured in the leachates of Part 2 of the soil column studies.

Fig. 4-55: Leaching of lead in the soil columns in Part 2.

As can be seen from Figure 4-55, column 8 had the highest peaks of extracted lead concentrations in comparison with the other three columns. The highest lead concentration detected in the leachate of column 8 was 124 mg/L on the 28th day. In addition, it can be seen from Figure 4-15 that column 8 had two other peaks in lead concentration with a value of 81.8 mg/L on the 42nd day and 48.4 mg/L on the 55th day. On the 39th and 47th experimental days, the lead concentrations in column 8 leachates decreased to 28.3 mg/L and 12.6 mg/L, respectively. After the 55th experimental day, as can be seen from Figure 4-55, the lead concentrations started gradually decreasing and reached values close to zero around day 88, which corresponded to the gradual increase in the pH values of the soil leachates. The peaks of the extracted lead concentrations on days 28 and 55 did not correspond to the pH of the soil leachates as the leachate pH was increasing at these times, while the peak in lead concentration on the 42nd day could be connected to the leachate pH. It can be seen from Figure 4-54 that the pH of the leachate was decreasing between days 40 and 47.

Column 5 had comparable lead concentrations with column 8 for the first 21 experimental days (Figure 4-55), however after reaching a peak value of 74.2 mg/L on the 25th day, the lead concentrations began decreasing with a small peak of 31.0 mg/L on the 33rd day, which corresponded to the increase in the leachate pH on day 25 (Figure 4-54). In column 6, smaller concentrations of lead were extracted from day 10 to 28, with the highest concentration of 27.0 mg/L on the 10th day. After the 28th day, the lead concentrations in the leachates were less than 10 mg/L with the exceptions of days 47 and 55, when 14.2 mg/L and 16.5 mg/L of lead were detected in the leachates. Column 7 had higher lead concentrations with 36.4 mg/L on the 28th day and 55.0 mg/L on the 42nd day

as compared to column 6. The lead concentration peaks of columns 6 and 7 corresponded to the decrease in the leachate pH of these columns (Figures 4-54 and 4-55).

It can be seen from Figures 4-54 and 4-55 that though the extracted lead concentrations in columns 5 and 8 were similar for the first 21 days, the leachate pH of column 8 was much lower than that of column 5 between the 10th and 21st day. And as mentioned before, not all the peaks of the extracted lead concentrations in column 8 corresponded with the leachate pH. Thus it can be concluded that the pH of the soil leachate was not only the parameter, which influenced the lead extraction in the soil columns.

The following subsections address the interaction between the pH of the column leachates, the lead concentrations detected in these leachates and the organic acid content of the rice filtrates flushed into the columns for each column individually.

Column 5

Figure 4-56 presents the pH of the column leachates and concentrations of lead detected in these leachates for column 5.

Fig. 4-56: Pb extraction and pH of the leachates in column 5.

From Figure 4-56 a clear relationship can be seen between the leachate pH and the extracted lead concentrations. The lowest pH values of the column leachates were observed between the 10th and 25th experimental days, and these days were characterized also with the highest lead extraction. For example, the lowest leachate pH was 4.7 on the 25th day, and on this day, the highest concentration of lead (74.2 mg/L) was extracted (Figure 4-56). The pH of the leachates started increasing on day 28, and the lead concentrations extracted after this day were very low, with the exceptions of days 33 and 35, when 31.0 mg/L and 17.2 mg/L of lead were extracted, respectively. Even though the leachate pH decreased to 5.6 on the 45th day, the lead extraction was very low (3.2 mg/L). It may be that the readily available lead may have already been extracted during the first 35 experimental days; therefore the lead was not available for extraction on day 45.

In Part 2 of the soil column studies, the organic acid content of the leachates was not monitored, therefore only the effect of the organic acids in the rice filtrates on the lead

leaching from the columns will be discussed in this part of the studies. Figure 4-57 presents the organic acid content of the rice filtrates and extracted lead concentrations in column 5.

Fig. 4-57: Organic acid concentrations in rice filtrates (a) and Pb concentrations in leachates (b) in column 5.

As can be seen from Figure 4-57, citric and oxalic acid were detected in the rice filtrates of column 5, and citric acid was in predominant concentrations. The highest concentrations of citric acid were detected in the filtrates for days 12 to 21, with a peak

on the 12th day, when 1131 mg/L of citric acid was detected in the filtrate. After the peak, the citric acid concentrations gradually decreased, and on the 28th day only 48.4 mg/L of citric acid was detected in the filtrate. Between days 31 and 35, oxalic acid was only the acid detected in the filtrates, with the highest concentration of 74 mg/L observed in the filtrate on day 33. After the rice addition on the 35th day, citric acid was detected again in the filtrates from days 39 to 49 in higher concentrations. For example, 753 mg/L of citric acid was observed in the filtrate on day 45.

The lead extraction in column 5 was the highest between days 14 and 28, which directly correlated to the days when the highest concentrations of citric acid were entering the column. The lead concentrations in the leachates on days 33 and 35 were moderately high, 31.0 and 17.2 mg/L, respectively, though the corresponding filtrates had small amounts of oxalic acid in them. It may be that the lead was leaching downwards at some rate, which did not correspond to the movement of the liquid. After the rice addition the citric acid concentrations increased in the filtrates, however not much lead could be detected in the leachates (Figure 4-57). This can be possibly explained by either the high pH inside the column at that time (Figure 4-56), which prevented the lead leaching or the readily available lead may have already been extracted. In addition, organic acid transformation inside the column may have occurred as demonstrated in Part 1 of the soil column studies. For instance, the citric acid may have degraded to some other organic acid inside the column at the higher pH.

Column 6

Figure 4-58 presents the pH of the column leachates and concentrations of lead detected in these leachates for column 6.

Fig. 4-58: Pb extraction and pH of the leachates in column 6.

As can be seen from Figure 4-58, when the pH of the leachates decreased to 5.3 on the 10th day and stayed at this level till day 31, the highest concentrations of lead (27.0, 26.3 and 26.7 mg/L on days 10, 17 and 21 leachates) were detected. When the pH of the leachates increased on days 31 to 39, the lead extraction was very low. On day 39, only 2.1 mg/L of lead was detected in the leachate. When the pH decreased on day 42 to 5.2 and stayed around this value for several days (Figure 4-58), the lead concentrations detected in the leachates increased, but they were lower than that of days 10 to 28, though the pH values of the leachates of days 42 to 47 were lower. Therefore, it can be concluded that the pH of the leachates was not the only factor affecting the extraction.

As it has been mentioned, the readily available lead may have been already leached out during the first part of the test.

Figure 4-59 presents the organic acid content of the rice filtrates and extracted lead concentrations in column 6. It should be mentioned that the organic acid content of the rice filtrates in Part 2 of the soil column studies was analyzed only from days 10 to 53.

Fig. 4-59: Organic acid concentrations in rice filtrates (a) and Pb concentrations in leachates (b) in column 6.

As can be seen from Figure 4-59, citric acid was the main acid in column 6 filtrates, and low concentrations of oxalic and tartaric acid were detected in some column 6 filtrates. The citric acid production pattern in column 6 was similar to that of column 5. High concentrations of citric acid were detected in the filtrates on days 12 to 21. The citric acid production during this period was higher in column 6 than in column 5 (Figures 4-57 and 4-59). The highest concentrations were 1175, 1185 and 1094 mg/L in the filtrates of days 12, 14 and 19, respectively. The citric acid concentrations decreased to 84.3 mg/L on the 26th day, and then no citric acid was detected in the filtrates until day 39. From the 39th day to 49th day, citric acid concentrations of 121 to 457 mg/L were detected in the rice filtrates as the additional rice layer was added and inoculated by a freshly prepared spore suspension on day 35. Low concentrations of tartaric acid (26 to 31.3 mg/L) were also detected in the filtrates of days 39 to 45.

As can be seen from Figure 4-59, the highest lead concentrations (26.3-27.0 mg/L) were observed in the leachates over the period 10-21. Although the citric acid concentrations in column 6 were higher than that in column 5 before the rice addition, the lead concentrations in column 5 during this period of time were almost two-fold higher as compared to that of column 6. One of the possible explanations may be the higher leachate pH in column 6 than in column 5, which was almost certainly due to the loading of the new soil to column 6 on day 10. The lowest lead concentrations, in the range of 2.1 to 5.2 mg/L, were detected in the leachates of day 31 to 39, which corresponded to the filtrates entering column 6 with no citric acid detected in them (Figure 4-59). After the 39th day, the lead concentrations in the leachates increased, with peaks on days 47 and 55, when 14.2 mg/L and 16.5 mg/L of lead were detected in the leachates, respectively.

After the 39th day, the lead concentrations from column 6 were higher than that of column 5, though the citric acid concentrations were similar in the filtrates of the both columns during this time. This demonstrated again the effect of pH on lead extraction as the leachate pH of column 6 was much lower than that of column 5 after 39th day.

Column 7

The pH of the column leachates and concentrations of lead in these leachates for column 7 are presented in Figure 4-60.

Fig. 4-60: Pb extraction and pH of the leachates in column 7.

As can be seen from Figure 4-60, no clear relationship between the leachate pH and lead concentrations could be observed in column 7. The periods of lowest leachate pH (4.1 for days 14 and 17) can be characterized by very low lead concentrations (5.5 and 5.3

mg/L). The highest lead concentrations for column 7 were 36.4 mg/L on the 28th day and 55.0 mg/L on the 42nd day, when the leachate pH was not the lowest. It can be seen from Figure 4-20 that the lead concentrations over or around 20 mg/L were extracted when the leachate pH values were less than 5.5.

The organic acid content of the rice filtrates and extracted lead concentrations in the leachates of column 7 are presented in Figure 4-61.

Fig. 4-61: Organic acid concentrations in rice filtrates (a) and Pb concentrations in leachates (b) in column 7.

Citric, oxalic, malic and tartaric acid were detected in the rice filtrates of column 7 as seen in Figure 4-61. As in the columns 5 and 6 filtrates, citric acid was the main acid, but the concentrations were much lower in column 7. The peaks in citric acid production were on days 10, 26 and 28, when 556, 761 and 634 mg/L of citric acid, respectively, were detected in the filtrates of column 7. After the 28th day, the citric acid concentrations decreased, and the new rice addition on the 35th day did not restart citric acid production in the column. As can be seen from Figure 4-61, after the 35th day, only gluconic acid with concentrations of 161 mg/L on day 39 and 63.5 mg/L on day 42 were detected in the rice filtrates. Oxalic acid was detected in the filtrates between days 21 and 35, with the highest concentration of 40.5 mg/L on the 35th day. Malic acid was detected in the filtrates on days 28, 31 and 33, with the highest concentration of 105 mg/L on the 28th day.

The highest lead concentration was in the leachate of day 42 (55.0 mg/L), however this did not relate to the organic acid concentrations as after day 31, little organic acids were detected in the filtrates. Also the leachate pH was not the lowest on this day either. It may be that there were different rates of downward movement through the column for the lead and liquid.

Column 8

Figure 4-62 presents the pH of the column leachates and concentrations of lead detected in these leachates for column 8.

Fig. 4-62: Pb extraction and pH of the leachates in column 8.

As has been discussed before, column 8 leachates had the lowest pH values among the columns. Through almost the whole experimental run, the pH values were below 5.0. The lowest pH values of column 8 leachates were observed between days 14 and 25, when the pH values were in the range of 3.8-4.6. The highest lead concentration was on the 28th day (123.6 mg/L) followed by day 25 (113.2 mg/L), which can be correlated to the low leachate pH of days 14 to 25. When the leachate pH increased from the 28th day to 39th day, as can be seen from Figure 4-62, the lead concentrations in the leachate gradually decreased to 28.3 mg/L on the 39th day. The leachate pH values decreased due to the new rice layer added on the 35th day. Though the lead concentrations increased for a while, for example, 81.8 mg/L of lead was extracted on the 42nd day and 64.2 mg/L on the 45th day, the concentrations were not as high as before the rice addition (Figure 4-62).

The lead concentrations then started decreasing and this was probably due to the increased pH values of the leachates, and the exhaustion of the readily available lead in the soil. The addition of the new rice layer on the 79th day resulted in an increase in the pH values of the leachates, and the lead concentrations after the 79th day were negligible as can be seen from Figure 4-62.

The organic acid content of the rice filtrates and extracted lead concentrations in the leachates of column 8 are presented in Figure 4-63. It should be noted that the organic acid content of column 8 rice filtrates was not determined between days 55 and 82. As can be seen from Figure 4-63, citric, oxalic, gluconic, succinic, malic and tartaric acids were detected in the rice filtrates of column 8. Citric acid was not the predominant acid as in the other three columns. The highest citric acid concentrations were in the filtrates of days 10 and 12, when 415 and 282 mg/L of citric acid were detected, respectively. Citric acid concentrations decreased to 16.6 mg/L on the 19th day, and then no citric acid was identified in the filtrates until day 39. From day 39 to 53, the rice filtrates contained citric acid, which was likely due to the new fungal growth on the fresh rice layer added on the 35th day. The highest concentrations of citric acid during this period of time were 278, 206 and 255 mg/L in the filtrates of days 39, 45 and 47, respectively. After the rice addition on the 79th day, citric acid was detected in the filtrates of day 88 (89.2 mg/L) and 90 (80.4 mg/L).

Fig. 4-63: Organic acid concentrations in rice filtrates and Pb concentrations in leachates in column 8.

As can be seen from Figure 4-63, gluconic and succinic acids were detected in concentrations comparable with the citric acid concentrations. Gluconic acid was detected in the filtrates of days 10, 12 and 21, with the highest concentration of 125 mg/L on the 10th day. Succinic acid was detected in the filtrates on days 17, 19 and 26 day,

with the highest concentration of 219 mg/L on day 19. After day 35, succinic acid was detected in all filtrate samples in concentrations ranging from 86.2 mg/L to 253.1 mg/L. Gluconic acid was detected only on days 35, 39 and 47 filtrates, with the peak concentration of 282 mg/L on day 39. After the rice addition on the 79th day, succinic and gluconic acid were the main acids in the filtrates. Gluconic acid had its peak on day 88 (295 mg/L), and succinic acid peaked on day 90 (186 mg/L).

Oxalic acid was detected in all the rice filtrates after day 33, but in much lower concentrations than citric, gluconic and succinic acids. The highest concentration of oxalic acid was 179 mg/L on the 35th day. Malic acid was detected in the filtrates on days 35 and 47, and small concentrations of tartaric acid were detected in the filtrates of days 33, 49 and 53.

Though the lead extraction was the highest in column 8 as compared to the other three columns, the organic acids were detected in much lower concentrations in the column 8 filtrates. For example, the highest extracted lead concentrations in column 8 were on days 25 and 28, while the corresponding filtrates had either no acid in them or very small concentrations of succinic or gluconic acid. As mentioned before, citric acid is considered as the strongest chelating agent in comparison with other organic acids. So it could be assumed that the lead was leaching out by the citric acid detected in the filtrates between days 10 and 19 at some different rate compared with the movement of the liquid. However, the citric acid concentrations in the column 8 filtrates were much smaller than that in columns 5, 6 and 7, while the lead extraction was the highest among the columns.

The rice quantity was doubled in column 8, and it was noticed that in addition to the spores, the column 8 filtrates were also full of rice starch. Also the filtrate pH values in

column 8 were very low. These could provide a suitable environment for organic acid production and transformation within this column. It may be that substantial concentrations of citric acid were generated inside the column.

Figure 4-64 presents the cumulative removal of lead from the contaminated soil in the soil columns.

Fig. 4-64: Cumulative lead removal in soil column tests of Part 2.

It can be seen from Figure 4-64 that column 8 achieved the highest cumulative lead extraction of 56.4 mg of lead. Though the extraction of lead in column 5 was lower than that in column 8 with 35.4 mg extracted, this was almost two-fold higher than that in columns 6 and 7. Columns 6 and 7 achieved the same cumulative extraction (17 mg).

The extraction efficiency of lead in the columns was calculated as the ratio of the cumulative lead extracted during the experimental time for each column to the total

amount of lead in the 20 g of soil. The results showed that after 61 leaching days, 21.6, 10.6 and 10.4% of the lead was extracted from the soil in columns 5, 6 and 7, respectively. Approximately 34.4 % of the lead was leached out of column 8 after 101 days.

The four soil columns in Part 2 of the soil column studies had the same column depth, but different culture conditions for rice fermentation were employed. As has been demonstrated in Figure 4-64, columns 6 and 7 had similar cumulative lead extraction, therefore it can be concluded that the Styrofoam addition in column 7 did not significantly affect the fermentation as the same type of rice and quantities were used in columns 6 and 7. However the fungal growth was different in these columns, and the organic acid production pattern, and subsequently the lead extraction pattern were different as well. For example, the greatest citric acid production and lead extraction in column 6 was observed before day 28, whereas in column 7, the highest concentrations of citric acid and extracted lead were detected after day 25. Also the fresh rice layer addition on the 35th day restarted the citric acid production in column 6, but not in column 7.

Column 5 had two-fold higher cumulative lead extraction than columns 6 and 7, but column 5 did not perform as well as column 8. Column 5 cumulative lead extraction was similar with that of column 8 until day 39, whereas after day 39, almost no lead was extracted in column 5. Column 5 was the column with the different rice type, and the rice of column 5 had a lower moisture content in comparison with the rice in the other three columns. Based on visual observations, the fungal growth in column 5 was the

strongest, though after the rice addition on the 35th day, the fungal growth was much less than that observed in column 8. One of the possible explanations is based on the pH of the filtrates. As has been presented in Figure 4-53, the filtrate pH on the 35th day was 7.8 in column 5, while the filtrate pH in column 8 was 3.8 on the day of the fresh rice addition. When the fresh rice layer was added, it contributed to the decrease in the filtrate pH in columns 5 and 6 due to the new fungal growth and the citric acid production on the surface of the fresh rice layer (Figure 4-53). However, the filtrate pH did not remain low for long as the old rice layer pH was very high and it affected the new rice layer fermentation, which resulted in the poor fungal growth, increased filtrate pH values and consequential poor lead extraction.

The pH was still very low in column 8, when the new rice was added as mentioned above. This can be explained by the rice quantity, which was doubled in column 8, so around the 35th day the rice fermentation in column 8 was still flourishing due to the presence of sufficient nutrients in this column. In columns 5 and 6, the nutrients were already utilized around that time, which was demonstrated by the increase in the filtrate pH and decrease in the citric acid production after day 25. It can be concluded that the rice quantity in column 8 was the most sufficient one. And it may be speculated that if column 5 had the same amount of rice as in column 8, the column 5 lead extraction would be competitive or higher than that in column 8.

The culture conditions in Part 2 of the soil column studies such as flushing frequency and mixing of rice in order to enhance the air flow were different from that of Part 1. In Part 1, the rice was not mixed and flushed every day, while in Part 2 the rice was flushed every other day and mixed before the flushing. As the extraction efficiency of lead in the

columns of Part 1 of the soil column studies was very low (less than 1% in most cases), it would appear that the reduced flushing frequency and the rice mixing positively affected the rice fermentation and subsequently the lead extraction. Also the fresh rice addition without mixing with the old rice layer and inoculation of the new rice with a freshly prepared spore suspension in Part 2 resulted in regeneration of organic acids in columns 5, 6 and 8. In Part 1 of the soil column studies, when the fresh rice was added, it was mixed with the old rice and was not reinoculated, which resulted in no additional organic acid production.

It should be noted that the fungal growth and fermentation were sensitive to the environmental conditions. This can be demonstrated with columns 6 and 8. All culture conditions were the same in these columns except the rice quantity, and this difference resulted in very dissimilar organic acid content of the rice filtrates between these two columns. In column 6, citric acid was the main acid produced, and only small concentrations of oxalic and tartaric acid were detected in some filtrates during the fungal fermentation. In column 8, six different organic acids were detected in the rice filtrates. Citric, gluconic and succinic acids were the main acids, and though malic, oxalic and tartaric acids were detected in quite low concentrations, their amounts were higher than that in column 6.

It is difficult to judge whether the different organic acid content of column 8 was the reason for the higher lead extraction in this column than in columns 5, 6 and 7. On the other hand, the concentrations of organic acids in column 8 filtrates were lower than that of columns 5, 6 and 7. As the data for organic acid content of the leachates in Part 2 of the soil column studies were not available, it might be assumed that organic acid

transformations occurred inside column 8, because of the different organic acid content of the filtrates, which resulted in the higher lead extraction in column 8.

As a final conclusion, it can be observed that the pH of the leachates and the organic acid concentrations in the filtrates had a profound influence on lead extraction from the columns. The effect of organic acid concentrations in the filtrates (mostly citric acid) was more clearly demonstrated in columns 5 and 6. The pH of the filtrates was also the parameter that affected the leachate pH and consequently the lead extraction.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The effect of different culture conditions on fungal production of organic acids from solid substrate was studied during the preliminary testing. The fungal filtrates of the preliminary experiments were analyzed for organic acid content by Analytical method A, and hence only citric, oxalic, and L-ascorbic acids were monitored in the filtrates. Only oxalic and ascorbic acids were detected in the filtrates. Citric acid could not be detected in the filtrates, which was likely due to difficulties with Analytical method A. When Analytical method B with a new HPLC column and a temperature control was employed in the further parts of the study, citric acid was detected in the filtrates generated under the same culture conditions. Thus, it was concluded that the results obtained by Analytical method A were suspect. In general, the different culture conditions did not substantially affect the organic acid content of the filtrates. The exception was in the case of 0.1 N NaOH pre-treated corncobs (pH=5), which resulted in the generation of the highest amount of oxalic acid, and ascorbic acid was not generated suggesting that initial substrate pH had an influence on fungal metabolism. This was also demonstrated on the corncobs acidified to the initial pH of 2. Though the composition of organic acids was the same in the filtrates from the untreated corncobs and acidified corncobs, *A. niger* NRRL 2001 produced higher concentrations of organic acids from the untreated corncobs.

The evaluation of organic acid production by different fungal species grown on various solid substrates demonstrated that different organic acids were produced from the different substrates by each fungal strain. Oxalic, citric, malic, succinic and formic acid were produced by *A. niger* NRRL 2001 from the corncobs. Oxalic, citric, tartaric,

succinic, and formic acid were produced by *A. niger* NRRL 2001 from the apple pomace, while from the boiled white rice, this strain produced only oxalic and citric acids. The hay fermentation resulted in oxalic and malic acid production. *A. niger* NRRL 2001 favored production of oxalic acid (undesirable fermentation product) on all substrates except for boiled rice. This may have been due to a different carbon source such as starch, which is the main constituent of rice. As oxalic acid concentrations were much higher than the concentrations of other acids produced, this may suggest that the high level of oxalic acid generated suppressed the production of other organic acids.

Corn cob fermentation by *A. foetidus* NRRL 337 resulted in the production of oxalic, citric and malic acid, while oxalic, citric, tartaric, succinic and gluconic acid were produced from apple pomace. Apple pomace fermentation by *A. foetidus* NRRL 337 resulted in the highest yield of succinic acid of the fungal strains employed. *A. foetidus* NRRL 337 generated only oxalic acid from the hay fermentation. Oxalic and citric acids were produced by this strain from the rice. *A. foetidus* NRRL 337 had the highest yield of citric acid from the rice as compared to the rest of the substrates and fungal strains. As the citric acid production from the boiled rice by *A. foetidus* NRRL 337 contributed about 94 % (mass basis) of the total organic acid production, it was concluded that this fungal strain favored citric acid production on boiled white rice.

In general *A. niger* ATCC 64065 had similar organic acid production behavior to *A. niger* NRRL 2001 on all substrates. The main difference was that *A. niger* ATCC 64065 did not favor oxalic acid production as much as *A. niger* NRRL 2001. On the hay and apple pomace, the oxalic acid production by *A. niger* ATCC 64065 was much lower than that by *A. niger* NRRL 2001.

A. aculeatus NRRL 358 was the weakest fungal strain for solid substrate fermentation as no organic acids were detected in the rice and hay filtrates. Oxalic, citric and malic acid were produced from the corncobs, and oxalic, citric, tartaric, succinic, formic and gluconic acid was generated from the apple pomace. The highest production was succinic acid from the apple pomace, while the production of the remaining acids from both substrates was negligible.

The effectiveness of fungal generated organic acids for leaching heavy metals from a real field soil was tested in batch metal extraction experiments. Through the general comparison of the lead extraction by the filtrates of four fungal strains generated on different solid substrates, it was clearly seen that the fungal filtrates generated during *A. foetidus* NRRL 337 fermentation of rice performed the best with respect to lead extraction. These filtrates had the highest concentrations of citric acid as compared to the other filtrates, therefore it was concluded that citric acid was the strongest metal chelator among the fungal organic acids produced.

The hay filtrates of all fungal species performed the lowest lead extraction. As the main acid of the hay filtrates was oxalic acid, it was concluded that oxalic acid had very low capacity for lead leaching. The corncob and apple pomace filtrates of *A. niger* NRRL 2001 and *A. foetidus* NRRL 337 leached out lead concentrations that were approximately 3-fold higher than those from the hay filtrates. Thus it was concluded that other fungal generated organic acids such as malic, succinic and tartaric acid were also capable of lead extraction, though they were much weaker chelators than citric acid.

Though the organic acid concentrations of *A. niger* ATCC 64065 filtrates were similar or higher than those observed in the *A. niger* NRRL 2001 and *A. foetidus* NRRL 337

filtrates, the lead extraction was much lower in the case of *A. niger* ATCC 64065 filtrates. Therefore, it was concluded that during *A. niger* ATCC 64065 fermentation of the solid substrates some unidentified metabolites were excreted, which affected the lead leaching from the soil.

A. foetidus NRRL 337 had the highest yield of citric acid from the rice as compared to the rest of substrates and fungal strains. Thus *A. foetidus* NRRL 337 and rice were selected as a fungal strain and solid substrate, respectively, for the further soil column studies.

The purpose of the soil column tests was to further evaluate the performance of the organic acid-containing flushing filtrates in soil columns that simulated in situ washing or a soil pile-leaching process. The impact of process variables on the leaching of lead in the soil columns was investigated.

Though all columns in Part 1 had the same culture conditions for fungal rice fermentation, the quantities and composition of acids produced differed from column to column demonstrating how difficult it is to control fungal fermentation. The addition of the fresh rice, which was mixed with the old rice and was not reinoculated, resulted in no additional organic acid production.

The effect of column depth on lead leaching was demonstrated in column 4. The cumulative organic acid production in column 4 was the highest among the columns, but due to the buffering effect of the increased quantity of soil in the deepest column depth, the pH of the leachates was higher. This column performed more poorly with respect to lead extraction than columns 1 and 2.

Column 2 performed the best cumulative lead extraction in Part 1, and the pH of the leachates of column 2 was the lowest among the columns. However the pH of the leachates was directly correlated to the organic acid content of the rice filtrates flushed to the columns and the leachates. It was demonstrated in column 3, where low leachate pH did not result in substantial lead extraction due to the poor organic acid content of the filtrates and leachates.

The impact of different organic acids on lead leaching was demonstrated in columns 1, 2 and 4, when the highest extracted lead concentrations were detected in the soil leachates with high citric acid concentrations. The leachates of column 3 had mainly gluconic acid and this column had the lowest lead extraction.

The acid transformation in the soil was clearly demonstrated in Part 1 of the soil column studies. The main acid of the rice filtrates was citric acid, whereas gluconic acid was in predominant concentrations in the soil leachates. It would appear that either citric acid degraded to gluconic acid inside the soil or gluconic acid was generated in soil as the soil pH favored gluconic acid production.

The four soil columns in Part 2 of the soil column studies had the same column depth, but different culture conditions for rice fermentation were employed. Columns 6 and 7 had similar cumulative lead extraction, therefore it can be concluded that the Styrofoam addition in column 7 did not significantly affect the fermentation as the same type of rice and quantities were used in columns 6 and 7. However the fungal growth was different in these columns, and the organic acid production pattern, and subsequently the lead extraction pattern were different as well.

Column 5 had a different rice type, and had two-fold higher cumulative lead extraction than columns 6 and 7, but did not perform as well as column 8. Based on visual observations, the fungal growth in column 5 was the strongest, though after the rice addition on the 35th day, the fungal growth was much less than that observed in column 8. One of the possible explanations was based on the pH of the filtrates as the filtrate pH on the 35th day was 7.8 in column 5, while the filtrate pH in column 8 was 3.8 on the day of the fresh rice addition. When the fresh rice layer was added, it contributed to the decrease in the filtrate pH in column 5 due to the new fungal growth and the citric acid production on the surface of the fresh rice layer. However, the filtrate pH did not remain low for long as the old rice layer pH was very high and it affected the new rice layer fermentation, which resulted in poor fungal growth, increased filtrate pH values and consequently poor lead extraction.

Column 8 had the highest lead extraction efficiency (34.4 %). This was explained by the rice quantity, which was doubled in column 8, so around the 35th day (the rice addition day) the rice fermentation in column 8 was still flourishing due to the presence of sufficient nutrients in this column. In columns 5, 6 and 7, the nutrients were already utilized around that time, which was demonstrated by an increase in the filtrate pH and decrease in the citric acid production after day 25. Thus it was concluded that the rice quantity in column 8 was the most sufficient one. It was speculated that if column 5 had the same amount of rice as in column 8, the column 5 lead extraction would have been competitive or higher than that in column 8.

The culture conditions in Part 2 of the soil column studies such as flushing frequency and mixing of rice in order to enhance the air flow were different from that of Part 1. In

Part 1, the rice was not mixed and was flushed every day, while in Part 2 the rice was flushed every other day and mixed before the flushing. As the extraction efficiency of lead in the columns of Part 1 of the soil column studies was very low (less than 1% in most cases), it would appear that the reduced flushing frequency and the rice mixing positively affected the rice fermentation and subsequently the lead extraction. Also the fresh rice addition without mixing with the old rice layer and inoculation of the new rice with a freshly prepared spore suspension in Part 2 resulted in regeneration of organic acids in columns 5, 6 and 8.

It was concluded that the fungal growth and fermentation were sensitive to the environmental conditions. This was demonstrated with columns 6 and 8. All culture conditions were the same in these columns except the rice quantity, and this difference resulted in very dissimilar organic acid content of the rice filtrates between these two columns.

As a final conclusion, it was also observed in Part 2 that the pH of the leachates and the organic acid concentrations in the filtrates had a profound influence on lead extraction from the columns. The effect of organic acid concentrations in the filtrates (mostly citric acid) was more clearly demonstrated in columns 5 and 6. The pH of the filtrates was also the parameter that affected the leachate pH and consequently the lead extraction. This was demonstrated in column 8, when the low filtrate pH resulted in the low leachate pH and the high lead extraction.

5.2 Recommendations

5.2.1 Fungal metabolism

The mechanisms of excretion of organic acids by fungi must be further studied to be better understood. The effect of different culture conditions on organic acid production must be better evaluated. Additional fungal strains could be screened in order to select the strain that favors the production of citric acid from the chosen substrate.

The optimum ratio of substrate quantity to soil weight should be determined to obtain the best metal extraction efficiency. The best time for fresh substrate addition, when the substrate pH favors regeneration of organic acids, requires further study.

5.2.2 Metal speciation and organic acid transformation in soil

As discussed in Section 4.4.1, organic acid transformation in the soil was clearly demonstrated during the soil column studies. A more detailed understanding of organic acid behavior inside the soil, and the effect of the soil parameters such as pH on the organic acid transformations is required.

The evaluation of metal extraction capacity of the commercially available organic acids such as succinic, tartaric, malic, formic and gluconic acid would be beneficial.

The metal extraction efficiency strongly depends on the metal speciation over the various fractions in the soil system. Thus an assessment of the properties of the contaminants and the soil should be determined as part of the leaching process.

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Appendix A: Spectrophotometric method for determining the number of spores in spore suspension

Experimental procedure:

1. Prepare a spore suspension by suspending pieces of PDA (potato dextrose agar) with a cultivated fungus on it in 10 ml of 0.01% Triton X-100 solution.
2. Filter the suspension through a glass wool to remove large mycelial fragments and clumps of agar.
3. Apply a serial dilution to 1 ml of the spore suspension with a dilution factor of 10 (6 dilutions).
4. Measure absorbance of each solution at 285 nm using distilled water as a reference.
5. Inoculate plates of PDA with 0.5 ml of original spore suspension and 6 dilutions.
6. After 5 incubation days, count the number of colonies growing on the plates, where it is possible.
7. Build a calibration curve of suspension absorbance vs. number of spores (colonies).

Figure A-1: Calibration curve: absorbance vs. number of spores/ml.

Appendix B: Raw Data Tables of Preliminary Testing Experiments

Table B-1: Experiment 1 raw data.

	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
<i>Incubation day</i>	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	51.4	419.7	ND
5	81.6	637.6	ND
6	91.4	725.7	ND
7	83	603.6	ND
11	82.5	683.6	ND
12	83.1	673.8	ND
13	68.8	631.2	ND
14	70.3	615.3	ND

Note: ND = not detected.

Table B-2: Experiment 2 raw data.

	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
<i>Incubation day</i>	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	66.5	140.1	ND
5	118.8	411.9	ND
6	86.2	508.9	ND
7	55.7	473.8	ND
11	67	621.8	ND
12	117.2	751.6	ND
13	137.7	1069.3	ND
14	59.5	659.1	ND

Table B-3: Experiment 3 raw data.

	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
<i>Incubation day</i>	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	200.7	359	ND
5	201.7	440.6	ND
6	175.6	428.8	ND
7	147.8	543.6	ND
11	150.8	759.9	ND
12	133.1	773.9	ND
13	128.5	786.6	ND
14	101.2	645.6	ND

Table B-4: Experiment 4 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	119.2	162.2	ND
5	113.2	255.5	ND
6	100	285.9	ND
7	93	332.8	ND
11	NA	NA	NA
12	187.4	749.4	ND
13	128.1	587.2	ND
14	98.3	569.7	ND

Note: NA = not available.

Table B-5: Experiment 5 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	11	185.8	ND
5	17.9	245.6	ND
6	30.8	349.5	ND
7	37.3	402.9	ND
11	21	325.1	ND
12	36.4	672.9	ND
13	43.7	914.2	ND
14	26.5	483.2	ND

Table B-6: Experiment 6 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	87.4	720.9	ND
5	65.4	503.1	ND
6	30.8	349.5	ND
7	57.2	283.9	ND
11	41	196.1	ND
12	139.1	568.6	ND
13	81.1	436.8	ND
14	54	331.4	ND

Table B-7: Experiment 7 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	94.9	320.6	ND
7	41.1	201.9	ND
11	146.6	753.6	ND
14	105.8	616.6	ND

Table B-8: Experiment 8 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	179	770.5	ND
7	113.5	677.8	ND
11	NA	NA	NA
14	116.2	1056.2	ND

Table B-9: Experiment 9 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	9.8	681.1	ND
5	ND	468.6	ND
6	ND	858.8	ND
7	ND	900	ND
11	ND	1695	ND
12	ND	1344	ND
13	ND	1240.03	ND
14	ND	1271.1	ND

Table B-10: Control 1 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	ND	ND	ND
5	158.4	87.1	19.3
6	116.7	246.5	ND
7	77.8	402.7	ND
11	83.8	580.8	ND
12	74	566.5	ND
13	77.2	739.5	ND
14	62.2	630.3	ND

Note: Contamination of control_W by fungal spores was followed by rapid growth of *A. niger* NRRL 2001.

Table B-11: Control 2 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method A		
	<i>L-Ascorbic</i>	<i>Oxalic</i>	<i>Citric</i>
4	ND	ND	ND
5	293.2	403.1	172.5
6	159.7	564	137.2
7	120.1	676.6	ND
11	85.5	614.9	ND
12	160.3	966.6	ND
13	95.7	829	ND
14	81.9	797.4	ND

Note: Contamination of control_M by fungal spores was followed by rapid growth of *A. niger* NRRL 2001.

Table B-12: Experiment 10 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method B				
	<i>Citric</i>	<i>Oxalic</i>	<i>Malic</i>	<i>L-Ascorbic</i>	<i>Succinic</i>
2	234.5	12.5	85.4	ND	ND
4	349.9	955.4	77.7	ND	25.3
7	145.2	1611.1	ND	411.2	18.9
8	85.6	2062.9	ND	ND	43.9
9	ND	994.6	ND	204.4	ND

Table B-13: Experiment 11 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method B				
	<i>Citric</i>	<i>Oxalic</i>	<i>Malic</i>	<i>L-Ascorbic</i>	<i>Succinic</i>
2	89.8	1.1	35.7	ND	14.35
4	122.7	399.5	7.1	ND	19.8
7	ND	403.5	ND	ND	ND
8	ND	740.3	ND	ND	ND
9	ND	492.1	ND	133.4	ND

Table B-14: Duplicate raw data of Experiment 10.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method B				
	<i>Citric</i>	<i>Oxalic</i>	<i>Malic</i>	<i>L-Ascorbic</i>	<i>Succinic</i>
2	221.3	17.9	60.1	ND	ND
4	339.6	1210	79.3	ND	33.6
7	76.5	1198.9	ND	212	ND
8	38.6	1520.7	ND	ND	26
9	ND	885.4	ND	ND	ND

Table B-15: Duplicate raw data of Experiment 11.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method B				
	<i>Citric</i>	<i>Oxalic</i>	<i>Malic</i>	<i>L-Ascorbic</i>	<i>Succinic</i>
2	78.7	1.4	24.2	ND	ND
4	93.3	231.1	ND	ND	ND
7	ND	351.9	ND	141.5	ND
8	ND	843.9	ND	ND	ND
9	ND	491.4	ND	121.4	ND

Table B-16: Control 3 raw data

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method B				
	<i>Citric</i>	<i>Oxalic</i>	<i>Malic</i>	<i>L-Ascorbic</i>	<i>Succinic</i>
2	858	40.7	439.9	180.2	166.4
4	509.2	256.4	160.3	ND	92.4
7	142.9	637.8	ND	612.3	64.7
8	106.5	1044.2	ND	454.6	ND
9	53.3	1083.8	ND	362.5	ND

Note: Contamination of control_10 by fungal spores was followed by rapid growth of *A. niger* NRRL 2001.

Table B-17: Control 4 raw data.

<i>Incubation day</i>	Organic acids (ppm) in fungal filtrates estimated by Analytical method B				
	<i>Citric</i>	<i>Oxalic</i>	<i>Malic</i>	<i>L-Ascorbic</i>	<i>Succinic</i>
2	179.8	1.5	62.5	ND	ND
4	141.3	5.3	14.2	ND	46.2
7	ND	17.6	ND	160.7	ND
8	ND	36.9	ND	166.7	ND
9	ND	60.6	ND	150.8	ND

Note: Contamination of control_11 by fungal spores was followed by rapid growth of *A. niger* NRRL 2001.

Table B-18: pH values of fungal filtrates (Exp. 10 and 11, Controls 3 and 4)

<i>Incubation day</i>	<i>Exp_10_1</i>	<i>Exp_10_2</i>	<i>Control_3</i>	<i>Exp_11_1</i>	<i>Exp_11_2</i>	<i>Control_4</i>
2	2.74	2.96	2.89	4.28	4.46	4.48
4	2.48	2.64	2.74	2.67	2.72	3.67
7	2.47	2.58	3.02	2.62	2.64	2.89
8	2.42	2.4	2.85	2.46	2.39	2.65
9	2.62	2.63	3.09	2.71	2.65	2.7

Appendix C: Raw Data Tables of the Experiments Conducted for Evaluation of Fungal Species and Solid Substrates

Table C-1: Experiment 12 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>	<i>Succinic</i>	<i>Formic</i>
2	1.2	342.9	1052.9	88.2	ND
5	533.5	83.9	ND	ND	37.8
8	1394.7	72.4	ND	10.8	57.1

Note: ND = not detected.

Table C-2: Duplicate raw data of Experiment 12.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>	<i>Succinic</i>	<i>Formic</i>
2	ND	196.9	564.1	66.6	ND
5	569.6	89.9	ND	ND	15.2
8	1186.7	ND	ND	ND	28.1

Table C-3: Experiment 13 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Succinic</i>	<i>Formic</i>
2	9.9	114.5	84.6	ND	ND
5	467.9	56.6	79.9	194.2	142.5
8	1230.6	67.3	152.5	136.7	118.1

Table C-4: Duplicate raw data of Experiment 13.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Succinic</i>	<i>Formic</i>
2	16.4	53.2	48.8	385.4	226.2
5	407.4	ND	49.7	150	99.8
8	1046.9	ND	145.9	67.5	56.2

Table C-5: Experiment 14 raw data.

Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>
2	ND	ND
5	16.2	ND
8	22.4	73.3

Table C-6: Duplicate raw data of Experiment 14.

Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>
2	ND	ND
5	7.5	ND
8	16.6	72.6

Table C-7: Experiment 15 raw data.

Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Malic</i>
2	ND	138
5	435	ND
8	532.7	ND

Table C-8: Duplicate raw data of Experiment 15.

Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Malic</i>
2	ND	126.9
5	234	ND
8	660.9	ND

Table C-9: Experiment 16 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B			
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>	<i>Formic</i>
2	ND	282.3	562.8	226
5	687.3	247.6	187.7	58.8
8	1045.4	72.3	ND	41

Table C-10: Duplicate raw data of Experiment 16.

	Organic acids (ppm) in fungal filtrates by Analytical method B			
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>	<i>Formic</i>
2	ND	308.4	679.7	109.9
5	465.1	157.5	ND	68.3
8	976.3	ND	ND	ND

Table C-11: Experiment 17 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Succinic</i>	<i>Formic</i>
2	4.1	140.6	66.4	283.9	366.7
5	112	86.7	73	166.3	223.3

Table C-12: Duplicate raw data of Experiment 17.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Succinic</i>	<i>Formic</i>
2	3.4	118.8	ND	524.1	290.2
5	90	108.3	61.9	322.9	278.1

Table C-13: Experiment 18 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B	
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>
2	ND	ND
5	11.7	21.5
8	89.1	339

Table C-14: Duplicate raw data of Experiment 18.

	Organic acids (ppm) in fungal filtrates by Analytical method B	
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>
2	ND	ND
5	1.6	ND
8	40.8	49.6

Table C-15: Experiment 19 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B
<i>Incubation day</i>	<i>Oxalic</i>
2	ND
5	38.3
8	77

Table C-16: Duplicate raw data of Experiment 19.

	Organic acids (ppm) in fungal filtrates by Analytical method B
<i>Incubation day</i>	<i>Oxalic</i>
2	ND
5	39.3
8	77

Table C-17: Experiment 20 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>
1	0.9	36.7	93.8
2	5.2	61.5	107.5
5	144.5	94.3	22.9
8	246.2	89.5	37.6
14	212	30.9	62.1

Table C-18: Duplicate raw data of Experiment 20.

	Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>
1	2.7	16.7	ND
2	11	83.7	169.8
5	198.5	120.1	51.4
8	219.8	43.6	39.5
14	171.6	ND	ND

Table C-19: Experiment 21 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Succinic</i>	<i>Gluconic</i>
1	ND	12.4	4.3	243.3	ND
2	ND	62	24.7	568	90.2
5	130.2	42.1	64.7	283.2	ND
8	233.5	33.9	74.2	138.5	ND
14	173.6	33.6	75.1	ND	ND

Table C-20: Duplicate raw data of Experiment 21.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Succinic</i>	<i>Gluconic</i>
1	ND	24	8.1	359.2	39.2
2	4	57.5	20.3	503.5	57
5	63.3	ND	16.6	193.3	ND
8	116.4	23.4	47	119.7	ND
14	134.6	25.5	59.4	ND	ND

Table C-21: Experiment 22 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B	
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>
1	ND	ND
2	ND	16.6
5	18.9	339.3
8	31.2	582
14	9.3	357.8

Table C-22: Duplicate raw data of Experiment 22.

	Organic acids (ppm) in fungal filtrates by Analytical method B	
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>
1	ND	ND
2	ND	ND
5	35.1	427.4
8	40.3	550.1
14	5.4	482.4

Table C-23: Experiment 23 raw data.

	Organic acids (ppm) in fungal filtrates by Analytical method B
<i>Incubation day</i>	<i>Oxalic</i>
1	ND
2	ND
5	76.4
8	114.2
14	67.5

Table C-24: Duplicate raw data of Experiment 23.

	Organic acids (ppm) in fungal filtrates by Analytical method B
<i>Incubation day</i>	<i>Oxalic</i>
1	ND
2	ND
5	137.3
8	95
14	10.9

Table C-25: Experiment 24 raw data.

Organic acids (ppm) in fungal filtrates by Analytical method B			
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>
2	ND	58.2	129.4
5	75.8	22.7	41.5
8	89.1	ND	ND

Table C-26: Duplicate raw data of Experiment 24.

Organic acids (ppm) in fungal filtrates by Analytical method B			
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>
2	ND	106	262.2
5	74.1	33.2	19.5
8	51.4	ND	ND

Table C-27: Experiment 25 raw data.

Organic acids (ppm) in fungal filtrates by Analytical method B						
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Gluconic</i>	<i>Succinic</i>	<i>Formic</i>
2	ND	46.3	23.3	46.2	411.9	27.7
5	10.7	20.3	15.6	ND	129.3	ND
8	ND	3	11.4	ND	ND	ND

Table C-28: Duplicate raw data of Experiment 25.

Organic acids (ppm) in fungal filtrates by Analytical method B						
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Gluconic</i>	<i>Succinic</i>	<i>Formic</i>
2	6.2	26.3	24	ND	511.2	ND
5	13.3	29.6	12.6	ND	61.2	ND
8	8.2	22.2	18.7	ND	ND	ND

Table C-29: pH values of *A. niger* NRRL 2001 filtrates (Exp. 12-15).

<i>Incubation day</i>	<i>Exp.12_1</i>	<i>Exp.12_2</i>	<i>Exp.13_1</i>	<i>Exp.13_2</i>	<i>Exp.14_1</i>	<i>Exp.14_2</i>	<i>Exp.15_1</i>	<i>Exp.15_2</i>
1	4.8	5.03	3.8	3.79	6.45	6.89	6.42	7.05
2	4.56	4.46	3.62	3.6	5.79	5.81	5.94	5.93
5	2.76	2.71	2.89	2.89	3.67	3.92	3.02	3.35
8	2.41	2.45	2.43	2.5	3.37	3.49	2.79	2.8

Table C-30: pH values of *A. niger* ATCC 64065 filtrates (Exp. 16-19).

<i>Incubation day</i>	<i>Exp.16_1</i>	<i>Exp.16_2</i>	<i>Exp.17_1</i>	<i>Exp.17_2</i>	<i>Exp.18_1</i>	<i>Exp.18_2</i>	<i>Exp.19_1</i>	<i>Exp.19_2</i>
1	4.27	4.37	3.69	3.72	6.71	6.87	6.4	6.42
2	4.04	4.12	3.56	3.57	5.66	5.8	6.77	6.07
5	2.84	2.91	3.48	3.55	5.6	5.23	5.24	4.81
8	2.72	2.77	2.5	2.5	3.34	4.17	4.34	4.15

Table C-31: pH values of *A. foetidus* NRRL 337 filtrates (Exp. 20-23).

<i>Incubation day</i>	<i>Exp.20_1</i>	<i>Exp.20_2</i>	<i>Exp.21_1</i>	<i>Exp.21_2</i>	<i>Exp.22_1</i>	<i>Exp.22_2</i>	<i>Exp.23_1</i>	<i>Exp.23_2</i>
1	4.11	4.15	3.5	3.56	5.81	6.06	5.98	5.99
2	4.01	4.12	3.55	3.6	4.98	4.8	5.83	6.15
5	3.26	3.24	3.25	3.32	3.11	3.02	3.65	3.44
8	3.06	3.14	2.98	3.06	2.88	2.86	3.26	3.19

Table C-32: pH values of *A. aculeatus* NRRL 358 filtrates (Exp. 24-28).

<i>Incubation day</i>	<i>Exp.24_1</i>	<i>Exp.24_2</i>	<i>Exp.25_1</i>	<i>Exp.25_2</i>	<i>Exp.26_1</i>	<i>Exp.26_2</i>	<i>Exp.27</i>	<i>Exp.28</i>
1	4.27	4.36	3.57	3.62	7.04	6.62	6.45	6.89
2	4.39	4.62	3.69	3.63	6.25	6.43	6.18	5.74
5	4.23	4.11	3.69	3.64	6.02	5.7	6.07	5.07
8	3.95	4.07	3.76	3.93	4.34	5.18	6.11	5.09

Appendix D: Raw Data Tables of the Batch Metal Extraction Experiments

In preliminary metal extraction tests, the effect of contact time on metal extraction was investigated. Soil slurries with *A. niger* NRRL 2001 filtrates (collected on the 2nd, 5th and 8th incubation day) were contacted for 3 days, and samples of the soil solutions were withdrawn for FAAS analysis each day. The soil solution samples for analyzed for Pb, Mn, Cu and Zn.

Table D-1: Extracted metal concentrations (ppm) by *A. niger* 2001 filtrates after 24 hr of contact time.

Substrate	Filtrates collected on the 2nd day				Filtrates collected on the 5th day				Filtrates collected on the 8th day			
	Pb	Mn	Cu	Zn	Pb	Mn	Cu	Zn	Pb	Mn	Cu	Zn
Control	0.71	0.03	0.01	0.00	0.38	0.04	0.02	0.01	0.58	0.00	0.04	0.03
corncoobs-1	2.08	0.36	0.21	0.82	1.04	0.34	0.10	0.66	1.55	0.00	0.18	0.79
corncoobs-2	2.08	0.39	0.20	0.81	1.30	0.44	0.15	0.82	1.65	0.00	0.18	0.81
apple pomace-1	7.85	0.39	0.17	1.80	4.20	0.44	0.10	1.39	6.68	0.06	0.26	1.92
apple pomace-2	5.65	0.32	0.15	1.45	2.36	0.37	0.09	1.09	6.77	0.06	0.24	1.95
rice-1	1.12	0.03	0.04	0.00	0.64	0.07	0.08	0.19	33.87	0.17	1.40	2.61
rice-2	1.12	0.06	0.06	0.01	0.77	0.13	0.10	0.36	24.19	0.13	1.61	2.36
hay-1	1.26	0.26	0.23	0.27	0.77	0.16	0.10	0.32	1.55	0.00	0.16	0.56
hay-2	0.98	0.16	0.17	0.16	0.77	0.10	0.08	0.40	1.55	0.00	0.15	0.53

Table D-2: Extracted metal concentrations (ppm) by *A. niger* 2001 filtrates after 48 hr of contact time.

Substrate	Filtrates collected on the 2nd day				Filtrates collected on the 5th day				Filtrates collected on the 8th day			
	Pb	Mn	Cu	Zn	Pb	Mn	Cu	Zn	Pb	Mn	Cu	Zn
Control	0.00	0.20	0.01	0.00	0.73	0.09	0.00	0.00	0.59	0.12	0.00	0.00
corncoobs-1	0.93	0.43	0.17	0.74	0.73	0.51	0.05	0.37	1.55	0.12	0.09	0.72
corncoobs-2	1.09	0.66	0.22	0.76	1.11	0.56	0.06	0.49	1.55	0.15	0.09	0.72
apple pomace-1	4.92	0.78	0.12	1.51	2.50	0.59	0.05	0.95	5.25	0.19	0.16	1.72
apple pomace-2	3.75	0.55	0.11	1.17	1.62	0.51	0.04	0.69	5.01	0.19	0.14	1.59
rice-1	0.10	0.08	0.02	0.01	0.73	0.14	0.01	0.11	31.39	0.36	1.13	2.65
rice-2	0.26	0.20	0.04	0.03	0.86	0.25	0.03	0.25	33.30	0.42	1.25	2.63
hay-1	0.93	0.55	0.19	0.17	0.47	0.20	0.06	0.10	1.31	0.09	0.10	0.39
hay-2	0.59	0.43	0.12	0.09	0.60	0.20	0.04	0.20	1.19	0.09	0.08	0.45

Table D-3: Extracted metal concentrations (ppm) by *A. niger* 2001 filtrates after 72 hr of contact time.

<i>Substrate</i>	Filtrates collected on the 2nd day				Filtrates collected on the 5th day				Filtrates collected on the 8th day			
	<i>Pb</i>	<i>Mn</i>	<i>Cu</i>	<i>Zn</i>	<i>Pb</i>	<i>Mn</i>	<i>Cu</i>	<i>Zn</i>	<i>Pb</i>	<i>Mn</i>	<i>Cu</i>	<i>Zn</i>
Control	0.50	0.00	0.02	0.01	0.25	0.05	0.02	0.00	0.23	0.12	0.02	0.00
corncoobs-1	1.33	0.56	0.14	0.53	0.70	0.48	0.03	0.29	1.43	0.05	0.07	0.42
corncoobs-2	1.33	0.71	0.12	0.55	0.81	0.53	0.05	0.38	1.31	0.05	0.07	0.39
apple pomace-1	4.09	0.71	0.07	1.34	2.05	0.56	0.05	0.89	4.41	0.19	0.11	1.39
apple pomace-2	3.40	0.53	0.09	0.98	1.15	0.48	0.03	0.62	4.29	0.42	0.10	1.31
rice-1	0.50	0.00	0.01	0.01	0.48	0.11	0.01	0.11	29.24	0.42	0.80	2.51
rice-2	0.50	0.04	0.03	0.04	0.48	0.27	0.01	0.22	22.20	0.39	0.86	2.29
hay-1	1.19	0.31	0.16	0.09	0.25	0.21	0.02	0.05	1.55	0.05	0.07	0.15
hay-2	0.91	0.22	0.11	0.07	0.25	0.24	0.02	0.22	1.43	0.05	0.06	0.16

Table D-4: pH of the soil solutions with *A. niger* NRRL 2001 filtrates of Day 2.

<i>Contact time</i>	<i>Control</i>	<i>Corn.-1</i>	<i>Corn.-2</i>	<i>Apple pomace-1</i>	<i>Apple pomace-2</i>	<i>Boiled rice-1</i>	<i>Boiled rice-2</i>	<i>Hay-1</i>	<i>Hay_2</i>
initial	7.08	6.42	6.47	5.64	5.96	7.05	6.96	6.76	6.91
24 hr	7.03	6.68	6.67	6.27	6.47	7.23	7.14	6.99	7.04
48 hr	7.27	6.84	6.84	6.56	6.64	7.34	7.24	7.2	7.24
72 hr	7.25	7.09	7.16	N/A	N/A	N/A	N/A	N/A	N/A

Note: N/A = not available.

Table D-5: pH of the soil solutions with *A. niger* NRRL 2001 filtrates of Day 5.

<i>Contact time</i>	<i>Control</i>	<i>Corn.-1</i>	<i>Corn.-2</i>	<i>Apple pomace-1</i>	<i>Apple pomace-2</i>	<i>Boiled rice-1</i>	<i>Boiled rice-2</i>	<i>Hay-1</i>	<i>Hay_2</i>
initial	7.27	5.81	5.49	5.35	5.54	6.55	6.32	5.92	5.97
24 hr	7.14	6.69	6.62	6.38	6.43	6.82	6.61	6.68	6.43
48 hr	N/A	6.89	6.81	6.76	6.85	7.11	6.93	6.94	6.75
72 hr	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table D-6: pH of the soil solutions with *A. niger* NRRL 2001 filtrates of Day 8.

<i>Contact time</i>	<i>Control</i>	<i>Corn.-1</i>	<i>Corn.-2</i>	<i>Apple pomace-1</i>	<i>Apple pomace-2</i>	<i>Boiled rice-1</i>	<i>Boiled rice-2</i>	<i>Hay-1</i>	<i>Hay_2</i>
initial	7.25	5.73	5.48	5.01	4.93	4.86	5.03	5.84	5.64
24 hr	7.27	6.75	6.7	6.37	6.37	6.38	6.6	6.88	6.82
48 hr	N/A	6.83	6.79	6.62	6.63	6.57	6.6	6.84	6.78
72 hr	N/A	7.06	7.1	6.73	6.88	6.69	6.79	7.45	7.48

Batch metal extraction tests with *A. foetidus* NRRL 337 filtrates had a contact time of 24 hours. The soil solution samples for analyzed for Pb, Mn, Cu and Zn.

Table D-7: Extracted metal concentrations (ppm) by *A. foetidus* NRRL 337 filtrates after 24 hr of contact time.

<i>Substrate</i>	Filtrates collected on the 2nd day				Filtrates collected on the 5th day				Filtrates collected on the 8th day			
	<i>Pb</i>	<i>Mn</i>	<i>Cu</i>	<i>Zn</i>	<i>Pb</i>	<i>Mn</i>	<i>Cu</i>	<i>Zn</i>	<i>Pb</i>	<i>Mn</i>	<i>Cu</i>	<i>Zn</i>
Control	0.40	0.00	0.02	0.09	0.71	0.00	0.00	0.00	0.71	0.00	0.00	0.00
corncoobs-1	2.47	0.08	0.13	0.48	1.71	0.00	0.12	0.02	3.29	0.00	1.48	0.27
corncoobs-2	6.47	0.25	0.22	1.63	2.14	0.00	0.21	0.20	3.29	0.00	0.96	0.39
apple pomace-1	7.80	0.13	0.13	1.04	7.71	0.05	0.21	1.74	6.14	0.00	0.80	1.42
apple pomace-2	4.25	0.04	0.08	0.78	2.57	0.00	0.10	0.73	3.29	0.00	0.40	0.61
rice-1	1.29	0.00	0.10	0.38	5.71	0.00	0.87	0.91	36.00	0.05	5.09	2.51
rice-2	1.88	0.00	0.13	0.54	6.86	0.05	1.23	1.19	34.00	0.03	5.65	2.30
hay-1	0.99	0.00	0.18	0.07	1.29	0.00	0.16	0.00	2.00	0.00	0.47	0.06
hay-2	1.29	0.00	0.22	0.09	1.57	0.00	0.14	0.00	1.43	0.00	0.35	0.08

Batch metal extraction tests with *A. aculeatus* NRRL 358 and *A. niger* ATCC 64065 filtrates had a contact time of 24 hours, and the soil solution samples for analyzed only for Pb.

Table D-8: Extracted metal concentrations (ppm) by *A. aculeatus* NRRL 358 filtrates after 24 hr of contact time.

	Extracted Pb concentrations (ppm)				
	<i>Control</i>	<i>Corncob filtrate</i>	<i>Apple pomace filtrate</i>	<i>Rice filtrate</i>	<i>Hay filtrate</i>
<i>Day 2-1</i>	0.27	1.3	2.43	0.27	0.68
<i>Day 2-2</i>	0.27	1.81	2.74	0.38	0.58
<i>Day 5-1</i>	0.32	0.55	3.73	0.08	0.08
<i>Day 5-2</i>	0.32	0.2	2.67	0.08	0.08

Table D-9: Extracted metal concentrations (ppm) by *A. niger* ATCC 64065 filtrates after 24 hr of contact time.

	Extracted Pb concentrations (ppm)				
	<i>Control</i>	<i>Corn cob filtrate</i>	<i>Apple pomace filtrate</i>	<i>Rice filtrate</i>	<i>Hay filtrate</i>
<i>Day 2-1</i>	0.13	0.87	6.47	0	0.24
<i>Day 2-2</i>	0.13	0.87	4.57	0	0.24
<i>Day 5-1</i>	0	0.76	0.66	0.45	0.34
<i>Day 5-2</i>	0.00	0.45	0.76	0.13	0.24
<i>Day 8-1</i>	0.00	0.36	0.00	0.86	0.45
<i>Day 8-2</i>	0.00	0.36	0	0.86	0.36

Table D-10: pH values of the initial soil solutions with *A.niger* ATCC 64065.

	<i>control</i>	<i>corn. filtrate</i>	<i>app.pom. filtrate</i>	<i>rice filtrate</i>	<i>hay filtrate</i>
<i>Day 2-1</i>	7.1	6.8	6.3	7.9	7.5
<i>Day 2-2</i>	7.1	6.6	6.6	7.9	7.6
<i>Day 5-1</i>	7.3	6.3	6.6	7.3	7.5
<i>Day 5-2</i>	7.3	6.4	6.4	7.5	7.5
<i>Day 8-1</i>	7.3	6.3	N/A	6.8	7.5
<i>Day 8-2</i>	7.3	6.6	N/A	7.2	7.5

Table D-11: pH values of the soil solutions after 24 hr with *A.niger* ATCC 64065.

	<i>control</i>	<i>corn. filtrate</i>	<i>app.pom. filtrate</i>	<i>rice filtrate</i>	<i>hay filtrate</i>
<i>Day 2-1</i>	7.0	6.3	6.0	7.3	7.4
<i>Day 2-2</i>	7.0	6.3	6.0	7.3	7.4
<i>Day 5-1</i>	7.1	6.7	6.6	6.9	7.1
<i>Day 5-2</i>	7.1	6.9	6.6	7.1	7.2
<i>Day 8-1</i>	7.23	7.0	N/A	6.8	7.3
<i>Day 8-2</i>	7.23	7.0	N/A	7.0	7.3

The organic acid content of *A. niger* NRRL 2001 filtrates (Table D-12) employed in the batch metal extraction tests differed from that of Section 4.2 as two different experimental runs (different flushing frequency) of *A. niger* NRRL 2001 solid substrate fermentation were conducted. Filtrates for the batch metal extraction tests were collected

during the *A. niger* NRRL 2001 fermentation, when the substrates were flushed on a daily basis.

Table D-12: Raw data of *A. niger* NRRL 2001 corncob fermentation.

	Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>
2	15.3	57.1	122.8
5	528.6	27.2	ND
8	562.2	ND	ND

Table D-13: Duplicate raw data of *A. niger* NRRL 2001 corncob fermentation.

	Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Malic</i>
2	10.1	66.6	173
5	524.8	32.8	28.8
8	1069.1	ND	53

Table D-14: Raw data of *A. niger* NRRL 2001 apple pomace fermentation.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Ascorbic</i>	<i>Succinic</i>
2	0.5	8	25.8	795.8	525.8
5	240.8	8.9	59.9	484.8	192.8
8	ND	8.7	ND	141.9	95.7

Table D-15: Duplicate raw data of *A. niger* NRRL 2001 apple pomace fermentation.

	Organic acids (ppm) in fungal filtrates by Analytical method B				
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>	<i>Tartaric</i>	<i>Ascorbic</i>	<i>Succinic</i>
2	ND	2.1	ND	572.1	362.2
5	236	6.4	42.8	473.6	176.8
8	854.3	19.1	107.7	630.7	199.2

Table D-16: Raw data of *A. niger* NRRL 2001 rice fermentation.

	Organic acids (ppm) in fungal filtrates by Analytical method B	
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>
2	ND	ND
5	23	5.7
8	321.4	390.6

Table D-17: Duplicate raw data of *A. niger* NRRL 2001 rice fermentation.

	Organic acids (ppm) in fungal filtrates by Analytical method B	
<i>Incubation day</i>	<i>Oxalic</i>	<i>Citric</i>
2	ND	ND
5	23.1	9.2
8	242.4	323.1

Table D-18: Raw data of *A. niger* NRRL 2001 hay fermentation.

	Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Tartaric</i>	<i>Malic</i>
2	ND	78.2	13.8
5	143.2	19.6	ND
8	373.2	ND	ND

Table D-19: Duplicate raw data of *A. niger* NRRL 2001 hay fermentation.

	Organic acids (ppm) in fungal filtrates by Analytical method B		
<i>Incubation day</i>	<i>Oxalic</i>	<i>Tartaric</i>	<i>Malic</i>
2	0.3	55.3	ND
5	126	5.8	ND
8	222.9	ND	ND

Appendix E: Raw Data Tables of the Soil Column Studies

Part 1**Table E-1: pH of the filtrates collected from the support apparatuses in Part 1.**

<i>Day</i>	<i>Column_1</i>	<i>Column_2</i>	<i>Column_3</i>	<i>Column_4</i>
4	4.48	4.69	5.14	4.56
5	3.15	3.51	4.15	3.66
6	2.87	2.76	2.95	N/A
7	2.89	2.75	3	2.73
10	2.83	2.96	3.25	2.92
11	3.01	2.99	3.07	2.72
12	2.76	2.75	2.78	2.67
13	2.81	2.79	2.89	2.74
14	2.73	2.75	2.7	2.63
18	3.27	3.29	4.44	3.48
19	3.88	3.5	3.85	3.15
20	3.38	2.99	3.46	3.09
21	3.25	2.96	3.12	3.05
25	5.67	3.37	3.45	3.76
28	7.28	6.55	6.95	7.21

Note: N/A = not available.

Table E-2: pH of the leachates collected from the soil columns in Part 1.

<i>Day</i>	<i>Column_1</i>	<i>Column_2</i>	<i>Column_3</i>	<i>Column_4</i>
5	6.91	6.97	7.02	7.44
6	7.41	7.34	7.02	7.32
7	6.89	6.27	5.67	7.56
10	5.47	4.99	4.84	6.11
11	5.31	5.49	5.44	5.32
12	5.59	5.67	5.27	5.28
13	6.26	5.4	6.08	6.45
14	6.93	6.11	6.61	7.09
18	5.28	4.47	5.9	6.41
19	5.34	6.65	5.29	7.96
20	5.62	7.69	5.47	7.88
21	6.28	7.23	5.6	7.98
25	6.67	7.18	5.68	7.83
28	6.59	6.7	5.84	7.84

Table E-3: The collected leachate quantity (ml) in Part 1.

<i>Day</i>	<i>Column_1</i>	<i>Column_2</i>	<i>Column_3</i>	<i>Column_4</i>
5	25	17	18	13
6	47	46	45	42
7	29	43	59	42
10	56	41	45	44
11	42	41	42	40
12	43	46	48	45
13	43	44	31	46
14	20	50	62	49
18	75	44	39	48
19	12	40	54	37
20	34	35	44	45
21	81	49	45	43
25	43	46	55	42
28	35	43	44	43

Table E-4: Lead concentrations (ppm) in the leachates in Part 1.

<i>Day</i>	<i>Column_1</i>	<i>Column_2</i>	<i>Column_3</i>	<i>Column_4</i>
5	0.67	0.44	0.21	0.00
6	1.60	1.02	0.67	0.32
7	4.39	7.18	5.09	3.23
10	6.60	15.44	5.44	5.79
11	10.95	17.36	9.58	13.70
12	5.34	9.46	4.54	10.26
13	1.00	7.22	3.22	5.56
14	2.11	7.44	2.22	4.56
18	19.18	20.41	2.56	15.27
19	14.62	4.68	5.90	1.17
20	2.23	0.76	0.76	1.34
21	1.77	0.63	0.42	0.84
25	0.53	0.11	0.22	0.22
28	1.03	0.11	0.03	0.20

Table E-5: Organic acid concentrations (ppm) in flushing filtrates and soil leachates in column 1.

Day	Filtrates		Leachates		
	CA	GA	CA	GA	SA
4	237.6	60.2			
5	677.5	81.9	115.8	372.1	N/D
6	898.3	103.8	N/D	744.9	N/D
7	N/D	61.2	N/D	482.2	N/D
10	205.9	12.2	N/D	227.8	131.4
11	495.2	N/D	N/D	302.3	N/D
12	297.3	N/D	N/D	67.8	N/D
13	375.7	N/D	N/D	73.9	N/D
14	N/D	N/D	N/D	N/D	N/D
18	N/D	N/D	809.5	40.2	145.2
19	N/D	N/D	N/D	N/D	N/D
20	N/D	N/D	N/D	N/D	N/D
21	N/D	N/D	N/D	N/D	N/D
25	N/D	N/D	N/D	N/D	N/D
28			N/D	N/D	N/D

Note: N/D = not detectable.

Table E-6: Organic acid concentrations (ppm) in flushing filtrates and soil leachates in column 2.

Day	Filtrates		Leachates		
	CA	GA	CA	GA	SA
4	150.6	451.2			
5	N/D	165.5	N/D	748	N/D
6	662.3	170.9	365.4	879.4	N/D
7	N/D	183.8	N/D	2213	N/D
10	N/D	18.7	N/D	832.9	168.9
11	612.1	N/D	N/D	199.4	255.1
12	550.3	N/D	N/D	57.9	N/D
13	485.3	N/D	396.4	3.6	N/D
14	1913.7	N/D	N/D	N/D	N/D
18	55.4	N/D	1549.5	N/D	N/D
19	N/D	N/D	N/D	N/D	N/D
20	N/D	N/D	N/D	N/D	N/D
21	511.3	43.4	N/D	N/D	N/D
25	N/D	N/D	N/D	N/D	N/D
28			N/D	N/D	N/D

Table E-7: Organic acid concentrations (ppm) in flushing filtrates and soil leachates in column 3.

<i>Day</i>	Filtrates		Leachates		
	CA	GA	CA	GA	SA
4	129	120.5			
5	720.4	561.2	81.2	181	N/D
6	N/D	153.1	N/D	472.2	N/D
7	N/D	53.8	N/D	1098.7	N/D
10	N/D	N/D	N/D	238	N/D
11	N/D	N/D	N/D	N/D	N/D
12	N/D	N/D	N/D	115.4	128.3
13	N/D	6.5	N/D	36.9	N/D
14	1117.2	73.8	N/D	N/D	N/D
18	N/D	N/D	N/D	N/D	N/D
19	N/D	N/D	N/D	N/D	N/D
20	N/D	N/D	N/D	N/D	N/D
21	N/D	34.3	N/D	N/D	N/D
25	N/D	N/D	N/D	N/D	N/D
28			N/D	N/D	N/D

Table E-8: Organic acid concentrations (ppm) in flushing filtrates and soil leachates in column 4.

<i>Day</i>	Filtrates			Leachates		
	CA	GA	OA	CA	GA	SA
4	139.3	38	N/D			
5	146.4	1054.1	82.2	N/D	N/D	N/D
6	N/D	87.6	N/D	N/D	201	N/D
7	N/D	177.4	N/D	N/D	2759.9	N/D
10	N/D	N/D	N/D	N/D	664.2	135.7
11	1441.7	56	N/D	N/D	495.4	425.9
12	795.6	N/D	N/D	N/D	14.2	263
13	973.9	N/D	N/D	N/D	N/D	N/D
14	3235.8	N/D	N/D	287	45.9	22.9
18	N/D	N/D	N/D	1331.7	184.2	N/D
19	410.5	N/D	N/D	N/D	N/D	N/D
20	N/D	N/D	N/D	N/D	N/D	N/D
21	219	44.5	N/D	N/D	N/D	N/D
25	N/D	N/D	N/D	N/D	N/D	N/D
28				N/D	N/D	N/D

Part 2

Table E-9: pH of the filtrates collected from the support apparatuses in Part 2.

Day	Column_5	Column_6	Column_7	Column_8
3	5.87	5.62	5.8	5.73
5	5.14	4.7	5.36	5.18
7	4.68	4.32	5.23	4.72
10	3.43	3.22	4.48	4.14
12	2.72	2.78	4.01	3.79
14	2.78	2.73	3.31	3.68
17	2.85	2.80	3.08	3.43
19	2.81	2.77	3.02	3.52
21	3.08	2.92	2.96	3.54
25	5.01	3.23	2.98	3.52
26	5.06	3.76	2.87	3.64
28	6.16	6.30	2.96	3.81
31	7.18	7.11	3.01	3.72
33	7.49	7.29	3.66	3.92
35	7.76	7.63	6.17	3.80
39	6.13	4.35	6.39	3.79
42	4.07	3.38	5.91	3.66
45	2.94	2.96	5.03	3.47
47	3.11	3.06	5.81	3.56
49	5.01	3.20	6.43	3.61
53	6.62	5.38	6.72	3.62
55	7.15	6.12	6.79	3.54
61	7.98	7.09	7.49	3.58
69	8.41	8.06	8.31	3.55
75				3.72
79				3.90
82				5.17
86				4.40
88				4.26
90				4.20
94				4.16
96				4.20
101				4.08

Table E-10: pH of the leachates collected from the soil columns in Part 2.

<i>Day</i>	<i>Column_5</i>	<i>Column_6</i>	<i>Column_7</i>	<i>Column_8</i>
5	6.55	6.87	7.09	6.13
7	5.93	5.98	5.81	5.05
10	4.95	5.29	5.52	5.06
12	5.09	6.06	6.29	4.74
14	5.01	6.31	4.08	4.07
17	4.78	5.39	4.11	3.78
19	5.03	5.23	4.95	3.87
21	4.84	5.43	5.44	3.91
25	4.77	5.28	4.86	4.01
28	5.53	5.45	5.03	4.58
31	6.55	5.8	5.1	4.72
33	6.88	6.24	5.05	4.78
35	7.97	6.93	5.78	4.7
39	7.13	7.04	5.47	5.04
42	6.7	5.21	5.51	4.26
45	5.59	5.02	5.1	4.14
47	6.78	4.99	7.86	3.94
53	6.88	5.72	6.82	4.62
55	8.01	5.44	5.18	4.79
61	7.63	6.98	7.96	4.69
65				4.74
69				4.65
75				4.99
79				5.41
82				5.24
88				8.31
94				5.15
96				4.97
101				7.14

Table E-11: The collected leachate quantity (ml) in Part 2.

<i>Day</i>	<i>Column_5</i>	<i>Column_6</i>	<i>Column_7</i>	<i>Column_8</i>
5	71	82	90	129
7	89	28	60	120
10	93	18.5	N/A	115
12	87	86	92	85
14	84	90	94	82
17	86	88	86	108
19	90	90	82	102
21	92	94	103	84
25	93	97	104	30
28	97	88	76	94
31	101	98	96	103
33	117	94	104	98
35	102	97	95	98.5
39	121	108	106	151
42	90	104	94	127
45	92	79	103	118
47	52	100	89.5	84
53	109	48	48	80
55	18	88.5	70	95.5
61	130	95	95	114
65				97
69				120
75				89
79				60
82				37
88				16.5
94				105
96				65
101				54

Table E-12: Lead concentrations (ppm) in the leachates in Part 2.

<i>Day</i>	<i>Column_5</i>	<i>Column_6</i>	<i>Column_7</i>	<i>Column_8</i>
5	0.00	0.06	0.06	1.13
7	0.80	1.37	6.13	12.68
10	1.72	27.04	3.25	15.07
12	8.36	6.12	2.11	21.40
14	37.76	18.62	5.50	26.03
17	44.79	26.57	5.35	27.19
19	48.49	22.72	10.75	49.50
21	62.38	26.11	11.22	58.68
25	74.19	16.08	20.25	113.24
28	44.00	18.13	36.40	123.64
31	10.86	5.16	24.17	74.82
33	31.02	3.40	25.16	38.72
35	17.22	2.59	10.00	31.74
39	4.52	2.14	7.85	28.32
42	4.47	7.92	55.03	81.75
45	3.23	8.22	15.98	64.17
47	7.09	14.17	11.29	12.57
53	3.18	8.46	6.16	42.35
55	1.94	16.49	2.39	48.39
61	1.10	6.21	4.45	21.00
65				13.12
69				11.05
75				6.00
79				1.46
82				4.18
88				0.10
94				1.01
96				0.55
101				0.03

Table E-13: Organic acid concentrations (ppm) in flushing filtrates in column 5.

<i>Day</i>	<i>OA</i>	<i>CA</i>
10	5.7	246.8
12	N/D	1130.7
14	N/D	728.4
17	N/D	809.4
19	N/D	696.6
21	N/D	460.5
25	5.3	154.3
26	9.5	281
28	N/D	48.4
31	37.4	N/D
33	74	N/D
35	60.3	N/D
39	3.8	67.3
42	N/D	57.6
45	N/D	753.4
47	2.3	436.9
49	3.3	302.5
53	3.2	N/D

Table E-14: Organic acid concentrations (ppm) in flushing filtrates in column 6.

<i>Day</i>	<i>CA</i>	<i>OA</i>	<i>TA</i>
10	34.8	4.9	N/D
12	1174.5	4.1	N/D
14	1185.4	N/D	N/D
17	921.7	3.7	N/D
19	1093.7	24.8	N/D
21	749.4	17.4	N/D
25	171.9	45.6	N/D
26	84.3	21	N/D
28	N/D	11.1	N/D
31	N/D	2.7	N/D
33	N/D	N/D	N/D
35	N/D	18.1	N/D
39	131.6	10.1	26
42	120.6	N/D	27.1
45	443.6	N/D	31.3
47	456.8	N/D	N/D
49	341.3	N/D	N/D
53	N/D	N/D	N/D

Table E-15: Organic acid concentrations (ppm) in flushing filtrates in column 7.

Day	CA	OA	MA	GA
10	555.6	N/D	N/D	N/D
12	N/D	N/D	N/D	N/D
14	140.3	N/D	N/D	N/D
17	269.5	N/D	N/D	N/D
19	370.3	N/D	N/D	N/D
21	54.7	18.3	N/D	N/D
25	344.1	11.8	N/D	N/D
26	761.2	23.3	N/D	N/D
28	634.1	34.3	104.6	N/D
31	101	39.5	43.3	N/D
33	61.9	24.4	51.3	N/D
35	N/D	40.5	72.4	N/D
39	49.9	N/D	N/D	161
42	N/D	N/D	N/D	63.5
45	N/D	N/D	N/D	N/D
47	N/D	N/D	N/D	N/D
49	N/D	N/D	N/D	N/D
53	N/D	N/D	N/D	N/D

Table E-16: Organic acid concentrations (ppm) in flushing filtrates in column 8.

Day	CA	OA	GA	SA	MA	TA
5	N/D	N/D	N/D	N/D	N/D	N/D
7	N/D	N/D	N/D	N/D	N/D	N/D
10	414.9	N/D	125.4	N/D	N/D	N/D
12	281.8	N/D	67.7	N/D	N/D	N/D
14	75.7	N/D	N/D	N/D	N/D	N/D
17	56.2	N/D	N/D	97.1	N/D	N/D
19	16.6	N/D	N/D	219.5	N/D	N/D
21	N/D	N/D	40.3	N/D	N/D	N/D
25	N/D	N/D	N/D	N/D	N/D	N/D
26	N/D	N/D	N/D	183.3	N/D	N/D
28	N/D	N/D	N/D	N/D	N/D	N/D
31	N/D	N/D	N/D	N/D	N/D	N/D
33	N/D	40	N/D	N/D	N/D	18.2
35	N/D	178.8	168.6	86.2	83.9	N/D
39	278.3	82.4	282.4	168.5	N/D	N/D
42	138.5	17.6	N/D	172.9	N/D	N/D
45	206.3	9	N/D	151.4	N/D	N/D
47	255.2	13.2	158.8	253.1	26	N/D
49	80.4	11.2	N/D	202.4	N/D	17.2
53	34.5	6	N/D	139.7	N/D	43.6
88	89.2	73.2	294.8	180.2	N/D	N/D
90	80.4	62.7	286.9	186.1	N/D	N/D
94	N/D	37.5	149.5	121.3	N/D	N/D
96	N/D	19.4	N/D	N/D	N/D	N/D
101	N/D	19.1	N/D	N/D	N/D	15.5

Appendix F: Initial metal concentrations of the soil employed in this study

Table F-1: Initial metal concentrations of the soil by ICP-OES analysis.

<i>Metal analyzed</i>	<i>Unit</i>	<i>Concentration</i>
Silver	µg/g	0.5
Aluminum	%	0.23
Arsenic	µg/g	5
Barium	µg/g	75
Beryllium	µg/g	0.4
Bismuth	µg/g	5
Calcium	%	18.4
Cadmium	µg/g	1
Cobalt	µg/g	7
Chromium	µg/g	7
Copper	µg/g	15
Iron	%	1.06
Potassium	%	0.21
Lithium	µg/g	6
Magnesium	%	0.86
Manganese	µg/g	193
Molybdenum	µg/g	1
Sodium	%	0.03
Nickel	µg/g	9
Lead	µg/g	8190
Tin	µg/g	20
Strontium	µg/g	829
Titanium	µg/g	86
Vanadium	µg/g	12
Yttrium	µg/g	5
Zinc	µg/g	124