ANALYSIS OF VARIOUS BIOREACTOR CONFIGURATIONS FOR HEAVY METAL REMOVAL USING THE FUNGUS *PENICILLIUM OCHRO-CHLORON*

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Abstract

Penicillium ochro-chloron (ATCC strain # 36741), a filamentous fungus with the capability for removing copper ions from aqueous solutions, was studied as a possible biological trap (biotrap) for remediation of heavy metal contaminants in industrial wastewaters. This research demonstrated that in shake flasks the fungus removed copper from surrogate wastewater with 100mg/L copper contamination by as much as 99%. These results did not translate to the bioreactor configuration of a packed bed column, as channeling occurred through the bed, shown by conductivity tracer studies. A fluidized bed configuration was studied and resulted in copper removal of 97%, with a capacity of 149 mg[Cu]/g dry weight biomass, under the conditions of 50% dissolved oxygen. For dissolved oxygen concentrations below the critical oxygen concentration for the fungus (20% saturation) there was minimal copper removal.

Mixing studies in the fluidized bed reactor showed that the system was diffusion limited. Mathematical modeling using first order kinetics associated with diffusion limited reactions resulted in rate constants for Cu^{2+} uptake of approximately 0.031 h^{-1}, which were dependent on the dissolved oxygen concentration. Modeling of the reaction with a second order kinetic equation showed that there are possibly factors regulating copper uptake besides oxygen. Electron microscopy showed that in some instances the copper removed was retained as large porous spherical extracellular precipitates. Energy Dispersive X-ray (EDX) analysis has shown similar complexes to be copper phosphate precipitates (Crusberg, 1994).

Removal of heavy metal contaminants from wastewater discharge is a necessity for many industries, due to environmental concerns and federal regulations. The use of a biological system for the removal and recycling of heavy metals could prove more economical than currently used physio-chemical processes.

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List of Abbreviations and Symbols

AFS	Advanced Fermentation Software
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
C	Bulk metal concentration (copper in this case)
°C	degrees Celsius
[Cu]	Copper concentration
dH_2O	deionized water
DNS	Dinirtosalicylic acid
EDX	Energy Dispersive X-ray analysis
F	F-ratio (comparison of variances)
GMS	Glucose Minimal Salts
hrs	hours
I.D.	Inner diameter
K	Rate constant
mL	milliliter
mM	millimole
mW	milliohm (unit of resistance)
nm	nanometer
NMM	New Minimal Media
OUR	Oxygen uptake rate
p	p-level (probability level)
pH	the negative decimal log of the hydrogen ions concentration
rmm	relative molecular mass (molecular weight)
rpm	revolutions per minute
W	Dry weight

Introduction

1. Problem Statement and Hypothesis.

Hazardous waste is constantly produced by industries all over the world. It has been estimated that the United States alone produces more than 275 million metric tons of hazardous waste every year. The majority of this waste is in the form of wastewater (90%), which needs to be detoxified and then released back into streams, rivers or the ocean (Hazardous Waste Management, 1994). Heavy metal removal from aqueous systems has, as a result, been a concern for industries and researchers for some time. The ingestion and retention of heavy metals by both animals and humans result in health problems, which can be lethal. Therefore; producing an efficient, cost effective and non-toxic method of removing metals from aqueous environments would improve the environment and public health. Biological systems have advantages over many chemical treatments, since they are usually more environmentally friendly and do not produce toxic waste. Treatments and processes used today in purifying wastewater are expensive and may themselves produce toxic waste (metal hydroxide sludge), with additional disposal costs (Crusberg, *et al.*, 1989). Designing a system, including bioreactor configuration and favorable conditions for the elimination of heavy metals from aqueous solutions could provide a possible solution for reducing wastewater contamination. Such a process could be used in wastewater treatment and, perhaps also in fields like bio-mining. In order for a metal-removal system to be feasible it would need to meet the stringent water quality standards enforced by the Environmental Protection Agency (the EPA). The current EPA water quality standards, for copper, in fresh water, is a maximum concentration of 17μg/L

and a continuous concentration of 11µg/L, in salt water the maximum concentration and the continuous concentrations are both 2.4µg/L. The maximum concentration means "the highest concentration of a pollutant to which aquatic life can be exposed to for a short period of time (approximately one hour) without delirious effects", and the continuous concentration is "the highest concentration of a pollutant to which aquatic life can be exposed to for an extended period of time (approximately four days) without delirious effects" (EPA, 1995). The existing standards for copper discharge in wastewater is a daily average of 2.7mg/L and no one day more than 4.5mg/L (CFR, 1998). In 1990-91 the highest copper concentration measured in a river in the United States was 8.89µg/L, and most rivers contained lower levels of copper (Windom *et al*., 1991). The reason that water sources are not more polluted is the diluting effect the large volumes of water has on the contaminants entering.

Heavy metal resistance and absorption is well documented in many microorganisms (Stokes, *et al.*, 1979). The fungus *Penicillium ochro-chloron* has been shown to have the ability to remove solubilized copper from aqueous solutions. Therefore; using this organism in a functional bioreactor configuration which compliments copper uptake, could provide a system for lowering the heavy metal concentration in solution. Should the fungus remove enough copper from the solution to meet the standards set by the EPA (above), and if this process could be scaled-up there is the potential for industrial use. The media conditions and physical environment in the bioreactor will affect the uptake achieved, therefore; changing and monitoring variables affecting the environment and media would provide research with the maximum heavy metal removal possible in any one configuration. Variables that may affect the removal

are: viability of the fungus, dissolved oxygen concentration, temperature, nutrients available to the organism, flow rate of the media, pH, ionic strength of the media, diffusion limitations, and residence time of the copper ions in the bioreactor (Stanbury & Whitaker, 1984).

2. Background Information and Significance

2.1. Motivation for Heavy Metal Removal from Water supplies.

Metals are essential minerals for all aerobic and most anaerobic organisms (Kirk *et al.*, 1979). However, it has been proven that large amounts of many heavy metals, such as copper, lead, cadmium or mercury seriously affect human health. The human body can not process and dispose of the metals, therefore the metals will deposit in various internal organs (Kirk *et al.*, 1979). Large deposits may cause adverse reactions and serious damage in the body. Excess of heavy metals can lead to diseases such as Wilson's disease, Argyria, cancer, heart and liver diseases, nervous system disorders and hypertension. Heavy metals form compounds in the body that can be carcinogenic and mutagenic even at very low levels (Ruiz-Manriques *et al.*, 1998). Therefore, not only the first generations are affected by heavy metals, but the effects can be passed on to second and third generations in the from of genetic abnormalities and birth defects. It is therefore essential for the present and future public health that people are not exposed to excessive levels of heavy metals in their diets.

Plants and crops also retain heavy metals if they are present in their growth environment, leading to accumulations of metals in food products. Today drinking water standards are enforced, but fishing waters, soil (Sims *et al*., 1990) and irrigation systems

may be exposed to large amounts of heavy metals. Using contaminated water for propagation (Crompton, 1997) lead to contaminated food products which may be distributed to the public and the end result still affects public health. This has happened even in the United States as recently as in the 1980's, with the run off selenium from farms in California (U.S. DI, 1997). Once these metals have entered into the environment they remain there indefinitely circulating, and will inevitably end up in the human food chain. To avoid the potentially deadly effects of heavy metal contamination the contaminants must be removed before they enter into the environment. Restricting the levels of heavy metals released in industrial effluent waters to safe levels is essential, and limiting discharges by total maximum daily loads prevents the initiation of a potentially deadly cycle.

2.2. The organism - *Penicillium ochro-chloron* ATCC 36741.

Penicillium ochro-chloron, a filamentous fungus in the ascomycete genus, was isolated in two different laboratories, one in Canada by P. M. Stokes, and one in Japan by M. Fukami. The organism was first isolated from electroplating baths containing high concentrations of copper and other metals (Fukami *et al*., 1983). The fungus is not known to be harmful to humans or animals, and therefore is not considered a biohazard. If cultured on agar plates it appears as a "closely interwoven basal felt of fine vegetative hyphae" (Raper, *et al.*, 1968). The white colonies appear cottony and floccose (Raper, *et al.*, 1968). Fungi which are grown in high osmolality are known to accumulate at least one intracellular solute having osmoregulatory characteristics (Gadd et. al., 1984). The fungus can be cultured on several types of agar and under many different growth

conditions with good results. *P. ochro-chloron* is known to have a high tolerance for a variety of heavy metals (Okamoto, *et al.*, 1977, Basu, *et al.*, 1955). Several studies show that *Penicillium ochro-chloron* not only can withstand high concentrations of heavy metals but it has a high copper requirement for optimal growth and sporulation (Gadd et al., 1984). It has also been shown that *Penicillium ochro-chloron* grown from spores from parent colonies challenged by copper in their growth medium, absorbs Cu^{2+} in aqueous media. The presence of copper in the growth medium for *P. ochro-chloron* has little to no effect on the growth rate of the fungus (Okamoto et al., 1974), but is required for optimum biomass increase; and the fungus has similar tolerance to zinc, manganese, iron, lead and cadmium (Okamoto et al., 1977).

P. ochro-chloron removes copper from contaminated aqueous solutions. A study was done where the fungus was grown for 7 days on samples of lake water supplemented with the components of glucose-mineral-salts medium (GMS). The copper concentration (0.02mg/L)in the water was reduced to zero, and the copper had accumulated in the fungus (3.637g total) (Stokes et al., 1979). *P. ochro-chloron* is often found in environments which contain high levels of copper that would normally be toxic to other organisms. Spores of *P. ochro-chloron* may in fact originate in the copper salt purchased from commercial suppliers. Fouling of high energy shock tubes filled with saturated $CuSO_4$ solution by *P. ochro-chloron* has plagued experiments at U.S. national physics laboratories.

2.3. Bioremediation as biosorption of heavy metals.

It is known that heavy metals are essential for the metabolism of many organisms as the constituents of metalloenzymes, but they can also be highly toxic above these trace concentrations (Kirk *et al*., 1979). Certain species of bacteria and fungi have the ability to grow in media containing heavy metals (Venkateswerlu, *et al*., 1989) and they also absorb the metal ions if exposed to them in aqueous solutions over a period of time (Mullen *et al*., 1989).

The use of biological systems and biological accelerants (when biological system acts as catalysts) is often referred to as bioremediation or natural attenuation (Nyer & Duffin, 1997). These concepts include phenomena like biodegradtion, dispersion, dilution, sorption, volatilization, and/or chemical or biochemical stabilization mainly of contaminants. Bioremediation can be used to effectively reduce contaminant toxicity, mobility, or volume to levels that are protective of human health and the ecosystem (Nyer & Duffin, 1997). Biosorption can be defined as the gathering of a substrate into a concentrated layer(s)or cluster, performed by a biological system. The actual pathway and mechanism for the removal of copper ions from solution by *Penicillium ochro-chloron* is not known. Influx of ions with diffusion into the fungus occurs, and there seems to be no energy-dependent uptake by *Penicillium ochro-chloron* (Gadd et al., 1985). Biosorption can be used to describe the result since there is a clear accumulation of copper complexes intertwined with the mycelia after copper challenge.

2.4. Bioreactors.

A bioreactor is a system which supports and may contain an organism during a desired process. Bioreactors can be used to produce biomass which in turn produce desired products. The bioreactors have defined and controllable environmental factors and are therefore an efficient way of exploiting capabilities of biological systems (Mueller *et al*., 1993). They have proven to be useful in both research and industry since the internal environment is usually easily manipulated and monitored.

In order for a process to be cost effective and feasible certain criteria have to be met. The final goal needs to be reached in a reasonable time frame and production cost should be as low as possible. Factors affecting the efficiency of a process and a system therefore have to be defined and optimized. Factors may be temperature, nutrient availability, oxygen supply and demand, pH, mixing, agitation and mass transfer. In simple systems (like shake flasks) the control of dissolved oxygen (DO) has proven difficult, as the mass transfer is dependent on the surface to liquid ratio and the availability of gas in the flask. Attempts to increase the surface area of the media and increase the flow of air proved to increase the DO in flasks (Tunac, 1989). In more advanced bioreactors the DO is controlled by directly adding gases to the media. Supplementing the medium with gases, along with good mixing greatly increases the oxygen transfer rate and thereby also the DO in the medium. Factors affecting the biological system can be controlled by using cooling water jackets (temperature), acid/base addition (pH), feed lines (supplies nutrients), and baffles/agitators (mixing and shear stress).

There are numerous bioreactors with varying configurations to meet different requirements, but there are two major groups of reactors – batch reactors and continuous reactors. Individual reactors can be classified in four different ways: 1. Based on the combination of the mode of substrate addition and the reactor geometry (i.e. continuous tubular packed bed), 2. Based on the configuration of the biomass in the reactor (i.e. freely suspended or immobilized), 3. The mode providing the mixing within the reactor (i.e. Mechanical agitation or gas agitation), and 4. Based on the type of biocatalyst or enzyme used in the reactor (i.e. aerobic microorganisms or anaerobic microorganisms) (Atkinson, 1991).

2.4.1. Packed-Bed Column Reactors

Packed-bed column reactors are commonly used in both industry and research for separation of molecules, determination of relative molecular mass, identification of substances or purification purposes (Wheelwright, 1991). These reactors can be used in both continuous and batch settings. Chromatography takes place in various columns using various characteristics of substrates. The packed-bed columns consist of the container and the bed contained within. The bed can be anything from glass beads to silicon or plastics. In some cases the bed consists of a substrate that is bound to the substance forming the bed, i.e. proteins attached to silicon beads. The target molecule or ion is then passed through the packed-bed, in hopes it will attach to the assigned ligand or substrate. The final step, in most cases, is to release the target molecule through, for example, a salt gradient (Scopes, 1994).

2.4.2. Fluidized-bed reactors

Fluidized bed reactors (bubble columns and air lift reactors) have several different phases to achieve mixing and mass transfer in the reactor. These reactors are generally used in batch settings. There is normally a liquid phase and a gaseous phase, and a third solid phase within the reactor. The solid phase may be biomass or binding substrate, generally attached to a substrate, like glass beads, but may be self-contained if the biomass can survive the physical environment. The general idea is to create mixing by allowing gases to rise through the liquid (Figure 1). The liquid phase generally contains the target molecule, and flows upward through the middle of the reactor and down along the edges, creating a fountain effect, as a result of the gaseous up-flow through the reactor. The target molecules adhere to the biomass or binding substrate, and once the reactor is harvested the target molecule is separated from the biomass or binding substrate.

2.5. Scale-up of Bioprocesses

The scaling up of biological processes for industrial use is accompanied by difficulties in reproducing the exact conditions used in the bench scale experiments. The requirements of the organism for nutrients and inorganic substrates needed for metabolism have to be maintained within the system and have to be accounted for in the scaling up process. There are also physical problems that need to be addressed such as the difference in the fluid motion larger reactors (Wheelwright, 1991). Other aspects include energy input and constant climate control in the reactor.

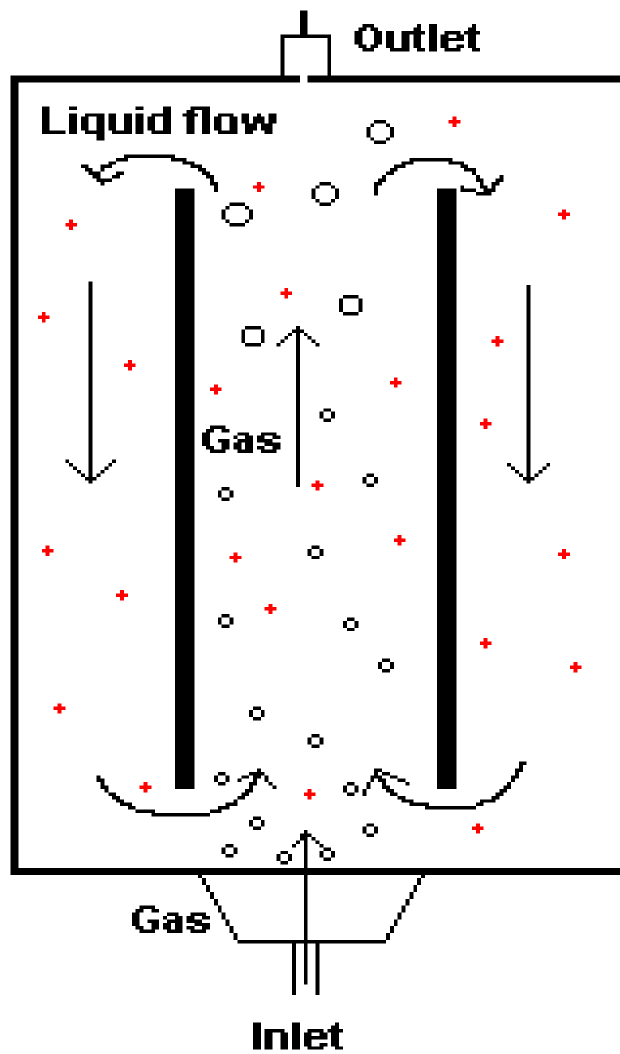

Figure 1. Schematic diagram of a fluidized bed (bubble column) reactor. The red particles represent the biomass or binding substrate.

3. Experimental Approach

3.1. General objectives

The main objective of this investigation was to investigate if *P. ochro-chloron* is a suitable model organism to use in the process of eliminating heavy metals from aqueous solutions. There is the possibility that either this fungus or another fungus has the ability to remove copper to meet the discharge standards required by federal regulations. The production of a basic functional and effective bioreactor design for the removal of copper in aqueous solution was the final goal. The reactor was based on an aqueous system containing Cu^{2+} ions in solution.

3.2. Specific Approach

The intention was to use *Penicillium ochro-chloron* or a product thereof, to produce a biological system for the removal of copper ions from aqueous solutions. The research was conducted using fungal beads and the main reaction vessel was the Bioflo III fermentation system. The Bioflo III was used to investigate variables and parameters that affect copper uptake, such as the DO concentration and temperature. The media contained all the essential nutrients for metabolism, along with copper. The first type of reactor investigated was a packed-bed column reactor, since previous work has been done using this system. The flow rate through the packed-bed was controlled and varied with a peristaltic pump. The media was pumped from the Bioflo III through the packed-bed column. Varying the flow rate would also change the time that any one molecule remains in the backed-bed. The residence time is affected by the flow rate in the following relationship:

$$\text{Residence time (sec)} = \frac{\text{Volume (L)}}{\text{Flow rate (L/sec)}} \quad \cdots\cdots\cdots\cdots\cdots\cdots (1)$$

Therefore:

$$\text{Residence time (sec)} \propto \frac{1}{\text{Flow reate (L/sec)}} \quad \cdots\cdots\cdots\cdots\cdots\cdots (2)$$

Changing the flow rate would allow the copper to remain shorter or longer time in the packed-bed and may affect the copper removal. Liquid samples were analyzed for copper concentration, nutrient concentration and pH. The ionic strength of the media was maintained throughout the different experiments. The assumption was made that the diffusion co-efficient of copper in the media remained the same over time. The investigation continued with a study of how different DO concentrations, varying biomass, varying copper concentrations and different reactor configurations affected copper removal.

Biosorption normally reaches an equilibrium, in this case where the amount of copper absorbed is equal to the amount that is released. Depending on the concentration of copper remaining in solution at the time the equilibrium was reached the suitability of the organism *P. ochro-chloron* for removal of copper ions could be concluded.

Materials and Methods

The chemicals used during the course of this work were all of analytical grade whenever available and were obtained from Sigma, Fisher, DIFCO or Mallinckrodt. The organism used for the copper uptake experiments was the fungus *Penicillium ochro-chloron* ATCC strain # 36741. Sterile technique in a laminar flow hood was used for the growth and maintenance of the fungus.

4. Media and Plates

4.1. Corn Meal Agar Plates with 400mg/L Copper

Corn Meal Agar plates were used to maintain the fungus and to produce spores. The growth medium contained 400mg/L copper as copper sulfate to challenge the fungus. Unless the fungus is challenged with the copper during sporulation, the spores produced will eventually lose their resistance to, and concomitantly, to remove copper from solutions when mature. The plates were prepared by dissolving 8.5 g Difco Corn Meal Agar in 500mL dH_2O and autoclaving for 20 minutes at 121°C. One mL of filter sterilized 10,000mg/L Cu^{2+} as $CuSO_4$ was added to each 100x15mm plate. Once the agar was cool enough to be handled, 24mL of the agar was pipetted onto each plate. The pipetting mixed the copper and agar sufficiently. The plates were cooled and stored under sterile conditions in 4°C until used.

4.2. Glucose Minimal Salts Media (GMS)

GMS, or glucose minimal salts, was used to grow the *P. ochro-chloron* beads. The media constitution was as follows: $NaNO_3$ 3.0 g/L, $MgSO_4 \bullet 7H_2O$ 0.75 g/L, KCl 0.75

g/L, KH_2PO_4 1.5 g/L, $CaCl_2$ 0.15 g/L, Tween-80 7.5mL/L. The Tween-80 was dissolved in 200mL of hot water before included in the media. Tween-80 is a detergent that causes the fungal spores to disperse, promoting the formation of fungal mycelia into beads. The media was sterilized by autoclaving for 25 min at 121°C.

4.3. New Minimal Media without Glucose (NMM-G)

NMM-G, or new minimal medium without glucose (-G), was the maintenance solution for *P. ochro-chloron* beads used in our lab. Essential nutrients for maintenance metabolism are provided, but at levels low enough to limit extensive growth. The media constitution was as follows: $NaNO_3$ 0.2 g/L, $MgSO_4 \bullet 7H_2O$ 0.5 g/L, KCl 0.5 g/L, KH_2PO_4 1.0 g/L, $CaCl_2$ 0.1 g/L. The media was sterilized by autoclaving for 25min at 121°C. NMM-G was the media used for all experiments, with the additions of copper sulfate, iron sulfate and glucose.

5. Fungal Spores and Beads

5.1. Spore Suspension Preparation

Cultures were maintained by using a plate on which a two-week-old culture of *P. ochro-chloron* had been grown and inverting it onto another, fresh Corn Meal Agar plate with 400ppm Cu^{2+}. Gentle tapping on the bottom of the culture plate transferred some spores onto the fresh plate. The plate inoculated with spores was placed in an incubator at 30°C, face up for at least two weeks. The plate with the two-week-old culture was flooded with 15 mL of sterile dH_2O. Flooding with water caused the hyphae to release the remaining spores into suspension. The spore suspension was transferred into a 15mL

sterile capped glass tube and stored at 4°C until ready for use. The spore suspension must be used within two to three weeks.

5.2. Testing the Spore Suspension

200mL GMS and 100mL of 18g/L glucose was added to a sterile 300mL Tunair shaker flask. Then 300μL of 10mg/mL $FeSO_4{\bullet}7H_2O$ and 250μL (5 drops) of spore suspension was added. The flask was then capped and placed in an orbital shaker at 30°C, agitating at 300rpm for two or three days. Once small beads appeared, an estimate of the number in the flask was made and the volume of spore suspension needed to obtain 40-60 beads per flask was calculated.

5.3. Growing Fungal Beads

In order to grow the number of beads required for an experiment, an estimation of the number of Tunair shake flasks needed was made, and 200mL GMS and 100mL 18g/L glucose was added to each flask, followed by 300μL 10mg/mL $FeSO_4{\bullet}7H_2O$ and the appropriate volume of spore suspension. The flasks were incubated in an orbital shaker at 30°C at 300rpm for four days. The beads used for all experiments were four-day-old beads unless otherwise noted.

5.4. Washing of Fungal Beads

After four days of growth, as much of the growth media as possible was decanted and discarded. A flamed metal mesh was placed over the opening of the Tunair flask and the remaining growth media was poured off. The beads were tapped back into the bottle and 150mL NMM-G was added. The flask was then incubated in an orbital shaker at

30°C, 200rpm for 30 minutes. This washing procedure was repeated twice for each flask. Finally all the beads were consolidated into a single flask and stored in a sealed conical flask at 4°C until used. Beads were not stored for more than one day before use.

6. Shake flask Experiments

6.1. Tunair Flask System

The shake flasks used for growth and experimental purposes were 300mL half baffled (2 baffles) Tunair flasks (Tunac, 1989). These flasks have a high aeration capacity as a result of their baffles. When the flasks are shaken, the baffles increase the surface area of the liquid by increasing the area at the liquid to gas interface. The flasks provide an effective method, allowing the use of smaller volumes of media for preliminary and basic studies. Generally, in a single sterile flask, 150mL or 200mL of total NMM media was used containing 2g/L glucose, 10mg/L $FeSO_4 \bullet 7H_2O$, varying $CuSO_4$ and varying the number of beads. The flasks were then placed in a rotary (orbital) shaker at 250rpm in the absence of light for the duration of the experiment. The shake was set at 30°C. However there was no cooling system in the shaker, it only prevented the temperature from dropping below 30°C.

6.2. Copper Uptake and the Effect on Removal with varying Biomass and Copper Concentration

To confirm that the fungus *P. ochro-chloron* did remove copper from aqueous solutions, the primary objective was to reproduce previous results. Beads were prepared and challenged with 100mg/L copper as copper sulfate in Tunair flasks with 150mL total

media. Samples of the media were taken at different time points for analysis of both copper and glucose content.

Secondly, an investigation of the possible effects of varying copper and/or biomass concentration on uptake was performed. Cultures of beads were grown and counted before being placed in Tunair flasks with 150mL total media. Different amounts of biomass corresponding to 5 beads (1mL), 25 beads (5mL), 50 beads (10mL) and 100 beads (20mL) were used. For each of the four conditions three different copper concentrations were tested; 20mg/L, 50mg/L and 100mg/L. Table 1 shows the variation in copper concentrations and biomass used.

6.3. Viability study

A primary concern for the studies to follow was the issue of whether the fungus needed to be alive to absorb copper. A viability study was conducted. Four cultures of equal bead number were grown. Three of the cultures were pretreated for two and a half hours with 0.5mM, 1mM and 2mM sodium azide, respectively. The fourth culture was pretreated in NMM only and served as the control. After pretreatment the cultures were transferred into shake flasks containing NMM with 100mg/L of copper, 2g/L glucose and 10mg/L iron sulfate. The reason the beads were removed from the presence of the azide was because of the possibility of the sodium azide complexing with the copper and affecting the assay results. Sodium azide inhibits the action of catalase and formate dehydrogenase at the third step in the respiration electron cascade. Since the fungus could not carry out respiration in the presence of azide, the results of this experiment would indicate if respiration is necessary. Liquid samples were taken to analyze the

Experiment	*Copper Concentration (mg/L)*	*Biomass*		
		Number of beads	*Volume (mL)*	*Dry weight (g)*
1	100	5	1	0.0123
2	100	5	1	0.0123
3	100	25	5	0.0615
4	100	25	5	0.0615
5	100	50	10	0.123
6	100	50	10	0.123
7	100	100	20	0.246
8	100	100	20	0.246
9	50	25	5	0.0615
10	50	25	5	0.0615
11	50	50	10	0.123
12	50	50	10	0.123
13	20	5	1	0.0123
14	20	5	1	0.0123
15	20	25	5	0.0615
16	20	25	5	0.0615
17	20	50	10	0.123
18	20	50	10	0.123
19	20	100	20	0.246
20	20	100	20	0.246

Table 1. Biomass and copper concentrations during shake flask experiments. Experiments with copper concentrations 100mg/L and 20mg/L were performed first. The 50mg/L experiments were later preformed with only two different amounts of biomass since the results of 5 and 25 beads were similar and 50 and 100 beads were similar.

changes in the copper concentration of the media over time. The experiment was maintained for 140 hours.

6.4. LA-lines Experiments

LA-lines were a series of experiments performed in an attempt to determine the significance of the genetic diversity of individual spores on copper uptake. Using plates that had been seeded 24 hours ahead of time, single colonies were picked from the plates and transferred onto fresh plates. The plates containing the single colony were then incubated for 7 days before being transferred onto fresh plates, which were then incubated for 14 days. The individual lines of fungus produced were cultured for a total of seven weeks before the spore solution was prepared. Theoretically, a single spore gave rise to an entire line of fungus since the single colonies were the result of the growth of a single spore. This way all the spores in any one solution prepared are assumed to be genetically identical. Five separate strains of *P. ochro-chloron* (LA1-LA5) were cultured in this manner. Shake flask experiments challenging beads grown from these spore solutions using media containing 100mg/L copper were performed to investigate if there was any effect on the absorption between the different lines.

6.5. Evaporation Study

In an attempt to account for the increase in the copper concentration noted in the LA-line experiments, an evaporation study was performed. Three Tunair flasks were weighed and then filled with 200mL of dH_2O and re weighed. They were then placed in under the same conditions in which the LA-line experiments had been kept under

(250rpm in an orbital shaker at 30°C), and the change in weight was monitored over time. The results of this study are reported in Appendix E.

7. Assays

7.1. Copper Assay (Bathocuproine Method)

7.1.1. Reagents

1+1 HCl. Mix half concentrated HCl and half dH_2O

$NH_2OH \bullet HCl$ (rmm= 69.49 g/mol). Dissolve **77.78g** in **700mL** dH_2O

30% Sodium citrate - $C_6H_5Na_3O_7 \bullet 2H_2O$ (rmm = 294.1 g/mol). Dissolve **210g** into **700mL** dH_2O

NaBathocuproine - $C_{26}H_{18}N_2O_6S_2Na_2$ (rmm = 564.5 g/mol). Dissolve **0.7g** into **700mL** dH_2O

7.1.2. Procedure

Tubes were acid washed and cleaned for this assay and all samples were be done in triplicate. The blank was 5mL dH_2O treated as a sample. Three standards were prepared by adding 5mL dH_2O into three tubes and then adding 10µL of 1000mg/L standard copper solution at pH 4. The theoretical volume of sample containing approximately 10µg of copper based on the original copper concentration was calculated. The total volume in each test tube needed to be 5mL. The calculated volume of sample was added to appropriate volume of dH_2O to give a final volume of 5mL. To each test tube, 100µL of 1+1 HCl, 500µL of $NH_2OH \bullet HCl$, 500µL of 30% Sodium citrate, and 500µL of NaBathocuproine were added. The test tubes were vortexed and incubated at room temperature for at least 10min, but not longer than 60min before reading the

absorbance at 484nm. Appendix A shows the calculations for converting the absorbance reading to amount of copper in mg/L.

7.2. Glucose Analysis using the YSI Glucose Analyzer

7.2.1. Calibration

The instrument was calibrated using a 1g/L glucose standard. The sample (200μL) was injected and if the reading deviated by more than 5% from the standard, the calibration was repeated.

7.2.2. Reading Samples

The syringe was filled with sample, once the membrane was rinsed and the display read zero/inject, the value was adjusted to zero and then the sample was injected. The syringe was rinsed repeatedly in dH_2O. The value read represents the glucose concentration in mg/dL of the sample. The membrane was cleared and the sample injection repeated for reproducibility. When the last sample had been analyzed the membrane was cleared and the machine turned to standby once the display read zero/inject.

7.3. Glucose Assay using the Colormetric Dinitrosalicylic Acid Method

7.3.1. Background

The DNS method tests the sample for the presence of any free carbonyl group (reducing sugar). Since the only reducing sugar in the media used was glucose there was no need to have a more specific assay. The method was achieved through the oxidation of the aldehyde functional group in glucose, while DNS was reduced to 3-amino,5-

nitrosalicylic acid under alkiline conditions (Miller, 1959). The sulfite was present to absorb the dissolved oxygen, which could interfere with glucose oxidation. Phenol was also added to the solution to enhance the color density.

7.3.2. Reagents

Dinitrosalicylic Acid Reagent Solution (1%) contains (per liter):
Dinitrosalicylic Acid 10g
Sodium Sulfite 0.5g
Sodium hydroxide 10g
Phenol 2g

40% by weight Potassium Sodium Tartrate solution

7.3.3. Procedure

Each sample (100μL) was diluted with 1.4mL dH_2O and 1.5mL 1% DNS reagent was added to each sample. The tubes were covered (not air tight), vortexed and placed in a 90°C water bath for 5-15 minutes. The tubes were promptly removed and placed in an ice-bath; 0.5mL of Potassium Sodium Tartrate was added to each tube to stabilize the color. All tubes were vortexed and read at 575nm in visible light within 60 minutes.

8. Packed Bed Experiments

8.1. Configuration and Preparation

Kontes Flex-Columns, I.D. 2.5cm, and height either 10cm or 20cm, were used for these experiments. The frits at the top and bottom of the column were removed, and replaced with a 1x1mm mesh to avoid clogging. The flow-adapter was drilled out to allow larger tubing to be used, also to avoid clogging. Both the columns and flow-adapters were acid washed, then surface sterilized using 95% ethanol. Prepared beads of desired volume were poured into the column immediately before the experiment was

started, and allowed to settle to the bottom of the column due to gravity, creating a packed bed. The flow adapter was inserted and a 1cm fluid head above the bed was created to allow beads to move slightly without clogging the inlet. A downward flow through the bed was used. A packed bed containing 60mL (or 10mL) of beads was used for uptake experiments. An acid washed 1L Bioflo III fermentor was used as the media reservoir for the column experiments. The Bioflo III system allowed for temperature control, aeration and agitation to control the dissolved oxygen tension in the media, and to ensure mixture of the media. The system also allowed for easy monitoring, sampling and sterilization.

Figure 2 shows the apparatus for the column experiments.

Measuring the dissolved oxygen (DO) concentration in the media flowing into and out of the column gave an indication of the activity of the beads in the bed. The inline DO (measured with a Microelectrodes, Inc. MI-730 oxygen electrode, 1/8") was measured by a voltmeter attached to a small DO probe, placed in the tubing exiting the column. The inlet DO was assumed to be the same as the DO in the Bioflo III reactor vessel (measured with a DO probe). The probes were zeroed by bubbling nitrogen gas into the media contained in the Bioflo (zero DO concentration) and pumping it through the bypass, past the inline DO probe. The probes were spanned to 100% saturation by bubbling air through the reactor until the DO concentration reached equilibrium. This value was set to 100%.

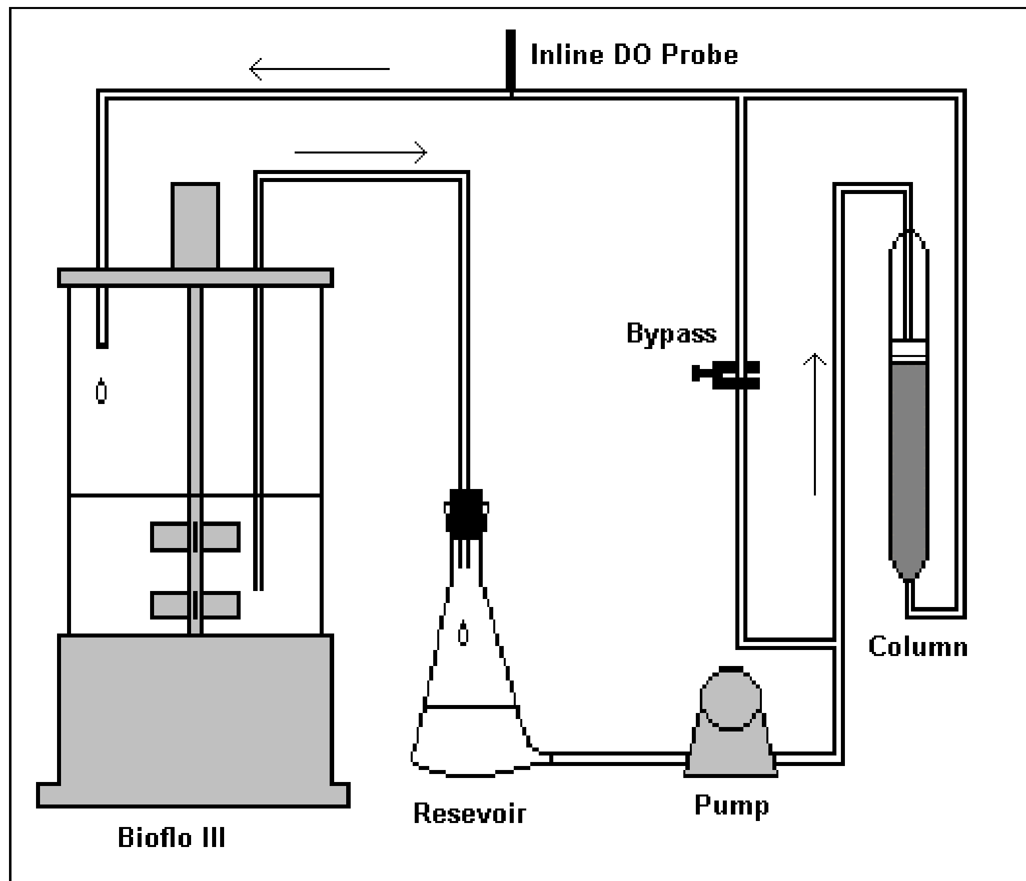

Figure 2. Schematic diagram of the packed bed column apparatus. The Bioflo III ensures mixing and uniform media, temperature control and DO control. The reservoir ensures no air bubbles enter the packed bed. The bypass allows for calibration of the inline DO probe without passing media through the column.

8.2. Experimental Setup

To evaluate the physical conditions affecting the copper removal of the fungus, a series of experiments were performed where temperature, DO, glucose concentration, media flow rate through the column, and copper concentration were varied and the effects monitored. Table 2 shows the different experiments and the physical aspects that were altered. Each experiment was continued for 69 hours, unless clogging forced the premature termination of the experiment due to large pressure drops across the column.

8.3. Conductivity Experiments

In order to investigate the channeling effects created in the packed bed column experiments, pulse chase tracer experiments were performed. The conductivity profile of a column of fungal beads after injecting a bolus of salt, compared to either solid or completely permeable phases would give an indication of the channeling effects in the packed bed. Three different phases were used to produce indicative conductivity profiles of the different substances. Polycarbonate beads are impermeable to water and were used to create a profile corresponding to complete bypass of the beads. Media with no beads was used to create a profile for no resistance and finally the *P. ochro-chloron* beads were used to create the profile for the packed bed experiments.

9. Fluidized bed experiments

9.1. Effect of Oxygen Concentration on Copper Removal

9.1.1. Configuration

Fluidized bed reactors consist of at least two different phases – liquid and solid (biomass). The configuration for copper removal by the fungal beads contained three

Experiment #	*DO* (%)	*[Copper]* (mg/L)	*Glucose* (g/L)	*Temperature* (℃)	*Flow rate* (mL/min)
1	90	100	0.2	20	25.5
2	90	100	2	20	25.5
3	90	100	2	20	25.5
4	90	100	2	20	25.5
5	90	100	0	20	25.5
6	90	1000	2	20	25.5
7	90	1000	2	20	25.5
8	90	1000	2	20	8
9	90	1000	2	20	8
10	90	200	2	25	0.56
11	90	100	2	25	8
12	90	100	2	25	8

Table 2. Physical conditions in packed bed column experiments. The 10cm packed beds consisted of 60mL beads (0.738g dry weight), and were run for 69 hours if conditions allowed. 1250mL media was used except for experiment 1 where 1000mL was used. The increase in volume was a result of the addition of the 'reservoir', which contained 200-250mL media.

phases – gas (air/nitrogen), liquid (media) and solid (biomass). The reactor was run in batch mode. The media containing nutrients and copper along with the fungal beads were all contained in the Bioflo III system fitted with a 1.25L vessel. The Bioflo III acted as the reactor, allowing for control and monitoring of physical factors (DO, pH, temperature and mixing).

9.1.2. Dynamic Gassing Out Experiments

The critical oxygen concentration is a key indicator of an organisms dependence on aeration for metabolism. Below the critical oxygen concentration oxygen becomes the limiting nutrient for metabolic rates. In order to estimate this value for *P. ochro-chloron* a series of experiments using the dynamic gassing out methods were performed. A fluidized bed reactor containing 70mL beads and 900mL media was allowed to equilibrate at a DO of 80%. The air flow was shut off and the DO in the media was monitored over time to determine the OUR for the culture. To determine the critical oxygen concentration the DO must be allowed to drop until the slope is no longer linear (Stanbury, *et al.*, 1995). The graph produced by this data allowed calculations based on the slopes produced estimating the OUR for the fungus. The OUR experiment was performed at three different times to evaluate if the oxygen demand changes over time.

9.1.3. Experimental Setup

To determine the effect of dissolved oxygen concentration on the removal of copper ions by the fungus, a series of experiments were conducted. The bioreactor was set up under identical conditions for each experiment, varying only the DO concentration in the media. Based on the critical oxygen concentration for *P. ochro-chloron*, five

different DO concentrations were used: 10, 20, 30, 50 and 80%. Each experiment contained the beads produced by six Tunair shake flasks (varying from 40mL to 70mL beads), 900mL media containing 100mg/L copper, 10mg/L iron sulfate and 2g/L glucose. The temperature was maintained at 25°C, the air flow at 1L/min and the agitation between 100 and 125 rpm. The experiments were maintained for at least 69 hours before termination.

9.2. Gas mixer and Advanced Fermentation Software - ASF

9.2.1. The Gas Mixer

As the name implies the gas mixer allows for two gases to be mixed before they enter the Bioflo III system. The system allows for user defined equations to control DO using the two gases. The gas mixer can be used where there is a high oxygen demand by supplying pure oxygen to the system or if a low DO is desired by supplementing air with nitrogen gas. The gas mixer was used to lower the DO of the system below 50% by using and mixture of air and N_2. The gas mixer uses the current DO value to adjust the amount of air and N_2 supplied to the Bioflo. This way a set DO may be maintained even if the oxygen demand in the reactor changed over time.

9.2.2. AFS – Advanced Fermentation Software

AFS was used to control the gas mixer input to the Bioflo III gas supply. The software allows the control of the gas mixture supplied to the Bioflo. The AFS allows the use of control equations to set the DO value desired and to leave an error margin. When in use, the equation allows for a 10% variation in the DO before changing the gas mixture used to aerate the system. The following are the specifications and equations used to control the DO. The settings and control equations used during the experiments were as follows:

Gas 1 = Nitrogen

Gas 2 = Air

Unit 1 = Bioflo

Unit 2 = Gas mixer

$$\text{Control equation for unit 1: AIR} = @\,2\text{GASA2.SP} \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (4)$$

$$\begin{aligned}&\text{Control eqation for unit 2:}\\&\text{GASA2.SP} = \text{LIMIT(GSA2.SP} = ((0.5 \times \text{CMP}\\&(\text{X}, @1\text{do2.cv})) - (0.5 \times \text{CMP}(@1\text{DO21.CV}, \text{Y}))), 0, 100) \cdots\cdots\cdots\cdots\cdots\cdots (5)\end{aligned}$$

where x=(desired DO-5), and y=(desired DO +5)

9.3. Maximum biomass capacity for fluidized bed

An experiment to determine if more biomass would remove the copper in less time than previously shown was executed. Using the fluidized bed reactor setup with 900mL media containing 100mg/L copper, 10mg/L iron sulfate and 2g/L glucose and with 110mL beads. The temperature was maintained at 25°C, the airflow at 1L/min, the DO maintained at 80% or above and the agitation between 100 and 125 rpm. The experiment was maintained for 70 hours, before being terminated.

9.4. Uptake of low copper concentrations using a fluidized bed

To validate the shake flask experiments performed at lower copper concentrations, an experiment in the fluidized bed was performed with lower copper concentration, to determine if uptake of copper at lower concentrations was in any way inhibited by the lack of oxygen. The experiment used the fluidized bed reactor configuration with 900mL total media, containing 20mg/L copper instead of 100mg/L. The media also contained 10mg/L iron and 2g/L glucose. Temperature, airflow and agitation were the same as in previous experiments.

9.5. Mixing or Diffusion Limitation

Mixing in the fluidized bed reactor is essential in order to make the assumption that the media is uniform throughout the reactor. The mixing conditions used for the fluidized bed experiments are relatively benign. If the fungus consumes oxygen at a higher rate than which it is being supplied, the copper removal could be limited as a result of the low mixing conditions. A series of experiments varying mixing conditions was setup to allow the calculation of the mixing time. The mixing conditions were altered mainly by changing the rate of aeration in the reactor and by lowering the agitation. To assess the mixing time of the reactor a change in pH was created by adding 0.5mL of 50% NaOH by weight to the media and monitoring the pH.

10. Dry weight Determination

An effective method of determining the dry weight of the fungus proved difficult to achieve. Fresh weight determination would be difficult since the beads contained varying amounts of water. Weighing the beads when dehydrated was the chosen method of determining the weight of the biomass. The beads were lyophilized to dehydrate them and then weighed. If the beads were weighed after the completion of an experiment the weight of the copper bound would be included. However, removing the copper from the beads by acid washing could possibly remove more than just copper and result in a depressed actual dry weight. A standard curve was created by plotting the dry weight of known volumes of untreated beads. The volume of the beads was determined using a graduated cylinder and allowing the beads to settle to the bottom due to gravity, while submerged in NMM. The beads were then washed in dH_2O several times to remove the

NMM and lyophilized (Virtis Freezemobile 12LS). Also, several beads with a diameter of 3mm were examined for variation in density and elasticity through drop and compression tests.

11. Electron Microscopy

To establish if the fungus removes the copper intracellularly or if the copper is retained extracellularly, scanning electron microscopy of treated beads was performed. Comparing the surface of untreated beads with that of copper challenged beads would allow for extracellular analysis. The beads were removed from a copper solution (original concentration 100mg/L), washed in dH_2O three times to remove any remaining media. The beads were then frozen either using liquid hexane (-80°C) or in a -20°C freezer, and lyophilized to remove fluids. The beads were then cut in half and mounted on 2cm stubs and coated with gold palladium, and viewed using a JEOL JSM-5200 scanning electron microscope at an accelerating voltage of 25kV.

Results

12. Shake Flasks

12.1. Copper Absorption Studies

Figure 3 shows the results of an experiment using fungus beads, challenged in medium containing 100mg/L of copper as copper sulfate. The purpose of this experiment, along with following experiments, was to establish a control for copper absorption by *Penicillium ochro-chloron*. Previous research had shown that the fungus removed copper from aqueous solutions over time (Okamoto, *et al.*, 1974 & 1977). This shake flask control would allow for a relative comparison of absorption using various bioreactor configurations. In the first trial the fungus removed the majority of the copper in solution and retained it over time. The following experiments investigated the effect of copper concentration on the absorbance potential. They included the investigation of the effects of biomass on absorption as well. Figures 4a-d show the effect of varying the biomass load at constant copper concentration. For biomass concentrations of 0.0123g dry weight/L, 0.0615g dry weight/L, 0.123g dry weight/L, and 0.246g dry weight/L the copper absorbed was 65%, 94%, 94% and 94% respectively. Figures 5a-c show the effect on copper removal, of varying the initial copper concentration. Overall the fungus absorbs copper well at higher concentration of copper (100mg/L), but less effectively at lower concentrations (50 and 10mg/L). Removal was 94% for 100mg/L and only 37% and 19% (at termination) for 50mg/L and 10mg/L, respectively.

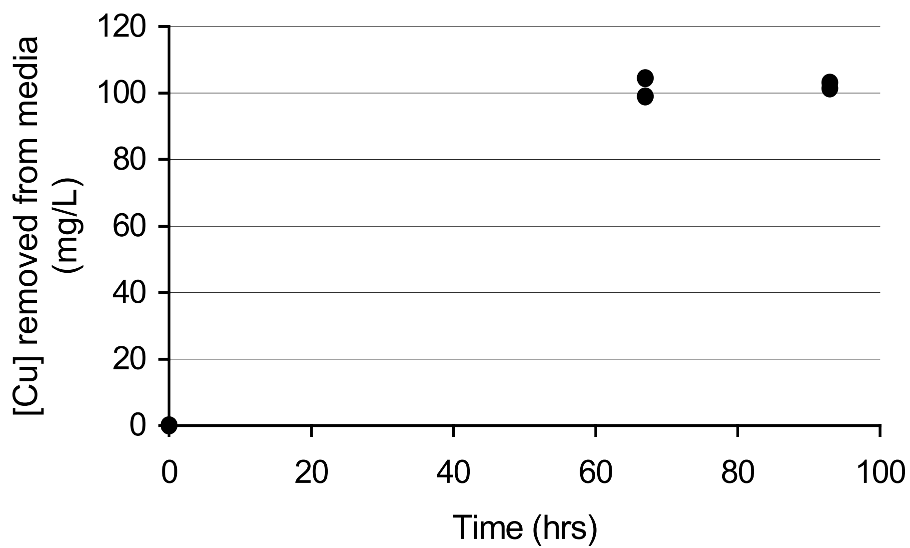

Figure 3. Absorption study using fungal beads prepared from original spores. The graph represents two experiments executed under identical conditions. The copper concentration at the beginning of the experiments was approximately 100mg/L. The biomass was approximately 0.62g dry weight. The absorbance capacity is approximately 165mg Cu/g dry weight with a residual copper concentration of 3.8mg/L (96.4% uptake).

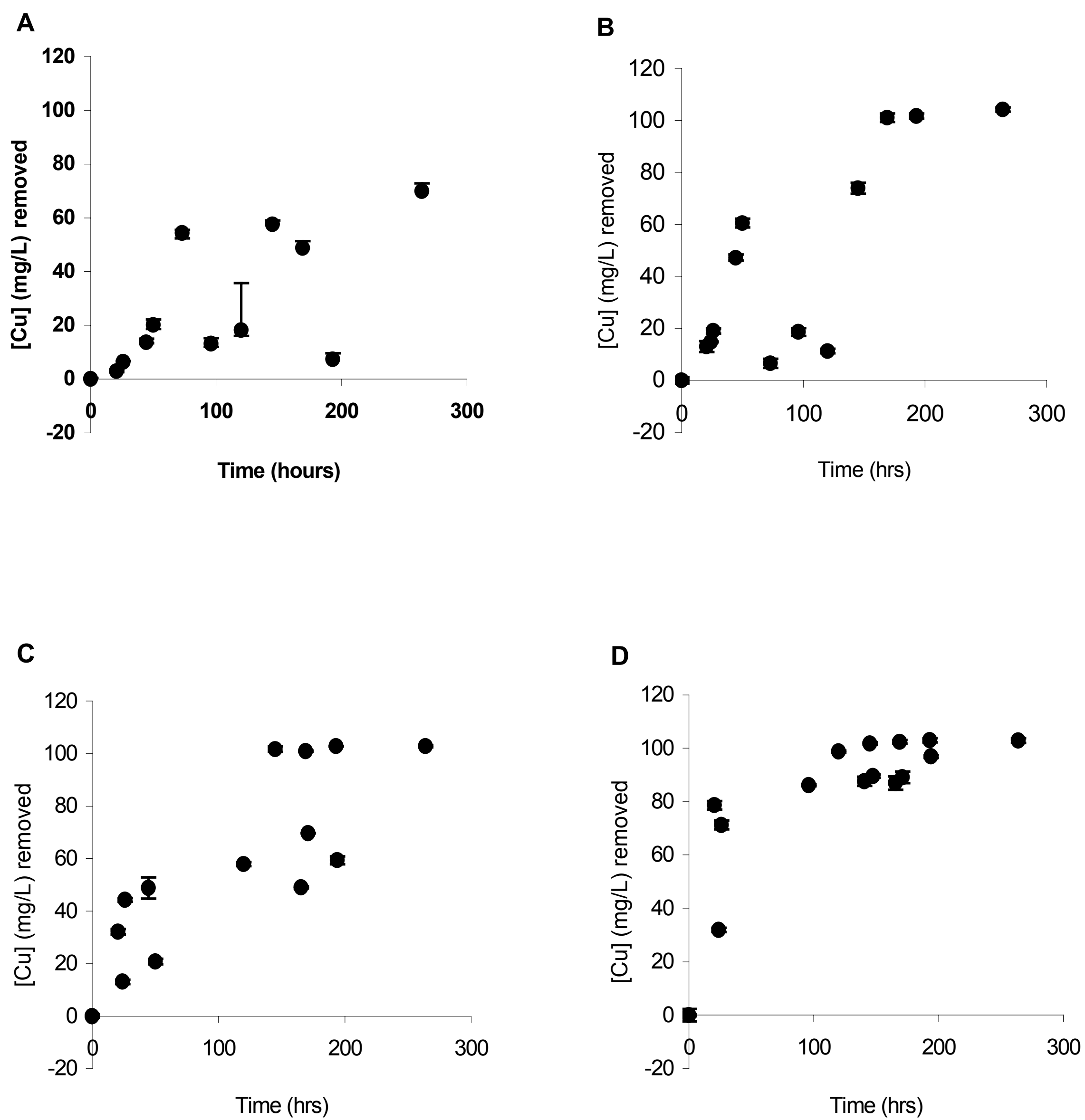

Figure 4. Copper removal from 100mg/L solution over time, **a**) 5 beads – 65% removed, **b**) 25 beads – 94% removed, **c**) 50 beads – 94% removed, and **d**) 100 beads – 94% removed.

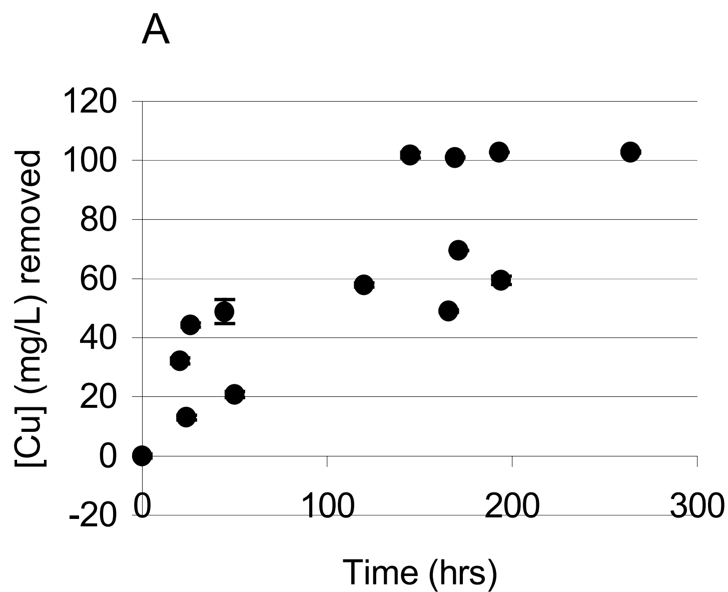

Figure 5a. Copper removal at 100mg/L copper concentration by 50 beads (dry weight ~0.12g biomass). At termination of the experiment the copper removed was 104.4mg/L (94% removed), which was also the maximum uptake.

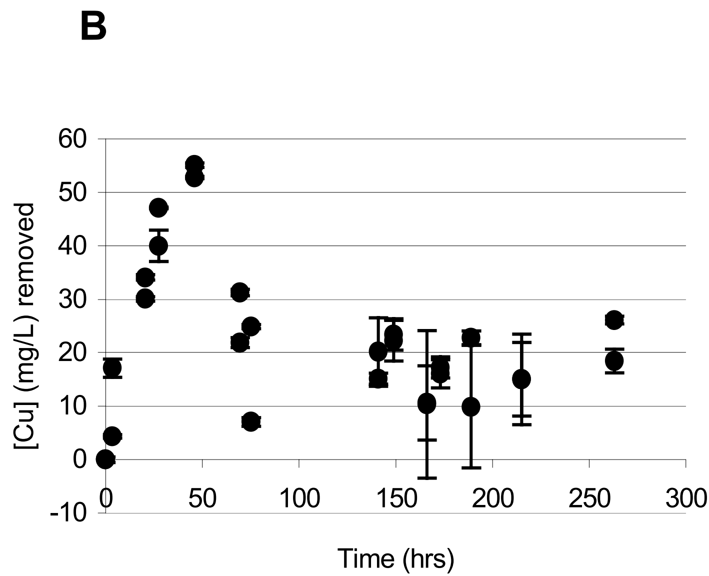

Figure 5b. Copper removal at 50mg/L copper concentration by 50 beads (0.12g dry weight). Initial burst of uptake, then approximately half was released again. At termination of the experiment the copper removed was 22.3mg/L (37% removal), while maximum uptake was 55.1mg/L (that is 91%).

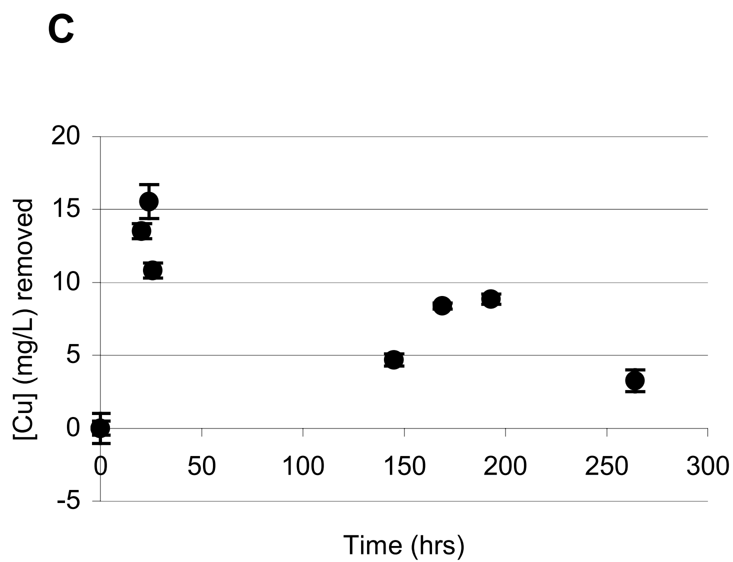

Figure 5c. Copper removal at 20mg/L copper concentration by 50 beads (0.12g dry weight). Also, initial burst of uptake, and release over time. At termination of the experiment the copper removed was 4.5mg/L (19% removal), while maximum uptake was 16.8mg/L (that is 70%).

12.2. Viability study

Figure 6 contains the summary of the viability study. As expected, for the first 20-25 hours beads pretreated with sodium azide did not remove copper as efficiently as the untreated beads. However, the inhibitory effects of the pretreatment seem to wear off over time, since the final copper removal for all flasks was approximately the same. This effect could be the result of dilution of the azide over time allowing respiration to resume. Recall that azide was not present in the medium during the copper challenge, since it would interfere with copper precipitation. Previous studies using conductivity within the beads proved that diffusion out of the beads takes several hours and would account for the lag time found before absorption occurred (Gudmundson).

12.3. LA-lines study

Copper uptake studies using LA-lines 1-5 indicate that the potential for significant genetic diversity exists in the *P. ochro-chloron* examined. The wide variation in uptake noted in Table 3 suggests that the potential exists within the population for poor uptake. LA-line 2 showed no uptake of copper whatsoever, indicating potentially a dramatic degree of diversity in the population. Whether this is a result of genetic diversity within the population or some other factor is not known and is beyond the scope of this study. An explanation for the slight increase in the copper concentration noted could be the result of evaporation of the media over time (see Appendix F).

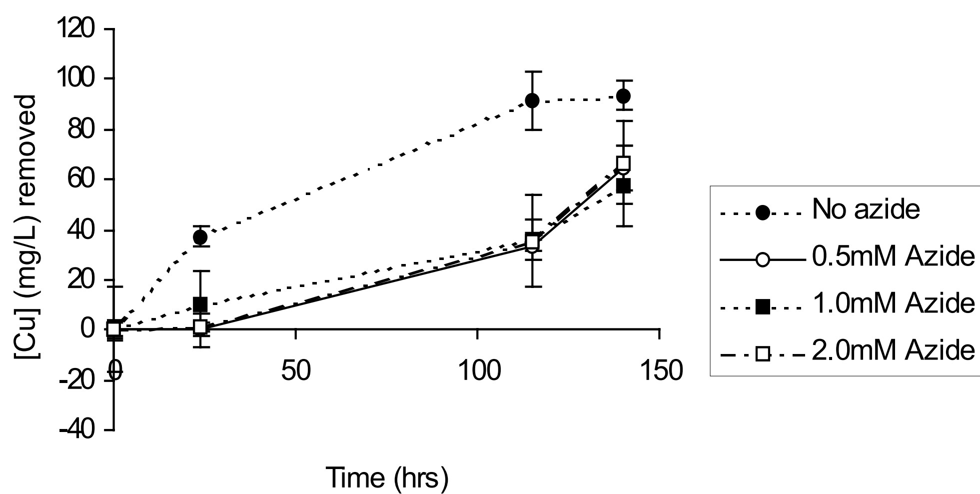

Figure 6. The effect of pretreatment with sodium azide on copper uptake. The beads pretreated with the azide show lag in the absorption of copper during the first 20 to 25 hours of the experiment. All samples pretreated show less uptake over time.

Time (hrs)	*LA 1*		*LA 2*		*LA 3*		*LA 4*		*LA 5*	
	[Cu] (mg/ L)	*Glu (g/L)*	*[Cu] (mg/ L)*	*Glu (g/L)*	*[Cu] (mg/ L)*	*Glu (g/L)*	*[Cu] (mg/ L)*	*Glu (g/L)*	*[Cu] (mg/ L)*	*Glu (g/L)*
0	98.7	1.91	98.7	1.91	98.7	1.91	98.7	1.91	98.7	1.91
40.5	76.7	0.9	77.5	1.35	50.5	1.02	58.6	1.22	50.9	1.05
64	110.6	0.145	94.0	0.84	99.0	0.19	109.3	0.57	98.1	0.33
88.75	115.2	0	122.0	0	99.7	0	96.1	0	107.4	0
160.25	59.6	0	-	-	50.5	0	56.3	0	76.9	0

Table 3. LA line shake flask results. LA lines 1, 2, 3, 4 or 5 in medium containing 100mg/L copper and 50 beads were used in each experiment. The overall trend was the same within the five lines, however; the copper removal patterns did not correlate to previous experiments under the same conditions. The slight increase in the copper concentration noted at times 64 and 88.75 hours could be the result of evaporation in the flasks. In LA2 there was no sample taken at time 160.25.

13. Packed Bed Column Experiments

13.1. Copper Absorbency

The packed bed was configured as shown in Figure 2 (Material and Methods). Varying conditions of medium flow rate and inlet D.O. concentration were tested to determine the effect on copper uptake. The trial run proved that copper absorption was possible in the packed bed, even though the uptake was considerably less than in the shake flask experiments. 36% removed for packed bed vs. 94% in shake flasks. The conditions were as follows: 10cm bed, 20ºC, 90% D.O., 700rpm agitation, 25.5mL/min flow rate through column. Figure 7 is a representative graph of the results of the packed bed column experiments, and table 4 is an overview of the results of the experiments. Experiments routinely had to be terminated prematurely due to clogging of tubing by mycelial build up. Varying the inlet DO and the medium flow rate seemed to have little or no effect on the overall copper removal by the fungus. At higher media flow rates there was a slight increase in copper absorbance, but the beads were compressed due to increased pressure drop across the column. It was theorized that the low D.O. values at the outlet was an indication that beads in the bed were not receiving enough oxygen for respiration.

13.2. Pulse-Chase Analysis of Flow Patterns

To investigate the flow patterns in the packed bed a comparative study between fungal beads and solid polycarbonate beads was performed. The pulse-chase procedure produced curves indicating that there are channeling effects in the fungal packed bed column. Figure 8 shows the profile curves produced by the conductivity experiments. The bell shaped curve indicates that there are channeling effects, since the polycarbonate

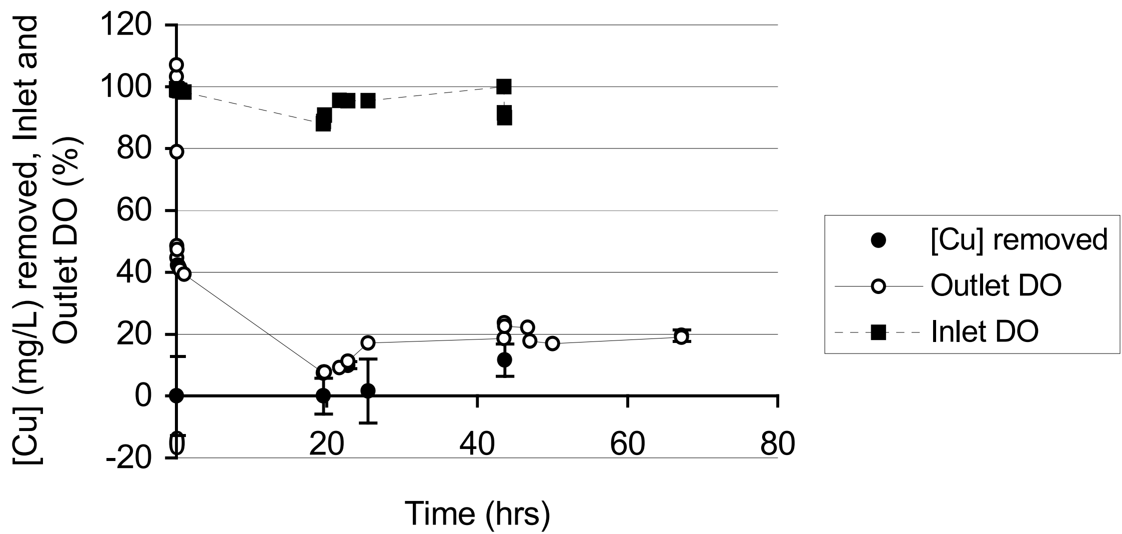

Figure 7. A representative example of the changes in physical conditions during a packed bed column experiment. The secondary Y-axis show the values for the pH and for the glucose concentration in the media.

[Copper] *(mg/L)*	*[Glucose]* *(g/L)*	*Temperature* *(°C)*	*Flow rate* *(mL/min)*	*Maximum uptake* *(mg/L)*	*Uptake capacity* *(mg [Cu]/g dry)*	*Lowest DO inline* *(%, sustained)*	*Average pH*	*Glucose consumption* *(g glucose/g dry wt)*
100	0.2	20	25.5	24.0	32.5	25.9	4.25	44.7
100	2	20	25.5	16.2	22.0	44.2	4.23	0.2
100	2	20	25.5	11.2	11.2	----	4.14	0.1
100	2	20	25.5	16.1	21.8	32.0	4.15	0.3
100	0	20	25.5	7.5	10.2	49.1	4.09	19.0
1000	2	20	25.5	27.9	37.8	46.0	3.69	0
1000	2	20	25.5	0	0	98.5	3.65	0.4
1000	2	20	8	0	0	----	3.68	0.4
1000	2	20	8	121.1	164.1	----	3.69	0.4
200	2	25	0.56	0.9	1.2	22.3	4.01	0
100	2	25	8	3.0	4.1	34.8	4.04	0
100	2	25	8	40.2	54.5	7.4	4.25	0

Table 4. Summary of 10cm column packed bed experiments. The maximum uptake is the amount of copper removed from the media, and the capacity is the amount of copper removed per gram dry weight of the fungus. The lowest inline DO reading is based on sustained readings after the first 30 minutes of the experiment.

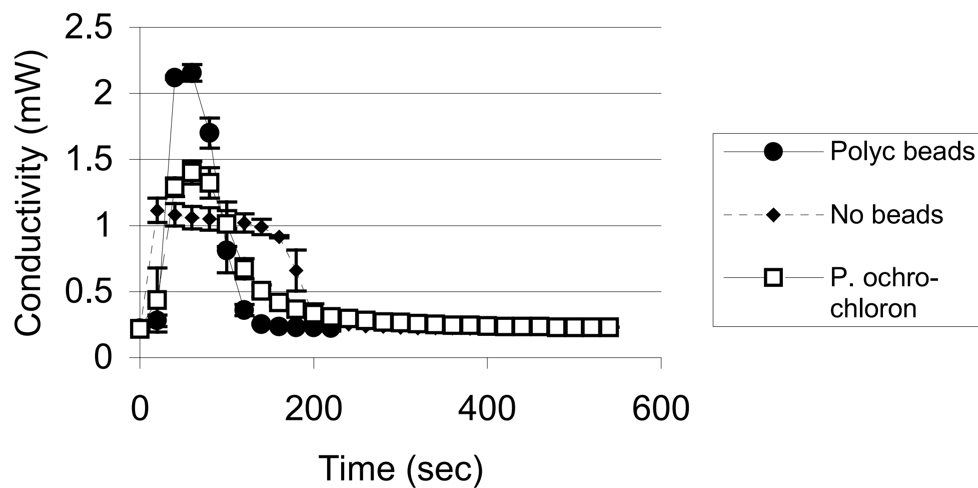

Figure 8. Conductivity profiles in a bed of Poly-carbonate beads, a bed of fungal beads and no bed, after pulse-chase injections. Conductivity is measured in milliohm (mW). The shape of the curve produced by the poly-carbonate beads and the fungus beads are similar indicating that the fungus packed bed experience channeling effects. The delay in the release (t≈110-200) in the fungus beads indicate some diffusion into the beads, but very limited.

beads produced this shape: i.e. the entire volume of liquid delivered bypasses the bead. The no resistance curve shows the effects of the mixing caused by the inflow of media. If the media actually was flowing through the fungus beads in the packed bed one would expect a prolonged decay to zero after the peak eluted. This result indicates that metal uptake is predominantly diffusion mediated.

14. Fluidized Bed Experiments

14.1 Effect of Varying Dissolved Oxygen Concentrations

The advantage of using a fluidized bed for this application is that the entire suspension (of beads) is exposed to a highly enriched environment in terms of oxygen availability. After observing the motion of beads at different agitation speeds and adjusting agitation accordingly, the first experiment performed proved to have better potential for copper removal than the packed bed configuration. The maximum copper removal was approximately 93%. To investigate the theory that the fungus requires oxygen to remove copper, the dissolved oxygen concentration was varied between 10% and 80%. These results are shown in Figure 9. The critical oxygen concentration of *P. ochro-chloron* is approximately 0.847mg O_2/L or 20% saturation in air based upon dynamic gassing out measurements (see methods, section 9.1.2). When the DO was maintained at or below the critical oxygen concentration, a decrease in copper absorption was noted. Using a D.O. higher than the critical oxygen concentration caused a drastic increase in the copper absorbed. Even though the 30% DO is above the critical oxygen concentration, poor mixing in the reactor to prevent shear damage to the beads results in non-homogenous conditions. Therefore the entire reactor sees an average D.O. of 30%,

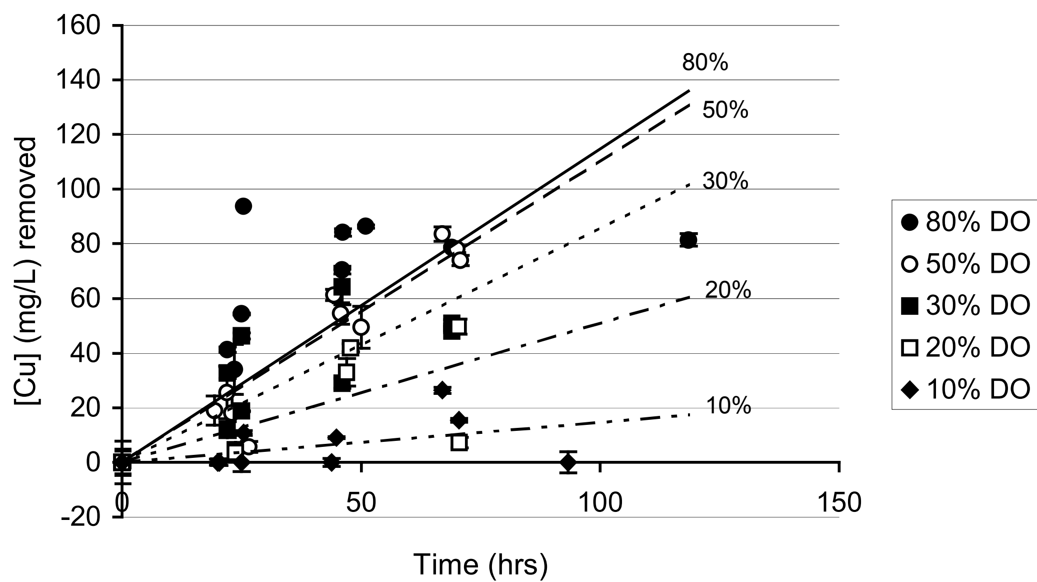

Figure 9. Overview of copper uptake from the fluidized bed experiments. The lines are the extrapolated best-fit lines with an Y-axis intercept of 0. The critical oxygen concentration of the organism is approximately 20% DO (based on air).

but some portions of the reactor will be below 30%. This effect is eliminated when the D.O. is at 50% saturation or higher.

14.2 Absorbency rate and the effect of increasing biomass.

The conditions used in the dissolved oxygen experiments had proven to require 70 hours or longer for optimum copper removal. A reduction in the process time would be preferred for development of practical applications of this technology. By increasing the biomass in the batch reactor the process time for optimum copper removal should be reduced. The results for a standard biomass load of approximately 0.615g dry weight (50mL beads) vs. 1.353g dry weight (110mL beads) are shown in Figure 10a, the goal was to maintain the D.O. at 80% saturation. Figure 10b is the overview of the conditions during the experiment, proving that the increased biomass requires higher oxygen transfer into the reactor since the DO dropped as low as 60% saturation. What is important to note in Figure 10a is that after an initial burst of uptake at 20 hours, the uptake then decreased in the experiment with the increased biomass load. This effect was probably due to some other nutrient being limiting with increased biomass. The NMM was optimized for a specific biomass concentration in shake flasks.

14.3 Effect of lower copper concentration on uptake.

Figure 11 shows copper uptake in a fluidized bed using a 10mg/L copper solution compared to shake flasks. In both cases copper is absorbed early in the experiment only to be released back into solution over time. Also, both systems achieve the maximum removal around 20 hours into the experiment and the removal is over 70%. However, in the fluidized bed configuration all of the previously bound copper was released, while in

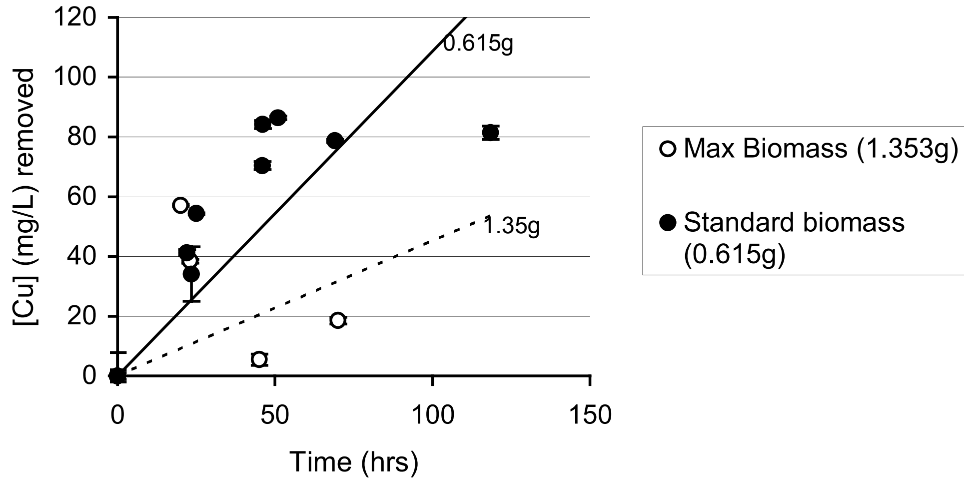

Figure 10a. A comparison between copper removal at 80% DO for two different biomass loads. The higher biomass load was poorly supported by the reactor as the dissolved oxygen concentration in the Bioflo III dropped well below 80%.

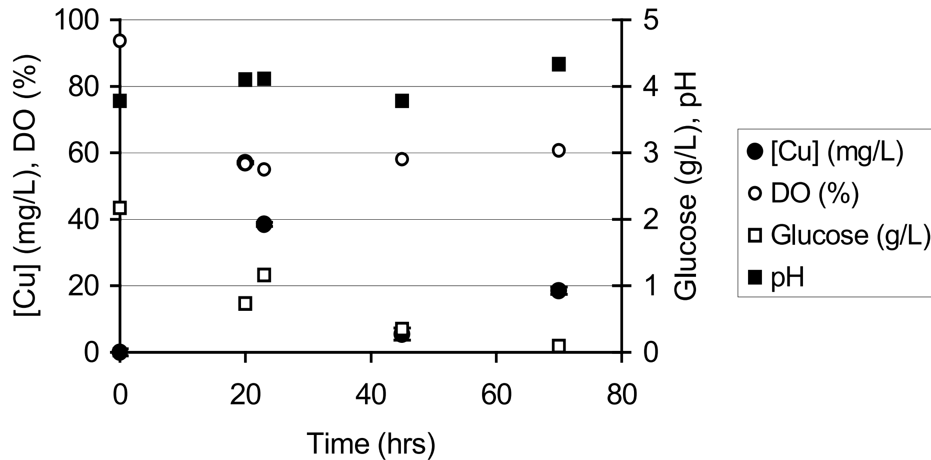

Figure 10b. An overview of the physical environment when using increased biomass. The glucose is completely consumed and the DO drops to about 60% increasing the agitation and shear stress on the biomass. Since the glucose is consumed the reduced copper removal could be a result of glucose limitation.

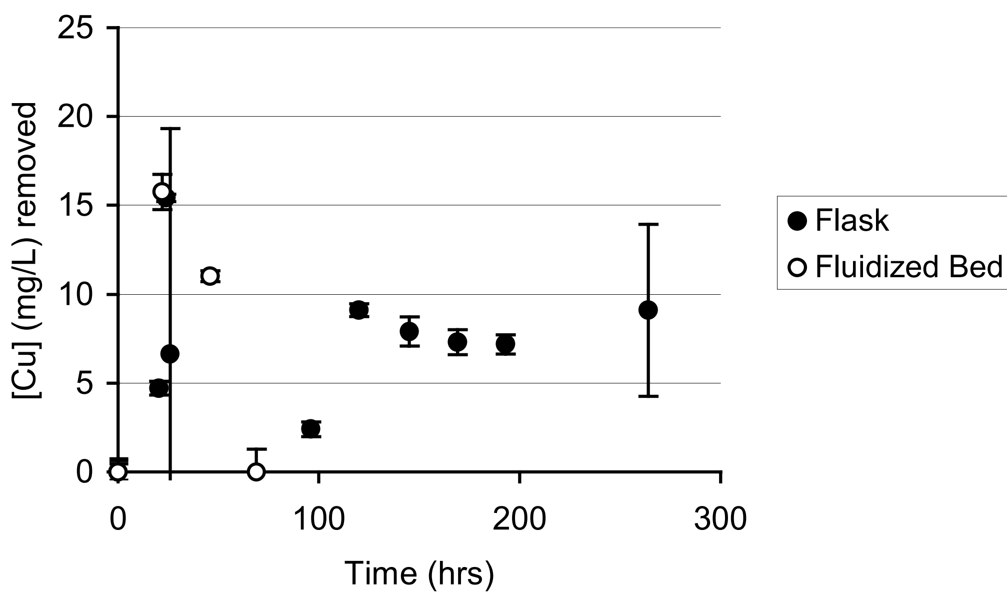

Figure 11. Comparison of copper removal between the fluidized bed and shake flask configurations. The overall trend for copper uptake between the shake flasks and the fluidized bed was similar. At approximately 20 to 25 hours into the experiment the maximum removal was achieved after which copper was released back into solution. In shake flasks approximately 73% removal was achieved and in the fluidized bed approximately 87% was removed.

the flasks some copper was retained (approximately 41.9% was retained, while the maximum copper absorbed was 72.4%).

14.4 Determination of oxygen uptake rate (OUR).

The OUR was determined by implementing the oxygen purge (dynamic gassing out, Materials and Methods section 9.1.2) to an otherwise stable system. The OURs at the three different time points are shown in Table 5a and vary over time as a result of the changing oxygen demand of the fungus. Calculations for the OUR and the mixing times are explained in Appendix E.

14.5 Homogeneity in the Fluidized Bed: Mixing Issues

Table 5b shows the different mixing times under varying aeration and agitation conditions. Mixing times ranged from two minutes to as high as three and a half minutes for the various conditions tested. Due to the strict requirement of low agitation speeds, the maximum agitation used was only 115rpm. Aeration had little effect on mixing in the fluidized bed.

15. Electron Microscopy (see also Appendix G)

Untreated beads were compared to copper challenged beads using the scanning electron microscope. Using the electron microscope it would be possible to visually determine if the fungus ingests the copper it removes, or if the copper is somehow precipitated extracellularly. The electron micrographs taken are shown in Figure 12a and b, where 12a is the cross section of an untreated bead and 12b is the cross section of a copper challenged bead. The copper exposed bead has large structures present nestled between the fungal filaments. The microspheres that appear in the challenged beads are

Time *(hrs)*	***Slope of Line*** *(mmol O_2/hrs)*	***Biomass*** *(g dry weight)*	***OUR*** *(mmol/hr g dry weight $^{-1}$)*
0	-0.0422	0.861	0.049
24	-0.1104	-	0.128
49	-0.239	-	0.278

Table 5a. Oxygen depletion times in the fluidized bed reactor. The slopes produced by plotting the decrease in the DO during dynamic gassing out against time and the OUR values at three different time point during a fluidized bed experiment. The oxygen demand increases over time as the copper removed increases proving that the fungus does need oxygen to continue removing copper from solution. Not much glucose was consumed during the experiment and the biomass is assumed to remain constant.

Condition	***Agitation*** *(rpm)*	***Aeration*** *(L/min)*	***Mixing time 1*** *(min)*	***Mixing time 2*** *(min)*	***Ave. mixing time*** *(min)*
Original	115	1	3-3.25	3	3
More Air	115	2.5	2.5	2.5	2.5
Max Air	115	4	2	1.75-2	2
Less Air	115	0.6	3.25-3.5	3	3.25
Agitation	50	0.6	3.5	3.5	3.5

Table 5b. Mixing times in the fluidized bed reactor. Increasing the agitation increased the shear stress and breakage of beads. Under all the mixing conditions the mixing time did not exceed four minutes. (Visual observation)

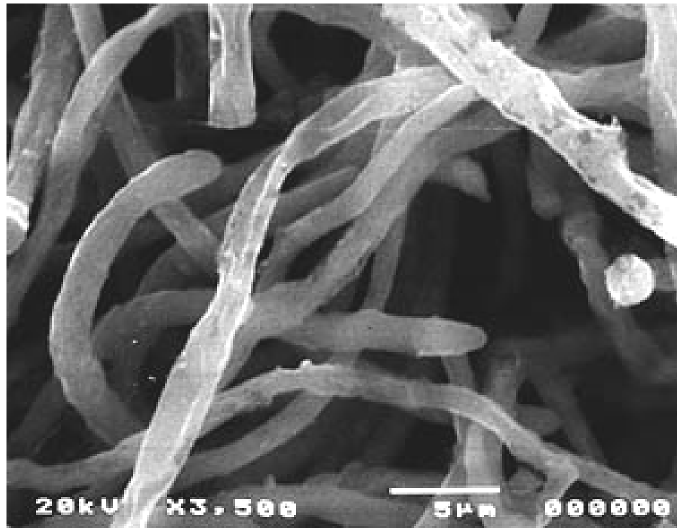

Figure 12a. Electron micrograph of the cross section of an untreated bead. The Filaments of the fungus are clearly visible, and large gaps between them make up the structure of the bead. The magnification is 3,500X and the accelerating voltage is 20kV.

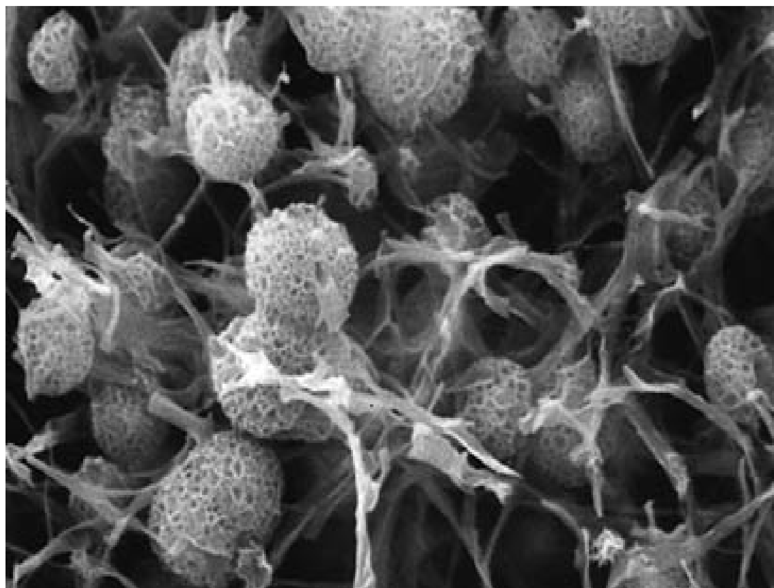

Figure 12b. Electron micrograph of the cross section of a copper challenged bead. The fungal filaments are visible with large spherical deposits between them. These deposits are thought to be either copper phosphate or copper oxalate. The magnification is 1,000X and the accelerating voltage is 25kV.

porous and vary between samples. The porous microspheres are thought to be copper oxalate complexes. By studying cross sections of beads from different time points, it was found that the microspheres are present most commonly in beads that have been exposed to approximately 20 to 25 hours of copper challenge. Qualitatively, bead samples from later in the process seem to have fewer microspheres.

Discussion

Penicillium ochro-chloron, a filamentous fungus, has the capacity to effectively remove copper from surrogate wastewater, if appropriate physical conditions are provided. The research proved that the fungus must be provided with oxygen above the critical oxygen concentration, for removal to occur efficiently. The fungus consumed some, if not all of the glucose supplied, while removing copper. The consumption of glucose along with the requirement for the presence of oxygen, indicate that the fungus is viable and it is undergoing aerobic respiration for copper uptake to occur.

The results from the shake flask study indicate that the organism has the capability to remove copper ions from aqueous solutions. The majority of these results showed effective removal and retention of the copper however, in the case of the LA-line study, this was not true. Table 3 shows an increase in the copper concentration, above the original copper level, at times 64 and 88.75 hours. This increase can be explained by the evaporation that occurs during the course of the experiment (see Appendix F). The decrease in the copper concentration later in the experiment, is the result of the fungus removing copper exceeding the effects of evaporation. The amount of dissolved oxygen available to the fungus was not studied, but may affect the overall performance of the fungus during the shake flask experiments. Many metal ion removal systems use dead biomass and dissolved oxygen concentration is not a concern. A number of studies use only rotary shaker experiments for the evaluation of the binding and removal capacity of organisms (Bosecker, 1993). These experiments are good measures of what an organisms' basic tolerance and binding capacity is, however this is not a suitable system for larger scale use.

Proving that genetic diversity of the organism is a factor affecting copper removal is a time intensive investigation. The experiments involving the genetically individual strains of *P. ochro-chloron* (LA1-5) proved that any conclusive results proving genetic diversity would require statistically significant, and for the purpose of this study, prohibitively large numbers of experiments. However, the results show that there is a possibility for poor metal ion removal within the population of the fungus, and the copper removal may be carried out by a select number of beads. It should be noted that this organism was first isolated from electro-plating baths with high levels of copper, and the organism must therefore be tolerant of the metal even though the entire population may not able to complex and retain it.

Although inconclusive, the results indicate that the fungus also may require a high copper concentration in the original medium when challenged in order for effective copper removal to occur. At concentrations lower than 100mg/L of copper the fungus did not remove copper as consistently as if the copper concentration was 100mg/L. Perhaps the fungus requires a certain level of copper ion concentration before the process resulting in copper removal is activated, or perhaps the lower levels of copper are not toxic enough to require active defense against it, since the organism is quite capable of growth and metabolism at 300mg/L copper. Further experimentation with more toxic metals might reveal different effective ranges of uptake bused or toxicity.

The packed bed column reactor experiments proved inefficient when using this organism for heavy metal removal. Previous research had also proven the static environment in packed bed reactors does not compliment organisms for the removal of heavy metals. There is poor mixing in these reactor designs and channeling often occurs

adding to the unfavorability of that design (White and Gadd, 1990). As a result of the static configuration of the biomass in the packed bed reactor, only a small amount of the fungus is supplied by oxygenated media. The dissolved oxygen concentration in the media at the outlet of the column is low, and together with the channeling effects in the column, halt copper removal. Also, as a result of channeling effects, other nutrients may not be supplied adequately to the viable biomass. The density of the fungal beads may increase the channeling effects in the column. Through physical examination it was found that the mycelial density and elasticity of individual beads varied. The reason for the difference in density was probably due to slight variations in growth conditions. Denser or more tightly wound beads naturally allow less media to flow through them, than does a porous bead.

In determining if the fluidized bed system was limited by diffusion or some aspect of poor mixing, several calculations were performed (Appendix E). Based on these calculations the shortest oxygen *depletion* time is 0.52 hours or 31 minutes while the longest *mixing* time is 3.5 minutes. Since there is ample time for the mixture to be replenished with oxygen, mixing is not the limiting factor. It is therefore concluded that uptake in the system is mainly diffusion mediated. This is true for the specific conditions used for the fluidized bed with a standard biomass load (Materials and Methods section 9.1.2).

The fluidized bed investigation rendered the most promising results for the use of this organism for heavy metal removal from aqueous solution. The fungus effectively removed large amounts of copper when the dissolved oxygen concentration was above the critical oxygen concentration of the fungus. To correct for the varying biomass in

each of the experiments the maximum copper removed per gram of biomass (dry weight) was calculated. The maximum removal achieved for the different dissolved oxygen concentrations are shown in Table 6, and in Figure 13. The trend shows an increase in the amount of copper that is removed per gram dry weight of the fungus with increasing dissolved oxygen concentrations. These data support the theory that the fungus requires oxygen for optimal Cu^{2+} removal. Considering that the system is diffusion limited the reaction taking place when removing the oxygen should fit a first order reaction. Using the data and the following rate equation:

$$\frac{dC}{dt} = -kCW \cdots\cdots\cdots\cdots\cdots (1)$$

where C is the bulk liquid concentration (mg/L), k is the rate constant, W is the biomass used in (g/L) and t is time (hours), k was calculated for each data point in every experiment. Equation (1) allows for correction for varying amounts of biomass used in different experiments. Figure 14 show the results from these calculations plotted against the dissolved oxygen concentration. This graph shows a clear decrease in the rate constant for the experiments at DO values of 10%, however there is no consistent increase in the k values with increasing DO concentrations. Further analysis with second order rate equations showed the data to have less correlation to the reaction kinetics (Appendix H), supporting the theory that the system is diffusion limited.

Statistical analysis (Appendix K) of the first order rate constants proved that analysis of variance was significant; $F_{(3,24)}=7.85$, $p<0.001$. F is the F-ratio which is a comparison of variances and p is the p-level. It should be noted that since the two experiments at 30% DO were very different, they were dropped for this statistical analysis. Analysis of 10% DO vs. the remainder of the experiments showed that the k

DO (%)	*Biomass used (g dry weight)*	*Max. uptake (mg [Cu]/g dry weight)*
10	0.615	36.0
10	0.492	48.4
20	0.492	82.9
20	0.738	56.6
30	0.677	98.2
30	0.369	93.4
50	0.861	73.3
50	0.554	148.7
80	0.861	99.1
80	0.615	102.8

Table 6. Maximum copper uptake during individual fluidized bed experiments. All DO values are based on air being 100% saturation using air. The values are corrected for the variation in the biomass used for each experiment to give a basis for comparison.

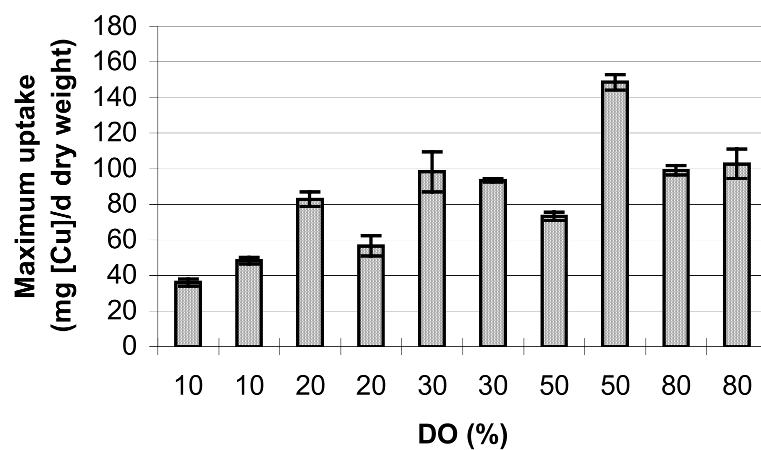

Figure 13. The effect of varying DO concentration on maximum copper uptake in a fluidized bed. The maximum uptake is corrected for the variation of biomass to give a basis for comparison. The overall trend is an increase in the maximum uptake with increasing DO concentration, and statistics show that the range 20-80% DO is significantly different from the 10% DO.

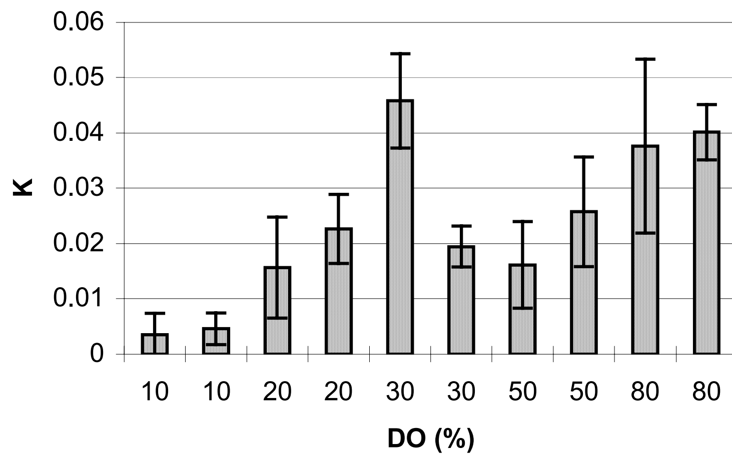

Figure 14. Copper uptake rate constants at different DO concentrations in a fluidized bed. The statistics indicate that the experiments with DO's 20-80% are within experimental error with the exception of one of the 30% DO runs. Statistics also show that with 99% confidence that the 10% DO values are significantly different from the other K values.

values are significant; $F_{(1,24)}$=15.6, p=0.0006. Further, it also showed that grouping *k* values at 10 and 20%DO and comparing those to a grouping of 50 and 80%DO, resulted in a finding that the 10 and 20% *k* values were significantly lower; $F_{(1,24)}$=15.5, p=0.0006. Both of these observations are consistent with the hypothesis that a DO concentration above the critical oxygen concentration is required for optimal copper removal, which suggests respiration is somehow vital to copper removal.

The analysis of data using second order kinetics indicates that there may be more than one reaction limiting the removal of copper by the fungus. The graph Figure H-1 (Appendix H) shows there is an optimal dissolved oxygen concentration for Cu^{2+} removal, since the reaction rate only increases till 30% DO and then drops at higher oxygen concentrations. The rate constants at 10% and 80% DO are similar suggesting that high levels of oxygen may actually decrease the rate at which copper is removed. However, since more copper is removed per gram dry weight at higher DO concentrations the rate may be affected by other variables than O_2 alone. Perhaps at higher concentrations of oxygen growth is more favorable than copper precipitation, hence decreasing the rate at which it occurs. Another possible explanation is that at lower concentrations of oxygen the preferred method for copper removal is through phosphate precipitation, while with more oxygen available oxalate precipitation is favored. The inconsistency in the *k* values may be the result of any of these factors or a combination of these factors.

When the copper concentration in the fluidized bed reactor was decreased to compare with the shake flask study at low copper concentration, similar uptake results were recorded. In both cases there was an initial burst of removal after which the copper

seems to be released back into solution. In the case of the fluidized bed experiment the copper is completely released. This could indicate that there is an active pathway for the removal of the copper. Perhaps, the defense mechanism against metal toxicity requires metabolic intervention and if the copper concentration is not high enough to be toxic the pathway is not turned on. The removal exhibited could be the result of the previously noted high copper requirement for optimum growth and sporulation (Gadd *et al.*, 1984), without a continuing uptake due to lack of nutrients required for growth and sporulation.

In the attempt to decrease the process time higher concentrations of biomass were used in the fluidized bed. For this experiment, there was an initial burst of copper removal during the first 20 hours, then the copper was completely released. The reason for this decrease in copper retention could be some other limiting nutrient in the medium, possibly glucose and/or phosphate. To increase biomass load efficiently, medium optimization and development would have to be performed. During the same experiment the oxygen demand of the biomass proved to be higher than the oxygen supplied. To eliminate the possibility for limitation of Cu^{2+} removal due to lack of oxygen, either an increase in the mixing or using pure oxygen instead of air might improve uptake. In either case this would change the conditions in the reactor significantly and introduce factors not yet considered but which need to be investigated. An increase in the mixing would probably cause shear damage to the biomass, and using pure oxygen would not be feasible in a large scale process. Other researchers have shown that increased biomass load in batch fermentors for the removal of copper, decreases the biomass capacity for copper removal (Ruiz-Manriquez *et al.*, 1998). It has been suggested that there are two reasons why increasing biomass may reduce that binding capacity, one being that an

increased number of cells increases the electrostatic interactions between the cells, and the other is that there is an optimum biomass/metal ratio. In the first case the cells can agglomerate which causes fewer available binding sites for the metal (Ruiz-Manriquez *et. Al.*, 1998). In the case of live biomass, such as *P. ochro-chloron*, the agglomerating theory does not apply since the number of cells in the beads are independent of how many beads are added to the reactor. The optimum bead/metal ratio may have some impact on the decreased copper removal at higher biomass loads. However, a more likely explanation is that there is a limiting nutrient causing the halt in copper removal. Precipitated copper in the fungus (as shown in electron micrographs, Figures 12a and b) is not normally soluble, unless some type of saturation metabolism caused by a limiting nutrient is forcing release.

The electron microscopy study showed the copper seems to be precipitates extracellularly and is held within the mycelia of the fugal beads. The binding of metal ions to extracellular components can be considered a biosorption process (Pradham, *et al.*, 1992). Since the metal is not taken into the cells this may be the way the organism copes with the toxicity at high concentrations of copper. The spherical porous complexes seen in Figure 12b are thought to be copper phosphate complexes. Depending on how long the beads have been challenged in copper containing media the number of these microspheres decreases. The most abundant number of spheres was found in samples taken after approximately 20 to 25 hours of copper challenge. The reason for the reduction in the number of spheres is not known, but is possibly due to a change from phosphate complexing of the copper to oxalate binding. The oxalate binding is thought to manifest itself as a coating of the mycelia rather than large free-standing complexes.

Conclusions

This work has yielded promising results using *Penicillium ochro-chloron* for the removal of copper ions from aqueous solutions. The results obtained from the study proved that the fungus has the potential to remove copper to relatively low levels if provided with favorable conditions. The fungus requires oxygen above its critical oxygen concentration for optimal removal. When comparing the levels of copper remaining in experimental media to the standards set by the EPA, it is clear that the fungus would meet at least some of those standards, if used in a recovery process. The current values for the discharge copper concentration for the metal finishing industry is an average of 2.7mg/L copper/day (40 CFR, 1998). However, for any fresh waterway, the maximum copper concentration is 17μg/L (0.017mg/L) (EPA, 1995), implying the fungus could not be used to detoxify existing bodies of water. The fungus can however remove copper at very high concentrations (100mg/L), and can therefore be valuable in industrial wastewater treatment applications. Comparing it to other organisms known to absorb and bind copper (Appendix J) it has a rather high binding capacity and can tolerate higher initial concentrations of copper than most shown. Potential use therefore, could be in the electro-plating industries or any other industry which produce high copper levels in wastewaters. Any potential use of the fungus as an industrial wastewater treatment would require further research into the mechanism of uptake beyond oxygen availability in order to guarantee sustained uptake and eliminate re-release back into the waste stream.

Future Work and Research

Since basic bioreactor conditions for copper removal by *P. ochro-chloron* have been established, future work could focus on other physical conditions that may affect the uptake. Aspects such as using oxygen instead of air to supplement the media, or using other sources of nutrients for the fungus need to be studied. A possibility would be to use spores, and allowing them to grow in the copper medium, hence eliminating the entire step of producing the biomass for the applications.

There are indications that the fungus removes other divalent cations than copper (Fukami, 1983). The copper experiments could serve as a model removal system for other toxic metal ions, such as lead and mercury. Future work could include investigations of how tolerant and effective the fungus is at removing other metal ions from solution.

Since the fungus seem to lack to ability to remove copper to low enough levels to comply with the EPA standards, research may look into how this system may be combined with other systems. Since the fungus tolerates high concentrations of copper, this system could be used as a precursor for a more sensitive system. It has previously been shown that systems containing several species has achieved metal removal as high as 99% (Erlich and Brierly, 1990, Jennett and Wixson, 1983).

However, if content with the accomplishments of the fungus to date, future research could focus on scaling up the process. The process needs to be able to handle large volumes of wastewater each day at a low cost. Efficient culturing and storage of the biomass, to allow shipping and long term storage needs to be optimized. Also, further medium development is required for higher biomass loads in batch reactors.

Finally, to more fully understand the mechanism of removal by the fungus, further investigation of the chemicals and enzymes involved in the copper complexing is required. Also, further investigation as to the complexing of copper as a defense mechanism or an affinity reaction would shed more light on how the fungus can be manipulated for optimum removal.

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Appendix A

The Bathocuproine assay results in absorbance readings for the samples and standards. In order to transform these results to quanteties in mg/L of copper the following calculations were perfomred:

Calculate the conversion factor as follows:

$$\text{Factor}\ (\mu\text{g/A}_{484}) = \frac{\text{Amount of Cu i standardsolution}\ (\mu\text{g})}{\text{Average A}_{484}\ \text{of the three Standard}} \cdots\cdots\cdots\cdots\cdots\cdots\cdots (3)$$

Average the A_{484} for each sample (three values).

$$[\text{Cu}]\ (\mu\text{g/mL}) = \frac{\text{Fator}\ (\mu\text{g/A}_{484}) \times \text{average A}_{484}\ \text{of sample}\ (\text{A}_{484})}{\text{Volume of sample added to each tube (mL)}} \cdots\cdots\cdots\cdots\cdots\cdots (4)$$

Appendix B – Dry Weight Determination

In order to accurately determine the absorbency capacity of the fungus a dry weight standard curve needed to be established. Since the amount of beads used was measured volumetrically the standard curve was based on the weight of varying volumes of lyophilized beads. The results are shown below as well as the curve produced and the equation of the line.

Volume (mL)	*Weight - Paper (g)*	*Combined weight (g)*	*Dry weight - beads (g)*
5	0.4096	0.4693	0.0597
5	0.41458	0.47681	0.06223
5	0.40703	0.46151	0.05448
10	0.41041	0.54134	0.13093
10	0.414	0.5186	0.1046
20	0.40626	0.63434	0.22808
20	0.41125	0.68054	0.26929

Table B-1. Original weights taken of the lyophilized beads. Only these three volumes were used since larger volumes would require two batches of beads being grown, and the four days time laps could introduce unknown errors to the weight determination.

Volume (mL)	*Average dry weight (g)*	*Standard deviation*
0	0	0
5	0.058803	0.003952
10	0.117765	0.018618
20	0.248685	0.02914

Table B-2. The average weights of the different volumes of beads and the standard deviation to allow error bars.

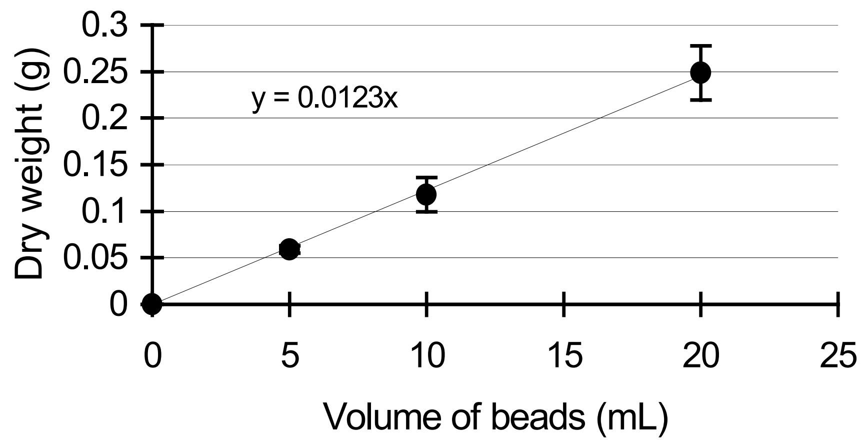

Figure B-1. Standard dry weight curve for *P. ochro-chloron*. The equation allows for estimation of the dry weight of beads of larger volumes as well.

Appendix C – Dynamic Gassing Out

In order to establish if the fluidized bed system was limited by mixing and limiting the oxygen supply the oxygen uptake rate (OUR) was required. An experiment using dynamic gassing out was performed to obtain a graph from which the OUR could be determined. 70mL beads were maintained in the fluidized bed at a DO of 80%. At three different time points (t=0, 24, and 49hours) the air supply was turned off and the DO drop monitored over time, once the DO reached below 70% or 20 minutes passed the air was turned back on with continued monitoring of the DO. The DO values were then plotted against time. The negative slope of the curve represents the OUR. These values were plotted separately to find the equation of the curve and the OUR of the system.

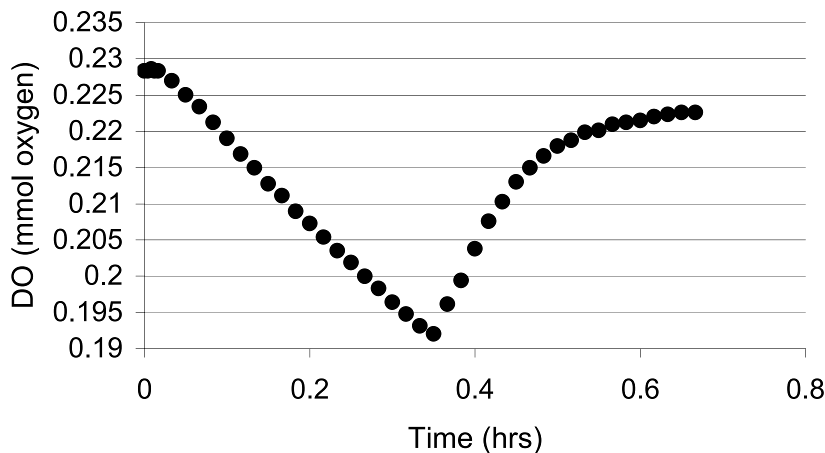

Figure C-1. Oxygen Uptake and Transfer rate curve for *P. ochro-chloron* during copper challenge after approx. 24 hours.

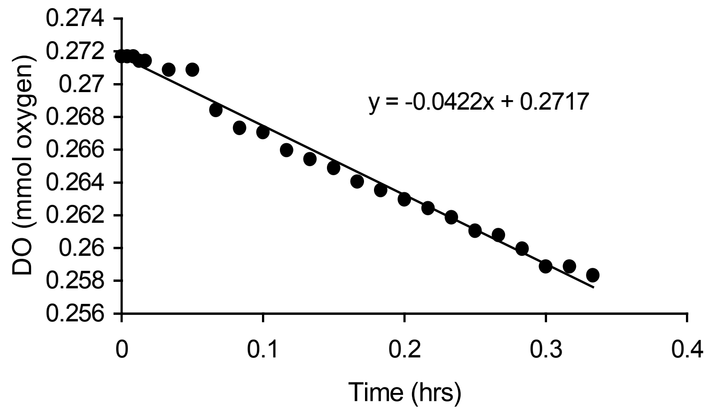

Figure C-2. The oxygen uptake rate at t=0. The decrease in the DO was minimal probably due to the fungus' need to acclimate to the new environment and begin metabolizing.

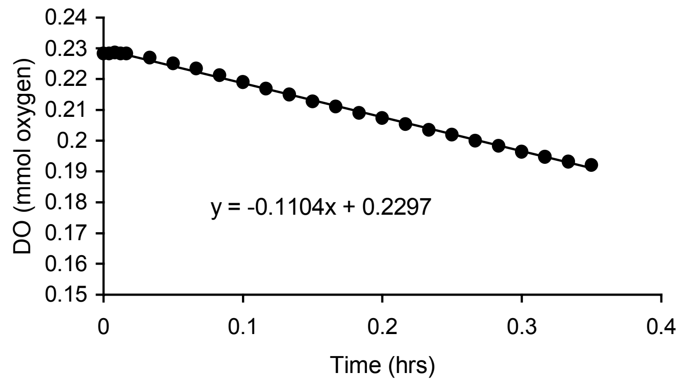

Figure C-3. The oxygen uptake rate at t=24 hours. At this point the fungus is metabolizing and removing copper from solution and the DO drop is considerably faster than at t=0.

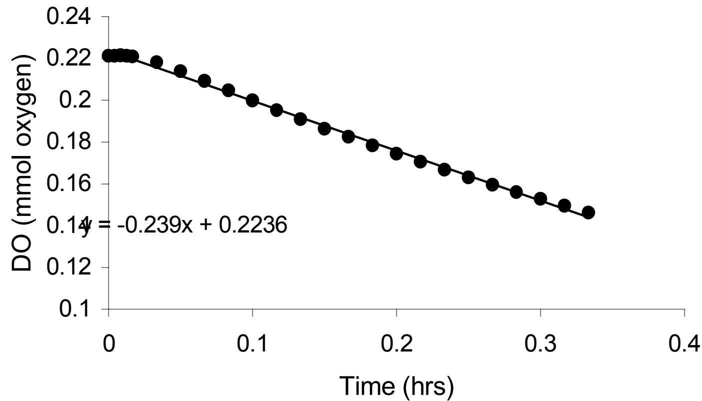

Figure C-4. The oxygen uptake rate at t=49 hours. Again the fungus is active and metabolizing. The metabolism may be slowed down as a result of the removal of copper.

Appendix D – Copper Solubility and pH

To determine the pH at which the experiments should be kept a precipitation study was performed on the media used. The concern was that the copper in solution would precipitate if the pH rose during the time of the experiment. A titration using NMM as in experiments with 100mg/L copper and NaOH (50% by weight). The precipitation was measured spectrophotometrically at OD600.

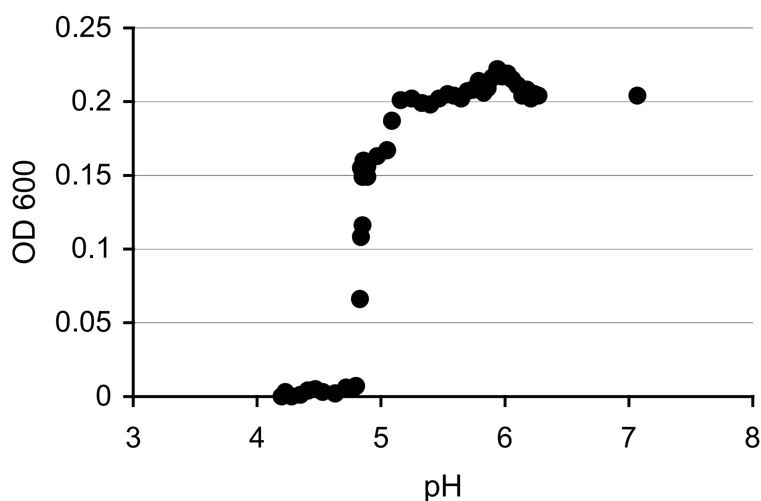

Figure D-1. Determination of the precipitation pH of copper in NMM. The original pH was 4.2 and the turbidity of the solution Increases drastically at pH approximately 4.8.

As the graph shows the turbidity of the NMM increases approximately at pH 4.8. The experiments using this media constitution were therefore kept at pHs below 4.8, optimally at pH 4.2 since that is the starting pH of the media.

Appendix E – OUR and Mixing times

When investigating if the fluidized bed system was mixing limited or diffusion mediated calculations to find the oxygen uptake rate (OUR) and the mixing times were performed. The amount of oxygen present in the media was calculated. The solubility of O_2 in water is 1.26mmol/L, and 21% of air is O_2, therefore the amount of O_2 in solubilized air in water is 0.2646mmol/L. The 100% dissolved oxygen (DO) is therefore the same as 0.2646mmol/L of oxygen. Since only 970mL of media was used in the Bioflo, the conversion of the DO readings would be:

$$\frac{\text{DO reading (\%)}}{100} \times 0.2646(\text{mmol/L}) \times \frac{1}{0.97(\text{L})} = \text{DO reading in mmol} \cdots\cdots\cdots\cdots (1)$$

The DO readings were converted to mmol of oxygen, time was converted from minutes to hours and the negative slope in the DO was plotted against time. The slope (mmol/hrs) of the line produced represents the rate of oxygen consumption in the reactor at that particular time (Slopes can be found in Appendix C). To find the OUR, the biomass as dry weight was calculated (Appendix B). In this case the volume of beads used was 70mL and the corresponding dry weight is 0.861g. The OUR can be found by dividing the slope of the lines with the biomass in dry weight. Table 5a show the OUR at three different times.

Finally the comparison between the time it would take the biomass to deplete the oxygen to the critical oxygen concentration and the mixing times would show if the system is mixing or diffusion limited. If the mixing times were longer than the oxygen depletion times the system would be limited by mixing, and vice versa. Since the critical oxygen concentration for the fungus is 20% DO and the system was maintained at 80%

DO there is 0.1434mmol oxygen to be consumed in 970mL of media. To find how long it would take the biomass to deplete the oxygen present, you should simply divide the amount of oxygen in the reactor by the OUR:

$$\frac{\text{Amount oxygen present (mmol)}}{\text{OUR (mmol/hr g dry weight}^{-1}\text{)}} = \text{Time to deplete oxygen (hr/g dry weight)} \cdots\cdots\cdots\cdots (2)$$

Appendix F – Evaporation Study in Shake Flasks

The results from the experiments involving the LA lines 1 through 5 showed copper concentrations in the medium increasing over time. The analysis indicated that the copper concentration increased to above the original concentration. This is not possible since there is no source of copper. To eliminate the possibility that the fungus contains some copper when introduced to the shake flasks, an experiment where beads were placed in 1*M* HCl. The vials were then placed in an orbital shaker for four days. The HCl was then analyzed for the presence of copper ions. In all cases there was no detectable copper released from the beads.

The reason for the increase in copper concentration was then suggested to be evaporation. The shake flasks are not sealed to allow gaseous exchange, so evaporation was a potential source of error. Therefore; three shake flasks were weighed and then 200mL water was added and weighed. The flasks were placed in the same orbital shaker at 250rpm as the experiments were originally performed, and their weight monitored over time. The results are shown in the graph below. It should be noted that this evaporation study was performed during cool and high relative humidity. If environmental conditions were different the loss may increase or decrease with temperature and humidity.

Using the equation of the line produced below the following shows the theoretical increase in the copper concentration with the same evaporation trend:

At t=64 hours then,

$$Y=Ct$$
$$Y=0.1347(64)$$
$$=8.6208\text{mL}$$
$$8.6208\text{mL}/200\text{mL}=0.043104$$

with original copper concentration of 98.7mg/L then,

1.043104*98.7=**103.0mg/L**

At t=89 hours then,

1.0559415*98.7=**104.6mg/L**

With these results it in mind once can theorize that the increase in the copper concentration may indeed be due to evaporation. The average increase at t=64 hours was 106.3mg/L and at t=89 hours 111.1mg/L. The larger increase may de due to different atmospheric conditions.

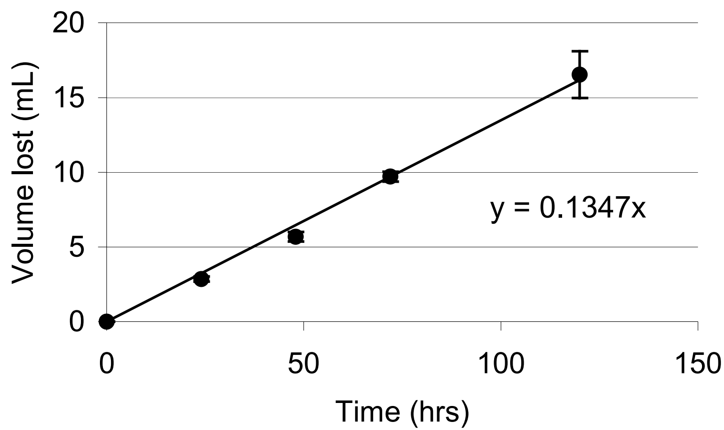

Figure F-1. The volume of water lost due to evaporation in a Tunair shake flask under standard experimental conditions. The rate of evaporation may vary with changing atmospheric conditions in the laboratory.

Appendix G – Electron Microscopy pictures

The electron micrographs taken as documentation that the copper seems to complex extracellularly in the fungus follow. The pictures are taken on a JEOL JSM-5200 scanning electron microscope at an accelerating voltage of 25kV.

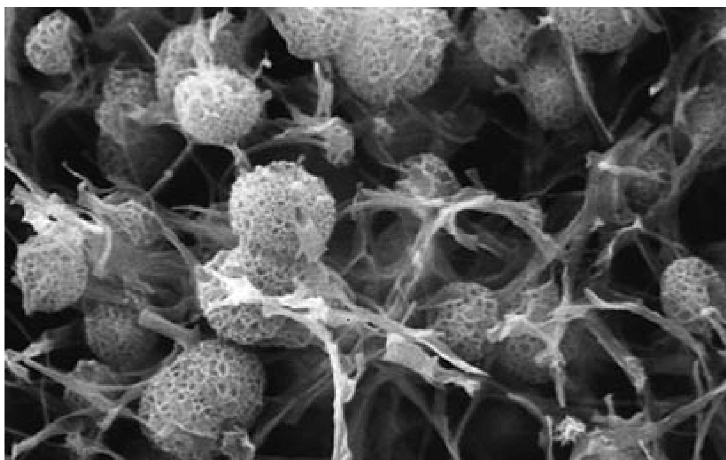

Figure G-1. Porous, round copper phosphate complexes are formed extracellularly within the fungus beads. These complexes appear after about 20 hours of copper challenge, but are not seem in specimens older than 35 hours. (X1000, 1cm=10μm).

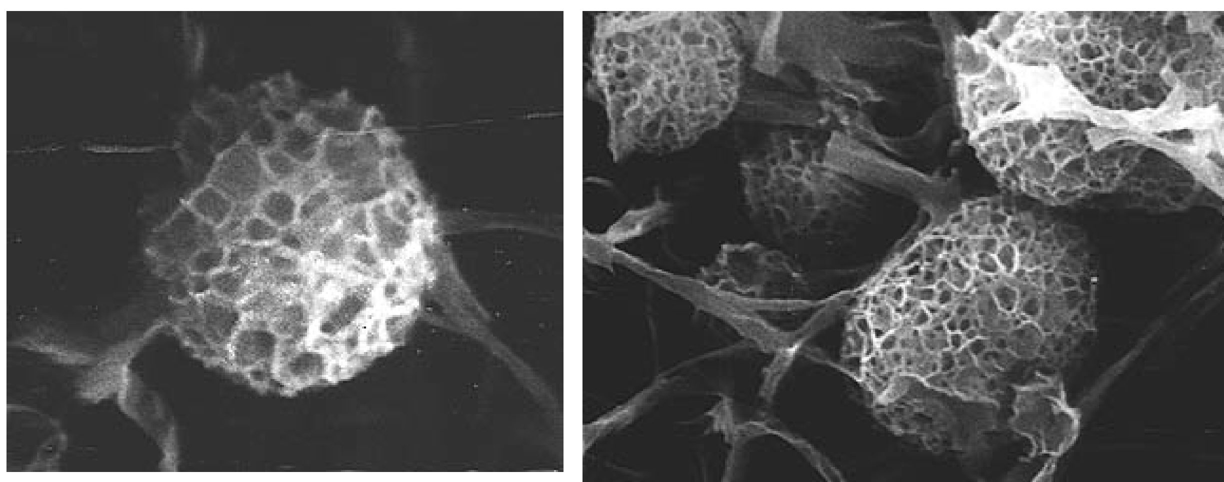

Figure G-2 & G-3. Higher magnification of the copper phosphate complexes noted after 20 hours of copper challenge. Figure F2 is X3500 (1cm=35μm) and F3 is at X2000 (1cm=20μm).

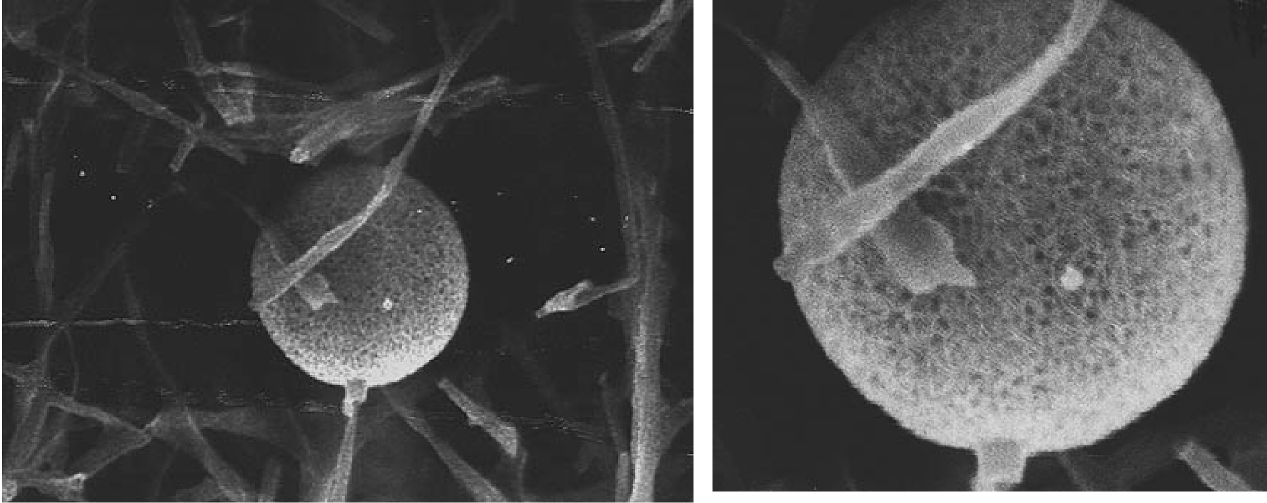

Figure G-4 & G-5. High magnification of what appeared to be a solid spherical precipitation in a sample which was 35 hours into copper challenge. This was the only such complex observed. It appears to be the same structure as the copper phosphate complexes observed in samples 20-25 hours into challenge, but much more closely packed. Figure F4 is at X2000 (1cm=20μm) and figure F5 is at X5000 (1cm=50μm).

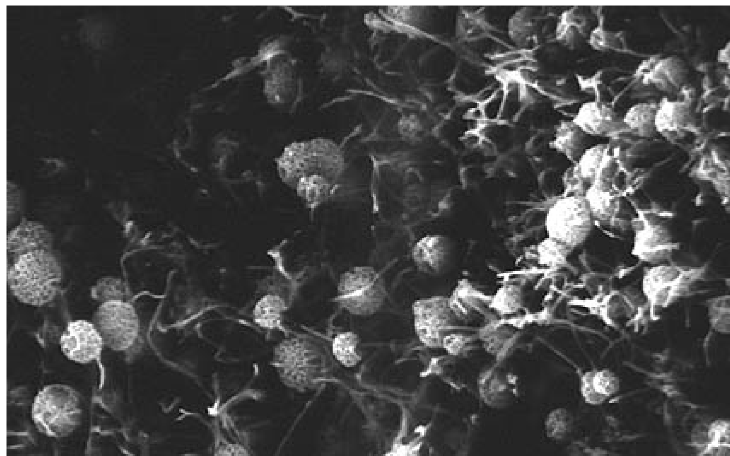

Figure G-6. This micrograph shows the abundance of microspheres observed in a sample 20 to 25 hours into copper challenge. This picture is taken at X500 (1cm=5μ m).

Appendix H – Mathematical Modeling

Part of the analysis of the data from the fluidized bed experiment included fitting to first and second order rate equations. The general equation for a first order (A→B) reaction is (2)

$$\frac{dC}{dt} = -kC \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (1)$$

where C is the concentration, k is the rate constant and t is time. However, to correct of the difference in the biomass used the equation used for the modeling was (1),

$$\frac{dC}{dt} = -kCW \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (2)$$

where W is the amount of biomass used in grams dry weight. The first order reaction fit the data well, but a second order (A+B→C) reaction was possible where the dissolved oxygen was the second variable. The equation is as follows (3):

$$\frac{dC}{dt} = -kC[DO] \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (3)$$

where C is the concentration, DO is the dissolved oxygen concentration, k is the rate constant and t is time. Again the equation was corrected for varying biomass and the equation used for the modeling was (4).

$$\frac{dC}{dt} = -kCW[DO] \quad \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots (4)$$

When solving for *k* for the data generated by the different fluidized bed experiments the following rate constants were found:

DO (%)	*k-values from Scientist calculations*
10	0.004145
10	0.005368
20	0.013389
20	0.009264
30	0.007656
30	0.018043
50	0.009475
50	0.003814
80	0.005925
80	0.005556

Table H-1. *k* values from Scientist calculations based on the biomass and the copper concentration from individual fluidized bed experiments.

These values should increase as the dissolved oxygen concentration increases, if the copper concentration and the DO are the two parameters affecting the uptake rate. As graph H-1 shows this is not true. The graph clearly shows and increase in the *k* values at DO concentrations at or slightly above the critical oxygen concentration for the organism. The reasons for this may be numerous, some of which are discussed in the Conclusion and Discussion section.

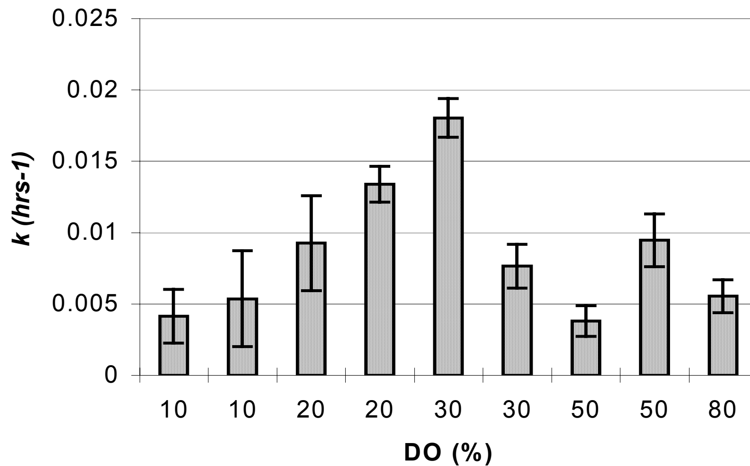

Figure H-1. Effect of varying DO on the *k* values from a second order reaction mathematical modeling. The error bars represent the standard deviation acquired from the calculations.

Appendix I – Copper absorbance

A table of Biosorption of copper from aqueous solution using microbial biomass. This table was first found a WPI masters thesis (Mark, 1997). The table is a summary of data reported in the articles by Kapoor and Viraraghavan (1995), Volesky and Holan (1995), and Veglio and Beolchini (1997). Areas in the table which are not filled imply that such data were not available to and/or not reviewed by the authors. References to the primary literature are given in the right-most column. CWP = Cell Wall Preparation. MP = Melanin Preparation.

Organism/ Biomass Type	***Biomass Class***	***Biosorption Capacity*** [†] ***(mg Cu/g)***	***Experimental Operating Conditions***				***Reference***
			pH	***T (°C)***	***C*** [‡] ***(mg/L)***	***Biomass (g/L)***	
Zoogloea ramigera	Bacterium	270	5.5		0-500 (e)	0.83	Norberg & Persson, 1984
Bacillus subtilis	Bacterium	152					Brierley & Brierley, 1993
Arthrobacter sp.	Bacterium	148	3.5-6	30	180 (e)	0.4	Veglio *et al.*, 1996
Penicillium notatum	Fungus	80					Siegel *et al.*, 1983
Candida tropicalis	Yeast	80					Mattuschka *et al.*, 1993
Active sludge bacteria	Bacteria	50	5	25	15-200 (e)	0.5	Aksu *et al.*, 1991
Chlorella vulgaris	Alga	42.9	4	25	10-260 (i)		Aksu *et al.*, 1992
Bacillus licheniformis (CWP)	Bacterium	32					Beveridge, 1986
Zoogloea ramigera	Bacterium	29	4	25	12-125 (i)		Aksu *et al.*, 1992
Pseudomonas syringae	Bacterium	25.4		22	0-13 (i)	0.28	Cabral, J.P.S., 1992
Cladosporium resinae (MP)	Fungus	25.4	5.5	25	1-320 (i)	1	Gadd & De Rome, 1988
Ganoderma lucidum	Fungus	24	5		5-50 (e)		Venkobachar, 1990
Rhizopus arrhizus	Fungus	19	5.5	25		1.05	Rome & Gadd, 1987
Cladosporium resinae	Fungus	18					Gadd *et al.*, 1988
Saccharomyces cerevisiae	Yeast	17	4-5	25	190 (e)	1	Volesky & May-Phillips, 1995
Rhizopus arrhizus	Fungus	16					Tobin *et al.*, 1984
Rhizopus arrhizus	Fungus	16					Tobin *et al.*, 1984
Cladosporium resinae	Fungus	16	5.5	25	1-320 (i)	1	Gadd & De Rome, 1988
Aspergillus oryzae	Fungus	13.6					Huang *et al.*, 1991
Pichia guilliermondii	Yeast	11					Mattuschka *et al.*, 1993
Saccharomyces cerevisiae	Yeast	10					Mattuschka *et al.*, 1993
Scenedesmus obliquus	Alga	10					Mattuschka *et al.*, 1993
Rhizopus arrhizus	Fungus	9.5	5.5	25	0.6-25 (i)		Gadd *et al.*, 1988
Penicillium chrysogenum	Fungus	9					Niu *et al.*, 1993
Streptomyces noursei	Bacterium	9	5.5	30	06-65 (i)	3.5	Mattuschka & Straube, 1993
Aureobasidium pullulans (MP)	Fungus	9	5.5	25	1-320 (i)	1	Gadd & De Rome, 1988
Aspergillus niger	Fungus	7.22					Rao *et al.*, 1993
Saccharomyces cerevisiae	Yeast	6.3					Brady & Duncan, 1993
Aureobasidium pullulans	Fungus	6	5.5	25	1-320 (i)	1	Gadd & De Rome, 1988
Streptomyces noursei	Bacterium	5					Mattuschka *et al.*, 1993
Bacillus sp.	Bacterium	5					Cotoras *et al.*, 1993
Aspergillus niger	Fungus	4	5		5-100 (e)		Venkobachar, 1990
Penicillium spinulosum	Fungus	3.6					Townsley & Ross, 1985
Penicillium digitatum	Fungus	3	5.5	25	10-50 (e)	6.5	Galun *et al.*, 1987
Aspergillus niger	Fungus	1.7					Townsley *et al.*, 1986
Trichoderma viride	Fungus	1.2					Townsley *et al.*, 1986
Saccharomyces cerevisiae	Yeast	0.8	4	25	3.2 (i)	2	Huang *et al.*, 1990
Saccharomyces cerevisiae	Yeast	0.4	4	25	3.2 (i)	2	Huang *et al.*, 1990
Penicillium spinulosum	Fungus	0.4-2					Townsley *et al.*, 1986
Penicillium ochro-chloron	Fungus	148.7	4	25	100	0.615	*This study*

[†] **Metal uptake as reported is not necessarily at maximum.**
[‡] **(i) = initial concentration; (e) = equilibrium concentration.**

Appendix J – Copper absorbance

A table of Biosorption of copper from aqueous solution using microbial biomass. This table was first found a WPI masters thesis (Mark, 1997). The table is a summary of data reported in the articles by Kapoor and Viraraghavan (1995), Volesky and Holan (1995), and Veglio and Beolchini (1997). Areas in the table which are not filled imply that such data were not available to and/or not reviewed by the authors. References to the primary literature are given in the right-most column. CWP = Cell Wall Preparation. MP = Melanin Preparation.

Organism/ Biomass Type	*Biomass Class*	*Biosorption Capacity*[†] *(mg Cu/g)*	*Experimental Operating Conditions*				*Reference*
			pH	*T (°C)*	*C*[‡] *(mg/L)*	*Biomass (g/L)*	
Zoogloea ramigera	Bacterium	270	5.5		0-500 (e)	0.83	Norberg & Persson, 1984
Bacillus subtilis	Bacterium	152					Brierley & Brierley, 1993
Arthrobacter sp.	Bacterium	148	3.5-6	30	180 (e)	0.4	Veglio *et al.*, 1996
Penicillium notatum	Fungus	80					Siegel *et al.*, 1983
Candida tropicalis	Yeast	80					Mattuschka *et al.*, 1993
Active sludge bacteria	Bacteria	50	5	25	15-200 (e)	0.5	Aksu *et al.*, 1991
Chlorella vulgaris	Alga	42.9	4	25	10-260 (i)		Aksu *et al.*, 1992
Bacillus licheniformis (CWP)	Bacterium	32					Beveridge, 1986
Zoogloea ramigera	Bacterium	29	4	25	12-125 (i)		Aksu *et al.*, 1992
Pseudomonas syringae	Bacterium	25.4		22	0-13 (i)	0.28	Cabral, J.P.S., 1992
Cladosporium resinae (MP)	Fungus	25.4	5.5	25	1-320 (i)	1	Gadd & De Rome, 1988
Ganoderma lucidum	Fungus	24	5		5-50 (e)		Venkobachar, 1990
Rhizopus arrhizus	Fungus	19	5.5	25		1.05	Rome & Gadd, 1987
Cladosporium resinae	Fungus	18					Gadd *et al.*, 1988
Saccharomyces cerevisiae	Yeast	17	4-5	25	190 (e)	1	Volesky & May-Phillips, 1995
Rhizopus arrhizus	Fungus	16					Tobin *et al.*, 1984
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Cladosporium resinae	Fungus	16	5.5	25	1-320 (i)	1	Gadd & De Rome, 1988
Aspergillus oryzae	Fungus	13.6					Huang *et al.*, 1991
Pichia guilliermondii	Yeast	11					Mattuschka *et al.*, 1993
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Scenedesmus obliquus	Alga	10					Mattuschka *et al.*, 1993
Rhizopus arrhizus	Fungus	9.5	5.5	25	0.6-25 (i)		Gadd *et al.*, 1988
Penicillium chrysogenum	Fungus	9					Niu *et al.*, 1993
Streptomyces noursei	Bacterium	9	5.5	30	06-65 (i)	3.5	Mattuschka & Straube, 1993
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Saccharomyces cerevisiae	Yeast	6.3					Brady & Duncan, 1993
Aureobasidium pullulans	Fungus	6	5.5	25	1-320 (i)	1	Gadd & De Rome, 1988
Streptomyces noursei	Bacterium	5					Mattuschka *et al.*, 1993
Bacillus sp.	Bacterium	5					Cotoras *et al.*, 1993
Aspergillus niger	Fungus	4	5		5-100 (e)		Venkobachar, 1990
Penicillium spinulosum	Fungus	3.6					Townsley & Ross, 1985
Penicillium digitatum	Fungus	3	5.5	25	10-50 (e)	6.5	Galun *et al.*, 1987
Aspergillus niger	Fungus	1.7					Townsley *et al.*, 1986
Trichoderma viride	Fungus	1.2					Townsley *et al.*, 1986
Saccharomyces cerevisiae	Yeast	0.8	4	25	3.2 (i)	2	Huang *et al.*, 1990
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Penicillium spinulosum	Fungus	0.4-2					Townsley *et al.*, 1986
Penicillium ochro-chloron	Fungus	148.7	4	25	100	0.615	*This study*

[†] Metal uptake as reported is not necessarily at maximum.

[‡] (i) = initial concentration; (e) = equilibrium concentration.

Appendix K – Statistical Analysis

As part of the mathematical modeling of the data from the fluidized bed experiments *k* values were calculated. Statistical analysis of the calculations was also performed using StatSoft's analysis program Statistica, Volume V. Analysis was performed on the *k* values from the modeling of the reaction with the first order kinetic equation. The *k* values were calculated in Scientist, to three significant figures, for every data point in all the experiments. Since the *k* overall values for the two experiments at 30% DO varied greatly, these two experiments were dropped for the purpose of statistical analysis.

First an least squares difference (LSD) test was performed on the data, with the *k* values being the variable. Table K-1 show the results of this test.

DO (%)	*{1}* 0.00125	*{2}* 0.0175	*{3}* 0.03	*{4}* 0.0414
10 *{1}*		0.129	**0.002**	**0.0001**
20 *{2}*	0.129		0.230	**0.033**
50 *{3}*	**0.002**	0.230		0.192
80 *{4}*	**0.0001**	**0.033**	0.192	

Table K-1. The table shows the LSD test performed on the *k* values from the first order reaction kinetics modeling. Values in **bold** are considered to be significantly different (either higher or lower than the value compared to). The variable are the *k* values.

The data shows that *k* values at 10% DO are significantly smaller than *k* values at 50 and 80% DO. It also shows that the *k* values at 10% DO are not significantly different from those at 20% DO. Nor is the *k* values at 50% DO and 20% DO. Overall this table shows that at *k* values at higher oxygen concentrations are higher than those at lower concentrations.

An Analysis of Variance (ANOVA) was performed on the data set. This showed to be significant ($F_{(3,24)}=7.85$, $p<0.001$), where F is the F-ratio (which is a comparison of variances), meaning that at one or more dissolved oxygen concentrations the *k* values are significantly

different from the others. As a result a planned comparison was made between likely values as follows:

DO (%)	*Comparison*
10	-1
20	1
50	1
80	1

Table K-2a. Summary of contrasts for comparison between 10% DO and the other experiments.

Univar test	*Sum of Squares*	*Degrees of freedom*	*Mean Square*	*F*	*p-level*
Effect	0.0045	1	0.0045	**15.6**	**0.000597**
Error	0.0068	24	0.0003		

Table K-2b. Planned comparison results of 10% DO vs. other experiments. Values in **bold** are considered most important.

DO (%)	*Comparison*
10	1
20	-1
50	1
80	1

Table K-3a. Summary of contrasts for comparison between 20% DO and the other experiments.

Univar test	*Sum of Squares*	*Degrees of freedom*	*Mean Square*	*F*	*p-lever*
Effect	0.0002	1	0.0002	**0.543**	**0.468**
Error	0.0068	24	0.0003		

Table K-3b. Planned comparison results for 20% DO compared to the other experiments. Values in **bold** are considered to be most important.

DO	*Comparison*
10	-1
20	-1
50	1
80	1

Table K-4a. Summary of contrasts for comparison between 10 & 20% DO and 50 & 80% DO.

Univar test	*Sum of Squares*	*Degree of Freedom*	*Mean Square*	*F*	*p-level*
Effect	0.0044	1	0.0044	**15.5**	**0.000625**
Error	0.0068	24	0.0003		

Table K-3b. Planned comparison results for 10 & 20% DO compared to 50 & 80% DO. Values in **bold** are considered to be most important.

P-level values of less than or equal to 0.05 is considered acceptable for biological experiments. In the cases of 10 vs. other and 10 & 20 vs. 50 & 80 the p-level values are less than the accepted meaning that in these two cases the *k* values are significantly different from each other. In the case of 20 vs. others the p-level value is higher than acceptable and this therefore shows that 20% DO *k* values are not significantly different from the other experiments.