BIOSORPTION SITES FOR LEAD [Pb (II)] IN PHANEROCHAETE CHRYSOSPORIUM

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BIOSORPTION SITES FOR LEAD [Pb (II)] IN *PHANEROCHAETE CHRYSOSPORIUM*

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ABSTRACT

BIOSORPTION SITES FOR LEAD [Pb (II)] IN *PHANEROCHAETE CHRYSOSPORIUM*

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Biosorption is a phenomenon involving the mechanisms that basically mediate heavy metal tolerance of microorganisms as well as sequestration of heavy metals from environment. Different classes of microorganisms have different biosorption capacities, as a result of the differences in composition and types of functional groups found on cell surfaces. The present study was undertaken to identify the molecular mechanisms for lead [Pb(II)] biosorption in the white-rot fungus, *Phanerochaete chrysosporium.* The methodology involved selective blocking of the functional groups known to participate in heavy metal biosorption and allowed us to determine their relative roles in Pb (II) biosorption in this organism. The relative concentrations of the Pb (II) sorbed from the aqueous environment and Mg^{2+} and Ca^{2+} ions released to the aqueous environment were measured and compared with both native and chemically-modified biomasses by using atomic absorption spectroscopy.

Fourier-Transform Infrared (FTIR) spectroscopy technique was used to monitor and analyze the molecular-level changes in both native and chemically modified cell surfaces upon Pb (II) exposure. Interactions of Pb (II) with the biomass surface was determined by observing the changes in wavenumber and absorbance of NH stretching and Amide I bands arising from the amine groups and C=O stretching band arising from the carboxyl groups. The roles of phosphate groups and lipids were also investigated.

Carboxyl groups seemed to be the most important functional groups for Pb (II) biosorption in *P. chrysosporium*, since the biosorption capacity dramatically decreased (by 92.8 %) in carboxyl groups-blocked biomass. Amine groups were found to play a secondary and minor role in Pb (II) biosorption, only a slight decrease (6 %) in Pb (II) biosorption was detected with amine groups-blocked biomass. Blocking of phosphate groups provided a small increase in biosorptive capacity and did not appear to have much significant role in biosorption. Upon chemical treatment with acetone to extract lipids of the cell surfaces, an increase of 20.3 % in the Pb (II) biosorptive capacity was determined.

It was concluded that carbonyl and carboxyl groups of chitin and glucan are the major sites and ion exchange via these groups is the main mechanism for Pb (II) biosorption in *P. chrysosporium*.

Key words: Lead [Pb (II)], Heavy metal biosorption, FTIR, Biosorption sites, *Phanerochaete chrysosporium*.

ÖZ

PHANEROCHAETE CHRYSOSPORIUM'DA KURŞUN [Pb (II)] İÇİN BİYOSORPSİYON BÖLGELERİ

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Biyosorpsiyon, mikroorganizmaların ağır metal toleranslarını ve çevredeki ağır metalleri bağlamalarını sağlayan mekanizmaları içermektedir. Değişik sınıflara üye mikroorganizmalar, hücre yüzeyinde bulunan fonksiyonel grupların kompozisyonu ve tiplerindeki farklılıklar nedeniyle farklı biyosorpsiyon kapasitelerine sahiptir. Bu çalışma, bir beyaz-çürükçül fungus olan *Phanerochaete chrysosporium*' da kurşun [Pb (II)] biyosorpsiyonunun moleküler mekanizmalarını tanımlamak üzere gerçekleştirilmiştir. Kullanılan metodoloji, ağır metal bağlanmasında rol oynadığı bilinen fonksiyonel grupların seçici olarak bloke edilmesini içermiş ve bu grupların organizmada Pb (II) bağlanmasındaki göreceli rollerinin anlaşılmasına olanak tanımıştır. Hem doğal, hem de kimyasal modifikasyona tabi tutulmuş biyokütleler için sıvı ortamdan alınmış ve hücrelere bağlanmış olan kurşunun ve sıvı ortama salınmış olan Mg^{2+} ve Ca^{2+} iyonlarının göreceli konsantrasyonları atomik absorpsiyon spektroskopisi ile ölçülmüş ve karşılaştırılmıştır.

Fourier-Transform Infrared (FTIR) Spektroskopi tekniği kullanılarak hem doğal biyokütlede, hem de modifikasyona uğratılmış biyokütlelerde Pb (II) ile etkileşimin neden olduğu değişiklikler moleküler seviyede analiz edilmiştir. Pb (II)'nin biyokütle yüzeyi ile etkileşimi, amin gruplarından kaynaklanan NH gerilme ve Amid I bandındaki ve karboksil gruplarından kaynaklı C=O gerilme bandındaki frekans kaymaları ve absorbans değişimlerinin gözlemlenmesi yoluyla belirlenmiş olup hücre yüzeyindeki fosfat gruplarının ve yağların rolleri de ayrıca araştırılmıştır.

Karboksil grupları bloke edilmiş biyokütlenin biyosorpsiyon kapasitesi dramatik biçimde düşmüş (92.8 %) olup, bu grupların *P.chrysosporium*'da kurşun biyosorpsiyonu için en önemli fonksiyonel grup oldukları belirlenmiştir. Amin grupları bloke edilmiş biyokütlenin Pb (II) biyosorpsiyonunda gözlenen sadece 6 % seviyesindeki düşüş, bu grupların organizmada kurşun biyosorpsiyonunda ikincil ve küçük bir rol oynadığını göstermiştir. Fosfat gruplarının bloke edilmesinin Pb (II) biyosorpsiyonuna önemli bir etkisi olmamış, sadece küçük bir artış kaydedilmiştir. Hücre yüzeylerindeki yağları uzaklaştırmak üzere kimyasal muameleden sonra Pb (II) biyosorptif kapasitesinde 20.3 %'lük bir artış belirlenmiştir.

P. chrysosporium'da kitin ve glukanda bulunan karbonil ve karboksil gruplarının en önemli Pb (II) bağlanma bölgeleri olduğu ve bu bölgelerdeki iyon değişiminin de Pb (II) biyosorpsiyonu için ana mekanizma olduğu sonucuna varılmıştır.

Anahtar sözcükler: Kurşun [Pb (II)], Ağır metal biyosorpsiyonu, FTIR, Biyosorpsiyon bölgeleri, *Phanerochaete chrysosporium*.

To my parents

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INTRODUCTION

1. General

1. 1. 1. Environmental Pollution and Lead

Industrialized and developed countries in the world are becoming increasingly concerned about the emerging effects of industrial activities which cause intensified environmental pollution and the breakdown of ecosystems. In these ecosystems, there has been an accumulation of pollutants such as heavy metals, synthetic compounds and wastewater nuclear liquids (Gadd, 2000). The detoxification of the heavy metal contaminants in wastewater and industrial effluents is gaining great significance and attention recently, due to the growing problems of limited water supply, possibility of damage to our current environment, need for more public health programs because of illness and/or diseases related to the environment and economical restraints.

Metallic species released into the environment tend to persist indefinitely, circulating and eventually accumulating throughout the food chain, thus posing a serious threat to the established environment and living systems. On the other hand, organic pollutants will eventually be diminished and destroyed in most cases. The mining industry effluents and metallurgical industry waste waters are the main sources of heavy metal contamination (Kratochvil and Volesky, 1998).

Lead is the metal which could be at the top of the environmental concerns. Lead is used as an industrial raw material for storage battery manufacturing, printing, pigments, fuels, photographic materials and explosive manufacturing (Yu *et al*. 1997).

Lead poisoning is the one of the most common pediatric health problems in the United States, affecting approximately 890,000 children nationwide at any given time. If not detected early, children with high levels of lead in their bodies can suffer from damage to the brain and nervous system, behavior and learning problems (such as hyperactivity), slowed growth, hearing problems, and headaches. Lead is also harmful to adults. Adults can suffer from difficulties during pregnancy, other reproductive problems, high blood pressure, digestive problems, nerve disorders, memory and concentration problems, muscle and joint pain (http://www.epa.gov/lead/leadinfo.htm#facts). The sources of this exposure are primarily leaded paint, which was not banned in the United States until 1978 and contaminated soil. Recent studies suggest that much of the existing soil contamination is probably a result of deposition from exhaust from cars that used leaded gasoline, in addition to leaded paint used on the exterior of buildings. Lead from these sources exists as or evolves into a variety of Pb^{2+} compounds, which are remarkably persistent in the environment. Unfortunately, these sources of exposure are often expensive to remediate, and the politics surrounding this issue are complex, suggesting that the legacy of lead poisoning will continue to plague mankind for many years to come (Lanphear, 1998).

Over the past five years, a quantum leap has been made in our understanding of the molecular mechanism of lead poisoning. Detailed biophysical studies have revealed that lead binds tightly to both zinc and calcium sites in proteins and alters their activity. However, lead binds to the 'best' (cysteine-rich) zinc sites (e.g., porphobilinogen synthase or called ALAD, the second enzyme in heme biosynthetic pathway) many orders of magnitude more tightly than to the 'best' (C2 domain) calcium sites. This tempts the chemist to say that effects of lead on zinc proteins are 'more important' than those of lead on calcium proteins (e.g., Synaptotagmin, which as a calcium sensor in neurotransmission) (Godwin, 2001).

The epidemiological evidence for an association between lead exposures and human cancer risk has been strengthened by the recent studies and new data on mechanisms of action provide biological plausibility for assessing lead as a human carcinogen. Both epidemiological and mechanistic data are consistent with a facilitative role for lead in carcinogenesis, that is, lead by itself may not be both necessary and sufficient for the induction of cancer, but at a cellular and molecular level lead may permit or enhance carcinogenic events involved in DNA damage, DNA repair, and regulation of tumor suppressor and promoter genes. Some of these events may also be relevant to understanding mechanisms of lead-induced reproductive toxicity. The kidney is a target organ for both acute and chronic lead toxicity. Toxic effects of lead to the kidney in both humans and experimental animals begins with changes in glomerular function, reflected in proteinuria, followed by acute morphologic changes that may slowly progress to a chronic irreversible nephropathy. The initial morphological changes include formation of lead–protein complexes called nuclear inclusion bodies and ultrastructural changes in cellular organelles, especially mitochondria.lead may act in the process of carcinogenesis by increasing the likelihood of fixed damage to DNA, either by inhibiting DNA repair or by displacing zinc in DNA binding proteins. Both of these events are important in protecting DNA from mutagenic insult. These mechanistic hypotheses would predict that lead is a "facilitative" or "permissive" carcinogen, permitting or augmenting the genotoxic effects of other exposures. Thus, sites for cancer in lead-exposed populations may vary, depending upon its interactions with the direct targets of genotoxic chemical co-exposures and/or on the organs in which lead is preferentially accumulated by the same physiological mechanisms that govern the metabolism of calcium or zinc, such as bone, liver, and prostate (Silbergeld, 2003)

1. 1. 2. Traditional Heavy Metal Removal Methods

Remediation of heavy metal contaminated waste water is generally accomplished by conventional methods like reverse osmosis, ion exchange and sludge separation, chemical oxidation/reduction, electrochemical treatment and also chemical precipitation processes e.g. with lime and biosulphide (H_2S produced by sulfate-reducing bacteria) and use of synthetic resins (Abraham *et al*. 2002,

Kratochvil and Volesky, 1998). These conventional methods are often either expensive or ineffective in large diluted wastewater applications (Texier *et al*., 1999, Jalali et al. 2002). The disadvantages of traditional metal removal techniques have led researchers to the investigation of microbial biomasses as biosorbents for heavy metal removal.

1. 1. 3. Alternative Techniques

Opposed to traditional removal systems, certain types of microbial biomass (living or nonliving cells) possess a high potential to sequester and accumulate inorganic ions which are present in aqueous solutions (Gadd, 2000). Microorganisms (bacteria, algae, fungi and yeasts) can effectively remove heavy metals (radionucleotides, organometallic compounds, and metalloids) from biotic or abiotic locations. Living and dead cells as well as cell-derived or isolated products such as cell walls, pigments and polysaccharides are all capable of metal removal. Therefore, active uptake or concentration of metal by living microbial cells could be defined as bioaccumulation.

1. 2. Heavy Metals Accumulation by Microbes

There are two separate mechanisms that are responsible for the accumulation of heavy metals in living systems: (i) adsorption to the outer structures of the cell (e.g., cell wall, capsule, slime layer) (Pighi et al., 1989) and (ii) intracellular uptake that can be either active (transport systems) or passive (diffusion) (Veglio and Beolchini, 1997). The selective intracellular accumulation is linked with physiological processes and therefore the microorganisms have to tolerate heavy metal accumulations. Figure 1.1 shows an overview of cellular location of major fungal transformations of metals and metalloids. The location of some processes, especially certain sequestration and transformation reactions, still remains to be uncertain. Possible involvements of other organelles such as mitochondria, endoplasmic reticulum, is not included (Gadd and Sayer, 2000).

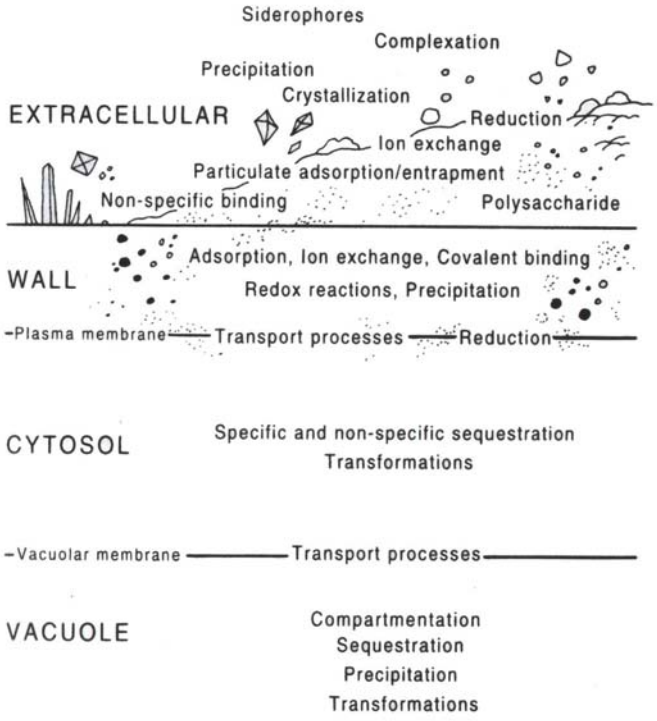

Figure 1.1 Mechanisms and cellular location of major fungal transformations of metals and metalloids (Gadd and Sayer, 2000).

1.2. 1. Metallothionein

Living organisms are able to respond with several different mechanisms to conquer the toxic effects of heavy metals. In 1957, the discovery of a cadmium-binding cysteine-rich protein marked the birth of a low molecular weight polypeptide superfamily, the metallothioneins (MT) (Margoshes and Valtee, 1957). These proteins are characterized by (i) low molecular mass (< 10 kDa), (ii) a high proportion of cysteinyl residues in the peptide chain (approximately 30% of the total amino acids) and (iii) complete absence of aromatic amino acids and disulfide bridges. The cysteinyl residues are in Cys-X-Cys or Cys-X-X-Cys sequence motifs (Kille *et al.,* 1994). The occurrence of these sequence motifs and the absence of hydrophobic residues predispose MT polypeptides to metal ion binding (Fischer and Davie, 1998).

1. 2. 2. Function of Metallothioneins in Heavy Metal Detoxification

MT proteins are able to control the intracellular concentration of metal ions. They also constitute effective defenses against metal-induced toxicity resulting from cellular accumulation of essential and nonessential metal ions (Kille *et al.,* 1994). The following three characteristics of MTs allow these molecules to be effective in metal ion detoxification: (i) MT binds metal ions tenaciously within polymetallic clusters (ii) MT has a tendency toward metal exchange reactions , and MT-bound Zn(II) ions are readily displaced by Cd(II) ions, resulting in sequestration of potentially toxic Cd(II) ions and (iii) the expression of MT genes in eukaryotes is metallo-regulated (Otvos *et al.,* 1993).

1. 3. Heavy Metal Biosorption

From the industrial sector's point of view, biosorption is important because of its ability to remove toxic metal waste from the environment and discarding it safely (Kefala *et al*., 1999). Biosorption is effective in decreasing the amount of heavy metal concentration to a lower amount with the use of inexpensive biosorbent materials (Yu *et al*., 1999). The biosorption process offers the advantages of low operating cost, minimization of the volume of chemical and/or biological sludge to be disposed of, high efficiency in detoxifying very dilute effluents, and no nutrient requirements. These advantages have served as the primary incentives for developing full-scale biosorption processes to clean up heavy-metal pollution. (Kratochvil and Volesky, 1998)

1. 3. 1. Definition of Biosorption

The term "biosorption" is used to describe the passive (i.e., not metabolically mediated) accumulation of metals or radioactive elements by biological materials. Usually, dead biomass serves as a basis for a family of biosorbents. Biosorption must be distinguished from bioaccumulation, which is an active process relying on metabolic activity and therefore occurs only in living organisms.

There are distinct advantages of using either living or dead biomass (Gadd, 1990; Brierley, 1990; Macaskie, 1990) The main advantages of using living biomass are that it is self-renewing, and active transport into the cell may lead to higher metal uptake levels, moreover excreted metabolic products can further contribute to metal removal. On the other hand, the use of dead biomass avoids problems with toxicity, no dangerous toxins will be discarded and the general public is not put into any kind of potential health danger (Zhou, 1999). Costs of cultivation of live biomass (e.g., nutrient supply) far exceed the costs of obtaining nonliving biomass. Waste products from fermentation processes or naturally abundant biomass can be used. Furthermore, biosorption by dead biomass is often faster, since only the cell-wall based binding, but not active transport into the cell do occur. Another advantage of using dead biomass is the easier and nondestructive recovery of bound metals which allows regeneration of the biosorbent material, making it available to use repeatedly. Metals accumulated intracellularly by living biomass can often be recovered only when the cell is destroyed. Dead biomass can be put into storage and used at a later time and its sorptive quality is unaffected upon storage. In most cases, working with dead biomass offers more advantages and is therefore focused on the majority of biosorption research (Schiewer and Volesky, 2000).

The determination of the metal uptake (q) by the biosorbent is most often based on the material balance of the sorption system: sorbate that disappeared from the solution must be in the solid. The sorption uptake, q, can be expressed in different units depending on the purpose of the exercise: for example, milligrams of metal sorbed per gram of the (dry) sorbent material (the basis for engineering process-mass-balance calculations), or mmol g^{-1} or meq g^{-1} (when stoichiometry and/or mechanism are considered). For biosorption process scale-up and applications, the uptake expressed per unit (reactor) volume is also important.

Metal uptake in system is calculated by the mass balance equation

q=(Ci-Cf).V/S where

Ci: initial metal concentration in solution (ppm-mg/L)

Cf: final metal concentration in soluion (ppm-mg/L)

V: Volume of aqueous sample (L)

S: weight of biomass used (g)

q: uptake of heavy metal of biomass (mg/g)

1.3. 2. Applications of Biosorption

Although many biological materials bind heavy metals, only those with sufficiently high metal-binding capacity and selectivity for heavy metals are suitable for use in a full-scale biosorption process. The first major challenge for the biosorption field was to select the most promising types of biomass from an extremely large pool of readily available and inexpensive biological materials. Although this task is not complete, a large number of biomass types have been tested for their metal-binding capability under various conditions, and a summary of metal-biosorption results has been published (Unz and Shuttleworth, 1996; Veglio and Beolchini, 1997). It should be noted that comparing results from different sources involves standardizing the different ways the sorption capacity may be expressed. The complex structure of the microorganism implies that there are many possible alternative sites for the metal to be captured by the cell.

Although several proprietary biosorption processes (such as AlgaSORB™ and AMT-Bioclaim™) were developed and commercialized in the early 1990s, a lack of understanding of the mechanism underlying the metal-sorption process has hindered adequate assessment of process performance and limitations, and thus the expected widespread application of biosorption. Consequently, the selection of industrial effluents for pilot testing has remained largely intuitive, as has the task of scaling up the process. The next real challenge for the field of biosorption was to identify the mechanism of metal uptake by dead biomass (Kratochvil and Volesky, 1998).

1.3. 3. Mechanisms of Biosorption

Knowledge of the biosorption mechanism was not easily obtained since biosorbent materials are not simple, clearly defined chemical compounds. Biosorbents comprise different types of cells with a highly complex structure whose various building blocks consist of a multitude of different molecules which in turn can display several binding sites. Moreover, even one binding site can participate in different binding mechanisms: carboxyl groups can, for example, engage in both complexation and electrostatic attraction of metal cations. Consequently, several

mechanisms often act in combination. As for overall metal binding mechanisms, main mechanisms are ion exchange, sorption of electrically neutral material (soluble metal-ligand complexes) to specific binding sites, and microprecipitation. These mail mechanisms are based on sorbate-sorbent or solute-solvent interactions, which in turn rely on some combination of covalent, electrostatic and van der Waals forces (Schiewer and Volesky, 2000).

The term "ion exchange" is used when the charge of ions taken up equals the charge of ions released (so that the charge neutrality of the particle is maintained), regardless of whether these ions are bound electrostatically or by complexation. The driving force of ion exchange is mostly the attraction of the biosorbent for the metal. Metals can be bound electrostatically or by complexation. Interactions between the metal and the solvent (usually water) play a role in so far as less hydrophilic molecules have a lower affinity for the liquid phase and are therefore sorbed more easily. The importance of ion exchange in biosorption has frequently been reported. The amounts of ions from the natural environment (Na^+, K^+, Ca^{2+}, Mg^{2+}, and H^+) and from biosorbent pretreatment (such as protonation) which are released during biosortion balance the heavy metal uptake by algae, bacteria, fungi, and peat (Fourest and Roux, 1994, Schiewer and Volesky, 2000).

The terms "adsorption" and "microprecipitation" are used to describe the accumulation of electrically neutral material which does not involve the release of a stiochiometric amount of previously bound ions. The difference between adsorption and microprecipitation is that in the former case, affinity between sorbent and sorbate (metal complex) and in the latter case limited solubility (i.e., an interaction between the solute and solvent) represents the main driving force. In microprecipitation, the metal cation and an anion (e.g., SO_4^{-2}, S^{2-}, oxalate, or HPO_4^-), itself often a metabolic product of certain biomass types, form insoluble aggregates (salts or complexes) such as sulfides, carbonates, oxides, oxalates, and phosphonates. Changed local pH or redox potential can also influence the occurrence of precipitation. Microprecipitation does not necessarily involve a bond between the biomass and metal. The process may, however, be nucleated by metal initially bound to active sites in the biomass. This means that a two stage process takes place where binding

to specific sites is followed by microprecipitation. The latter process is not limited by the number of binding sites but can occur in multiple layers (Macaskie *et al*., 1992).

1.3. 4. Electrostatic Attraction and Complexation

Ligands in the biomass (such as carboxyl groups) can form complexes with metal ions. Chelation, i.e., binding of one metal ion to two coordinating atoms in the same biomolecule, may also occur. Complex formation involves both covalent and electrostatic components whose relative contribution can be estimated by investigating how specific binding is. When purely electrostatic attraction occurs, the binding strength should correlate with the charge density. (z^2/r_{hyd}). Ions of the same charge (z) and hydrated radius (r_{hyd}) should therefore be bound with equal strength. Major deviations of the binding strength from the z^2/r_{hyd} correlation indicate a tendency toward a covalent bond character (Schiewer and Volesky, 2000).

The nature of the ions released provides information about the bond type. Electrostatically bound ions cannot displace covalently bound ions. It was observed that proton release occurred only during heavy metal uptake, not during light metal uptake. Since protons are mainly bound covalently, the biniding of heavy metals must have been more covalent than that of light metal ions. Similarly, the more Na^+ (which binds only electrostatically) reduces the uptake of other ions, the higher is the contribution of electrostatic attraction in the binding of those ions (Schiewer and Volesky, 1997).

The bond character in biosorption can partially be explained by Pearson's concept of hard and soft acids and bases. So-called hard ions such as alkaline earth ions and Mn^{2+} participate in ionic bonds. Easily polarizable soft ions such as Ag^+, Au^+, Hg^+, Hg^{2+}, and Cu^+ tend to form covalent bonds. Many transition metals (e.g., Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, and Cd^{2+}) as well as protens have intermediate characteristics, whereby Zn^{2+} tends toward hard and Pb^{2+} tends toward soft. Amond ligand atoms, O anf F are considered hard; S, P, ans As are considered soft; where N is classified as intermediate (Schiewer and Volesky, 2000).

Different correlations have been proposed to describe the increase of ionic-bond character with an increasing difference in the electronegativity between the two bonding atoms. For typical elements in biological ligands (O, N, and S), the ionic-bond character therefore increases with the electronegativity of the metal. It follows from the concept of hard and soft acids and bases that Pb^{2+} and Cu^{2+} are expected to display more covalent bond character and consequently stronger binding than are the hard ions Na^{+} and Ca^{2+}.

1.3.5. Binding Sites on the Cell Surface

Numerous chemical groups have been proposed to contribute to biosorptive metal binding by e.g., algae, bacteria, or biopolymers, including hydroxyl, carbonyl, carboxyl, sulhydryl, thioether, sulfonate, amine, imine, amide, imidazole, phosphonate, and phosphodiester groups. Whether any given group is important for biosorption of a certain metal by a certain biomass depends on factors such as the quantity of sites in the biosorbent material, the accessibility of the sites, the chemical state of the site (i.e., its availability), and the affinity between the site and the metal (i.e., the binding strength). For covalent metal binding, even an occupied site is theoretically available. To what extent the site can be used by the metal in question depends on its binding strength and concentration compared to the metal already occupying the site. For electrostatic binding, a site is only available only if it is ionized (Schiewer and Volesky, 2000).

The major binding sites in biosorption are acidic. Many groups like hydroxyl, carboxyl, sufhydryl, sulfonate, and phosphonate are neutral when protonated and negatively charged when deprotonated. When the pH of the solution exceeds its pK_a, these groups become mostly available for the attraction of cations. Amine, imine, amide and imidazole groups, on the other hand, are neutral when deprotonated and positively charged when protonated. Therefore, they attract anions if the pH is lowered such that the groups are protonated. The structural formulae and pK_a values of binding groups are summarized in Table 1.1.

For the freshwater alga *Chlorella*, the charge was positive (probably due to amine groups), favoring anion binding, at pH<3 and negative (mostly carboxyl groups contribute) at higher pHs, so that electrostatic attraction of cations occurred. The charge of biosorbent does not depend exclusively on the pH value. Covalent binding of metals can "consume" negatively charged groups. Groups become charge neutral that would otherwise have been negatively charged in metal-free solution of the same pH (Schiewer and Volesky, 1995).

Table 1.1 General heavy metal binding groups of microorganisms.

Group	**Structural Formula**	**pK_a**	**Ligand atom**	**Occurrence in selected biomolecules***
Hydroxyl	-OH	9,5-13	O	PS, UA, SPS, AA
Carbonyl (ketone)	>C=O		O	Peptide bond
Carboxyl	-C(=O)-OH	1,7-4,7	O	UA,AA
Sulfhydryl (thiol)	-SH	8,3-10,8	S	AA
Thioether	>S		S	AA
Sulfonate	-S(=O)(=O)-OH	1,3	O	SPS
Amine	$-NH_2$	8-11	N	Cto, AA
Secondary amine	>NH	13	N	Cti, PG, peptide bond
Imine	=NH	11,6-12,6	N	AA
Amide	-C(=O)-NH_2		N	AA
Phosphonate	-P(=OH)(-OH)-OH	0,9-2,1 6,1-6,8	O	PL
Phosphodiester	>P(=O)-OH	1,5	O	TA, LPS

PS: polysaccharides, UA: uronic acids, SPS: sulfated PS, Cto: chitosan, Cti: chitin, PG: peptidoglycan, AA: amino acids, TA: theicoic acid, PL: phospholipids, LPS: lipopolysaccharides. (Schiewer and Volesky, 2000)

1. 4. Recent Studies on Microbial Biosorption

1.4. 1. Bacteria

Bacteria are excellent biosorbents because of their high surface-to-volume ratios. Bacterial biomass (e.g., *Bacillus, Streptomyces, Citrobacter*) can be obtained as waste products from fermentation industries. Microprecipitation is a common phenomenon in metal binding by bacteria, while complexation by extracellular substances or by N and O ligands in the cell wall, as well as electrostatic attraction to charged groups present in the cell wall, may also occur. The walls of gram-positive bacteria are efficient metal chelators and the carboxyl group of the glutamic acid of peptidoglycan is the main metal deposition site (Brierley, 1990).

In gram-negative bacteria, most metal deposition occurs at the polar head regions of the outer membrane. In many bacteria metabolism-independent biosorption may be responsible for a large proportion of the total uptake. In gram-positive bacterial cell wall teichoic acids (TA) and teichuronic acids (TUA) can constitute up to 80% of the wall. Peptidoglycan is a linear polymer of alternating glucosamine and muramic acid with peptide side chains. These side chains bear one carboxyl group at the terminal amino acid and additional functional groups on certain intermediate amino aicds like asparagine, lysine, cysteine, or aspartic acid. TA contains phosphodiesters, and TUA feature carboxyl groups; both of which contribute to the negative charge of the biomass and enable ion exchange. Gram-negative have a much more thinner peptidoglycan layer which makes up approximately 10% of the weight of the cell wall, containing no TA or TUA. Therefore, they offer less negatively charged carboxyl groups which is a reason for their lower biosortive capacity (Remacle,1990)

Extracellular polymers of the capsule or slime layer contain carboxyl and occasionally phosphonate or sulfonate groups. It has been demonstrated that *Citrobacter* sp. can accumulate heavy metals as cell-bound heavy metal phosphates. (Macaskie et al., 2000). These organisms, together with *Klebsiella pneumonia,* were shown to remove uranium and lanthum from challenge solutions supplemented with phosphatase substrate (Macaskie *et al.,* 1994). Carboxyl groups of the call wall of *Bacillus subtilis* were shown to be primary metal deposition site via ion exchange,

whereas chemically modified amine groups did not reduce the metal uptake (Beveridge and Murray, 1980). For the filamentous bacterium *Thiothrix*, major metal sorption mechanism for nickel and zinc was shown to be ion-exchange within a time interval of less than 10 minutes. Calcium and magnesium effectively competed with heavy metals for the binding sites, moreover adsorbed nickel and zinc could be desorbed by treating the metal-laden biomass with $CaCl_2$. (Shuttleworth and Unz, 1993). Hydrochloric acid treatment was observed to be effective for desorption of uranium from pre-loaded biomass of *Bacillus* and *Micrococcus* species (Cotoras *et al*., 1992).

1.4. 2. Yeasts

Yeasts are among the major industrial waste products. The surface of yeast cells can also act as an ion-exchange resin with rapid reversible binding of cations. It has been demonstrated that polyphosphate groups and carboxyl groups on the cell surface of *S. cerevisiae* are active in metal complexation (Strandberg *et al.,* 1981). These phosphoryl groups appear to form stable complexes with uranium while the carboxyl groups become involved only when the phosphoryl groups are saturated.

Saccharomyces cerevisiae, extensively used in the food and beverage industry, was shown to readily replace H^+ with Sr^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+} and Tl^+ via covalent bonding at low metal concentrations, while weaker electrostatic interactions involving Mg^{2+} and Ca^{2+} displacement became primary ion-exchange mechanism (Avery and Tobin, 1993). Baker's yeast demonstrates slightly higher cadmium uptake than brewer's yeast. Aerobic baker's yeast biomass from the exponential growth phase is able to accumulate more cadmium than dry nonliving yeasts. For protonated baker's yeast carboxyl and amine groups present in sugar polymers in the cell wall involve in nickel biosorption, which has reached up to 25.0 mg/g upon treatment (Vasudevan *et al.*, 2003).

It has been postulated that increased level of biosorption might be a result of fermentation products adsorbed onto yeast cells (Omar *et al.,* 1996).

1.4. 3. Fungi

Fungi can be cheaply available as industrial waste products. *Aspergillus niger* has been used in the production of citric acid and of the enzyme glucamylase, *Rhizopus arrhizus* produces the enzyme lipase, just to name a few examples of fungi that have been employed in biosorption studies (Schiewer and Volesky, 2000). Filamentous fungi have higher metal adsorption capacities in comparison to yeast or algae. Some filamentous fungi such as *A.niger* grow as pellets, which aids the recovery of the metal-laden biosorbent. Other types of fungi can create problems of solid/liquid separation and are not easily filterable. Another potential disadvantage of the use of fungi is that of impurities due to adhering fermentation broth residue that may affect metal uptake.

Similar to algae and bacteria, the cell wall is the main site of metal deposition in fungi. Polysaccharides constitute up to 90% of the fungal cell wall. Figure 1.2.a shows the fungal cell wall architecture. The inner microfibrillar layer of the wall usually consists of chitin., but cellulose or, in rarer cases, noncellulosic β-glucan (in Hemiascomycetes) can take its place, depending on the taxonomic group. Figure 1.2.b shows the structure of chitin. The outer, more amorphous layer is made up of mostly α-glucans but can also contain mannans, galactans, chitosan (Zygomycetes e.g., *Mucor* and *Rhizopus*), or glycogen. The functional groups, which may be involved in the biosorption of heavy metals include phosphate, carboxyl, amine and amide groups (Guibal *et al.,* 1995; Akthar *et al.,* 1996).

Phosphated polysaccharides might be present; the phosphate content in *Mucor* can exceed 20% of the cell wall dry weight. The phosphate and carboxyl groups are thought to be responsible for the negative charge in the fungal wall, whereas the amine groups of the chitosan create a positive charge. Apart from electrostatic attraction to these charged groups, complexation with N or O donors (e.g., of chitin) may occur. Since the protein content the fungal cell wall is only about 10%, the importance of amino acid functional groups in metal uptake is small (Schiewer and Volesky, 2000). As in the case of bacteria, released metabolites can lead to

microprecipitaiton (oxalates due to oxalic acid, sulfides due to H_2S) or chelation (citric acid, siderophores).

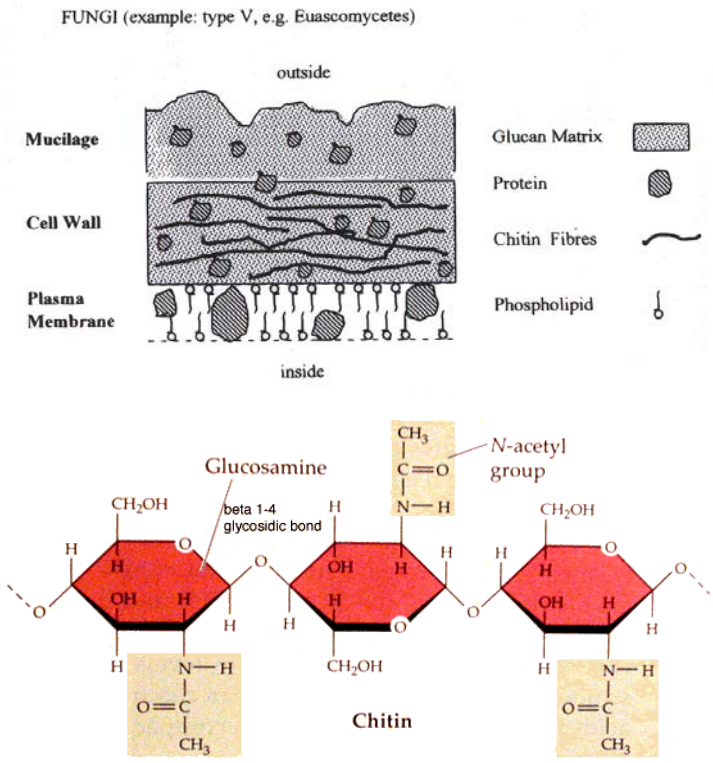

Figure 1.2 a) Cell wall structure in fungi (e.g., Euascomycetes; Moore-Landecker, 1996)
b) Structure of chitin (N-acteyl-glucosamine).

In general, members of the genus *Rhizopus* are good biosorbents. Deactivated *R. arrhizus* mycelium is able to accumulate up to 1/4 of its dry weight with uranium from a pure solution buffered at pH 4. In *R. arrhizus* carboxyl, and phosphate groups were observed to be responsible for biosorption of heavy metals and chemical treatments were found to cause reductions in metal uptake capacities as high as 60% (Tobin *et al.*, 1990). The dead biomass of *Rhizopus nigricans* was found to be a potent biosorbent for Cr(VI) ions. Native and immobilized biomass could bind to approximately 50 mg Cr/g biomass from aqueous solution at acidic pH and pretreated biomass was shown to remove up to 212 mg Cr/g. Blocking of amino

groups on the cell wall reduced the Cr uptake drastically, whereas carboxyl group modification resulted in an initial lag. (Bai and Abraham, 2002).

Aluminum is an element present in uranium mining wastewaters and can suppress the uranium uptake capacity of the immobilized microbial biomass (Tsezos and McCready, 1991). Aluminum interferes with the uranium biosorptive uptake capacity of *R. arrhizizus.* Dry cells of *R. arrhizus* are also potential alteratives in removing iron (III), lead (II) and cadmium (II) ions from industrial wastewater (Özer *et al.,* 1997, Yin *et al*., 1999). *Cladosporium cladosporioides* have gold uptake values up to 96.6 mg/g whole mycelium and 94.2 mg/g for isolated cell-wall preparation, being 44.5 mg/g and 42.8 mg/g for silver, respectively. Since no release of alkali metals were observed, complexation and electrostatic attraction mechanisms were suggested for biosorption (Pethkar *et al*., 2001). Regarding *M.rouxii,* chitosan contributed to most of the biosorptive capacity, 0.96 mmol Cu/g biomass obtained for the mycelium of this organism (Baik e*t al.*, 2002).

There are some studies that have been performed in the presence of multiple metals in solutions (Aksu *et al.,* 1997). The simultaneous adsorption of nickel (II) and lead (II) to *R. arrhizus* from binary metal mixtures were also demonstrated (Sağ and Kutsal, 1997). The effect of lead (II) ions on the uptake of nickel (II) ions was found to be antagonistic, whereas the total interactive effects of nickel (II) and lead (II) ions on the biosorption of lead (II) ions by *R. arrhizus* can be considered to be adsorbed selectively from the binary metal mixtures.

It has been found that the carboxyl groups on *Aspergillus* cell surface are crucial in the biosorption of copper (Akthar *et al.,* 1996). *A. niger* is capable of removing lead, cadmium and copper from aqueous solutions. Pretreatment of *A.niger* biomass using NaOH, formaldehyde, dimethyl sulfoxide resulted in significant improvements in biosorption of lead, cadmium and copper (Kapoor and Viraraghavan, 1997). Biosorption of lead and cadmium replaces Ca^{++}, Mg^{++} and K^{+} ions present on the biomass surface, indicating that biosorption takes place as a result of an ion-exchange process and include carboxyl and amine groups as active binding sites (Kapoor *et al.,* 1997). Acid washing is the most promising method for the

enhancement of metal adsorption capacity of *A. oryzae* mycelia (Huang and Huang, 1996). The treated cells can be reused after acid regeneration without losing or creating binding sites. Jianlong *et al.* (2001) have shown that lead-loaded *A.niger* biomass can be effectively regenerated by 0.1 M nitric acid. Previous calcium saturation of *Mucor miehei* and *Penicillium chrysogenum* biomass increased zinc binding capacity without requiring any pH control, and amine groups were reported to be the major binding site in uranium biosorption in above mentioned species (Roux *et al.*, 1994, Guibal *et al*., 1995). Tsezos *et al*. (1997) reported localization of palladium, silver, yttrium and nickel in the cells and on the cell surface bu using Transmission Electron Microscopy (TEM) and Energy Dispersive X-Ray microanalysis (EDAX).

1.4. 4. Algae

It has been well-documented that seaweed has impressive capacity of heavy metal biosorption (Kuyucak and Volesky, 1990). Brown and red algae are suitable for binding metallic ions such as Cu (III), Co (II), and Cd (II) due to their polysaccharide cell wall content and other sites with varying biding capacities of organism (Hamdy, 2000). According to some researchers (Crist *et al.,* 1988), the biosorption of heavy metals occur in two phases: (i) a fast (< 4s) surface reaction and (ii) slower metal uptake (2 h). The first phase is attributed to surface adsorption, mainly based on an ion exchange with the participation of the carboxyl groups of uronic acids. The second phase represents the diffusion of metal ions into the cell.

The biomass of *Sargassum* species was shown to possess excellent biosorbent properties by binding gold selectively at low pH (Kuyucak and Volesky, 1989a) and also cadmium, copper, nickel, lead, and zinc cations up to 20% of the biosorbent dry weight (Leusch *et al*., 1995). *S. hystrix, S. natans* and *Padina pavonia* were observed to remove lead rapidly within 30 minutes of contact. High degree of metal recovery was observed (95%) through elution with 0.1 nitric acid. Moreover, in repeated use of biomass experiment the lead uptake was constantly retained (98%) and no significant biomass damage took place after 10 sorption-desorption cycles (Jalali et al., 2002) *Ascophyllum* was found to bind cobalt (Kuyucak and Volesky, 1989b), cadmium (Holan et al., 1993) and lead (Holan and Volesky, 1994) and contain a

unique mixture of polysaccharides for binding. Nonliving biomass of *A.nodosum* was capable of accumulating Co^{2+} to the extent of 160 mg/g. Carboxyl groups found in alginates of the cell wall were found to play important role in Co^{2+} binding.

Micro-algae *Chlamydomonas reinhardtii* showed maximum loading for Cd as 145 mg/g (1.3 mmol/g) at initial pH 7. Cd-binding, which was not significantly affected by the presence of Ca^{2+} and K^{+}, was dominated by complexation to carboxylic functional groups (Adhiya *et al.*, 2002)

Oscillatoria anguistissima showed a very high capacity for Zn biosorption, being 641 mg/g dry biomass, which was comparable to the commercial ion-exchange resin IRA-400C. The biosorption of zinc by *O. anguistissima* was an ion-exchange phenomenon where a large concentration of magnesium ions were released during Zn adsorption (Ahuja *et al*., 1999).

Amino groups and lipid fraction was shown to be important in biosorption of Cu, Pb, and Cd in non-living biomass of *Pycnoporus sanguineus* (Mashitah, Zulfadhly, and Bhatia, 1999); In this study, also the release of Ca^{2+} ions during Pb uptake was reported.

1.4.5. 1. *Phanerochaete chrysosporium*

Taxonomy of *P. chrysosporium* is as follows:

Kingdom:	Fungi
Divisio:	Basidiomycota
Class:	Hymenomycetes
Subclass:	Homobasidiomycetes
Ordo:	Aphyllophorales
Family:	Corticiaceae
Genus:	Phanerochaete

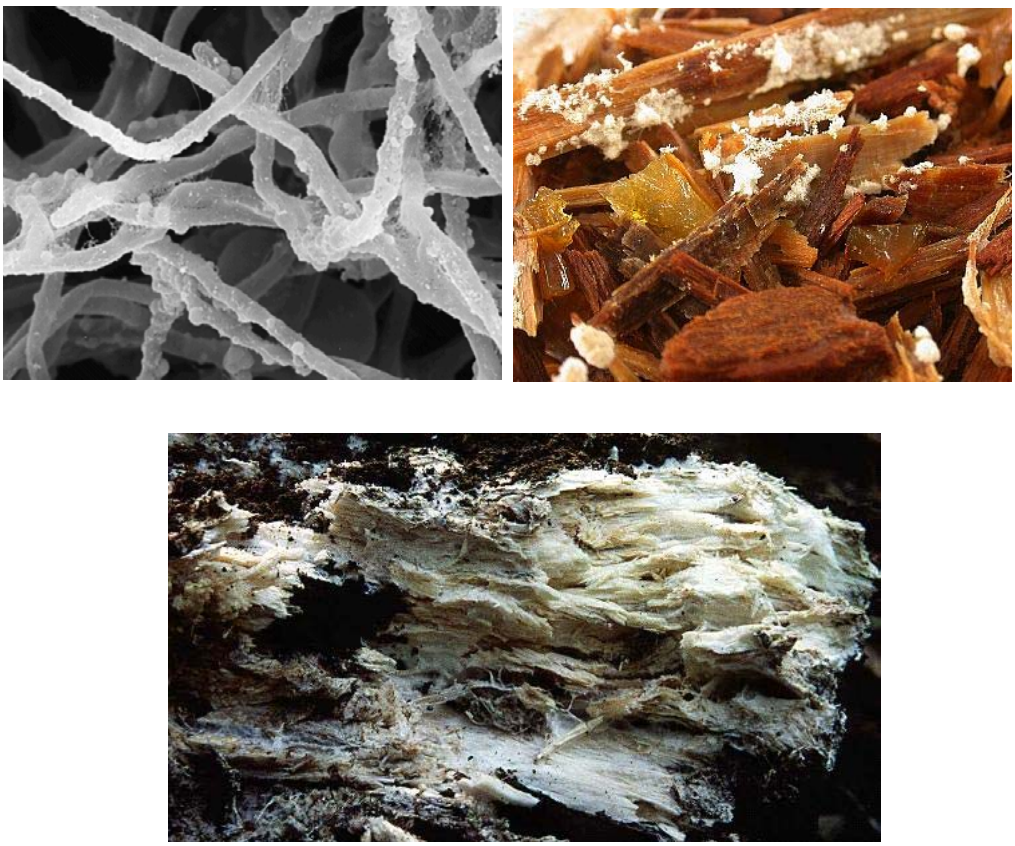

Figure 1.3 a) Mycelia of *P.chrysosporium*. Image size is 40 microns.(http://commtechlab.msu.edu/sites/dlc-me/zoo/microbes/microbemonth.htm)

b) Fruiting body of *P.chrysosporium* on wood.

c) *P.chrysosporium* rotting wood on a tree bark. Lignin is degraded, white-coloured cellulose remained untouched. (http://botit.botany.wisc.edu/toms_fungi/may97.htm

P. chrysosporium was described briefly by Burdsall and Eslyn (1974) (http://botit.botany.wisc.edu/toms_fungi/may97.html). It is one of those resupinate, or crust fungi, that decays wood. These fungi never form a mushroom for reproduction, but form effused, very flat, fruiting bodies that appear as no more than a crust on the underside of a log. The *P. chrysosporium* genome is approximately 30 Mb, organized in 10 chromosomes (Martinez *et al.*, 2004). Its genome sequencing is almost complete. This effort has been carried on by DOE Joint Genome Institute and the organism first basidiomycete genome sequenced.

P. chrysosporium has several features that might make it very useful. First of all, unlike some white rotters, it leaves the cellulose of the wood virtually untouched. Secondly it has a very high optimum temperature (about 40 ^{0}C). These characteristics point to some possible roles in biotechnology. In the subclass Homobasidiomycetes where *P.chrysosporium* belongs, the major component of the cell wall is glucan, constituting more than 50% of the cell wall dry weight. Chitin content is approximately 20%, mannose content is 5%, and other sugars e.g., galactose, galactosamine, xylose and arabinose are found in trace amounts (<0,5%) (Herrera, 1992). It has been investigated by many laboratories as a possible biobleaching and biopulping agent to replace the harsh chemicals that are being used in conventional paper bleaching. The organism produces lignin peroxidases (LiPs), a family of extracellular glycosylated heme proteins, as major components of its lignin-degrading system. These enzymes, along with others capable of degrading toxic waste have great environmental importance. Some of the lignin-degrading enzymes of *P. chrysosporium* will also degrade toxic wastes, such as PCB's, PCP's and TNT.

1.4.5. 2. Heavy Metal Biosorption Studies Using *Phanerochaete chrysosporium*

White-rot fungi are characterized by their unique ability to degrade lignin and a number of structurally similar organic compounds. In addition, these organisms are able to accumulate heavy metals at high levels, providing an opportunity for their use for *in situ* bioremediation of organic pollutants accompanied by heavy metal ions (Baldrian, 2003). The white-rot fungus *Phanerochaete chrysosporium* (Basidiomycete) has been proposed for bioremediation of complex waste materials due to its ability to degrade a number of pollutants (Bumpus *et al.,* 1985). *P. chrysosporium* produces laccases and peroxidases that are involved in the degradation of lignin which is the most complex polyaromatic substance. Because this wood-decomposing fungus can degrade many different pollutants, it has been widely evaluated as a means of enhancing pollutant degradation in soil (Ali and Wrainwright, 1994).

P. chrysosporium was employed for the removal of Cd, Cu, Pb, Mn, Ni, Co and Hg from the culture media (Dhawale *et al.,* 1996; Yetiş *et al.,* 2000). In the presence of high external metal concentrations, mycelial growth was severely inhibited (Falih,

1997). There was a strong indication that metals should be transported in high amounts into the cells. In another study, the equilibrium adsorptive capacity order of *P. chrysosporium* was determined as Pb(II)>Cr(III)>Cu(II)=Cd(II)>Ni(II) (Yetiş *et al.,* 1998). Resting cells of P.chrysosporium were shown to uptake Pb(II) up to 80 mg/g dry cell. Live and dead cells uptake 9 mg Pb(II)/g, and 20 mg Pb(II)/g dry biomass, respectively (Yetis *et al.*, 2000). Compared with ion-exchange resin which showed a quite consistent adsorption capacity in a pH range of 2,5-5, the living fungal mycelium had an optimal adsorption capacity for copper in a quite narrow pH range around 6. All the studies indicated that this fungus has potential for metal biosorption in emerging bioengineering technologies for treating industrial effluents and contaminated waters and soils (Sing and Yu, 1998).

1.5. 1. Basis of Infrared Spectroscopy

The term "infrared" covers the range of the electromagnetic spectrum between 0.78 and 1000 μm. In the context of infrared spy, wavelength is measured in "wavenumber".

Infrared (IR) region is divided into three sub regions (Figure 1.4) (Smith, 1999):

Region	Wavenumber range (cm^{-1})
Near	14000-4000
Middle	4000-400
Far	400-4

The atoms in a molecule are constantly oscillating around average positions. Bond lengths and bond angles are continuously changing due to this vibration. A molecule absorbs infrared radiation when the vibration of the atoms in the molecule produces an oscillating electric field with the same frequency as the frequency of the incident IR light. The molecule will only absorb radiation if the vibration is accompanied by a change in the dipole moment of the molecule. A dipole occurs when there is charge separation across bond. If the two oppositely charged molecules get closer or move further apart as the bond bends or stretches, the moment will change.

In a molecule, atoms vibrate and rotate relative to their center of mass. If the vibration energy is small, the motion can be approximated to Simple Harmonic Motion, SHM. These vibrational and rotational phenomena are used to determine the compounds of matter and its structure. The vibrational frequency (ν_{vib}) of the spring-like bond is given by;

$$\nu_{vib} = ½ [\sqrt{} (k / \mu)]$$

where k is the force constant the stiffnes of the bond and μ is the molecular reduced mass of atom A (with mass m_A) and atom B (with mass m_B), and is defined as;

$$\mu = m_A m_B / (m_{A+} m_B)$$

All of the motions can be described in terms of two types of molecular vibrations. One type of vibration, a stretch, produces a change of bond length. A stretch is a rhythmic movement along the line between the atoms so that the interatomic distance is either increasing or decreasing. The second type of vibration, a bend, results in a change in bond angle. These are also called scissoring, rocking or wigwag motions. Each of these two main types of vibration can have variations. A stretch can be symmetric or asymmetric. Bending can occur in the plane of the molecule or out of plane; it can be scissoring, like blades of a pair of scissors, or rocking, where two atoms move in the same directions (Volland, 1999). Figure 1.4 demonstrates the main types of variations schematically.

An infrared spectrum usually consists of a plot of the absorption of radiation as a function of wavenumber. As each different material has a unique combination of atoms, no two compounds produce the exact same infrared spectrum. Therefore, an infrared spectrum can result in a positive identification of every different kind of material.

The value of infrared spectrum analysis comes from the fact that frequencies and intensities are sensitive to local structure, orientation, physical state, conformation, temperature, pressure and concentration (McDonald, 1986).

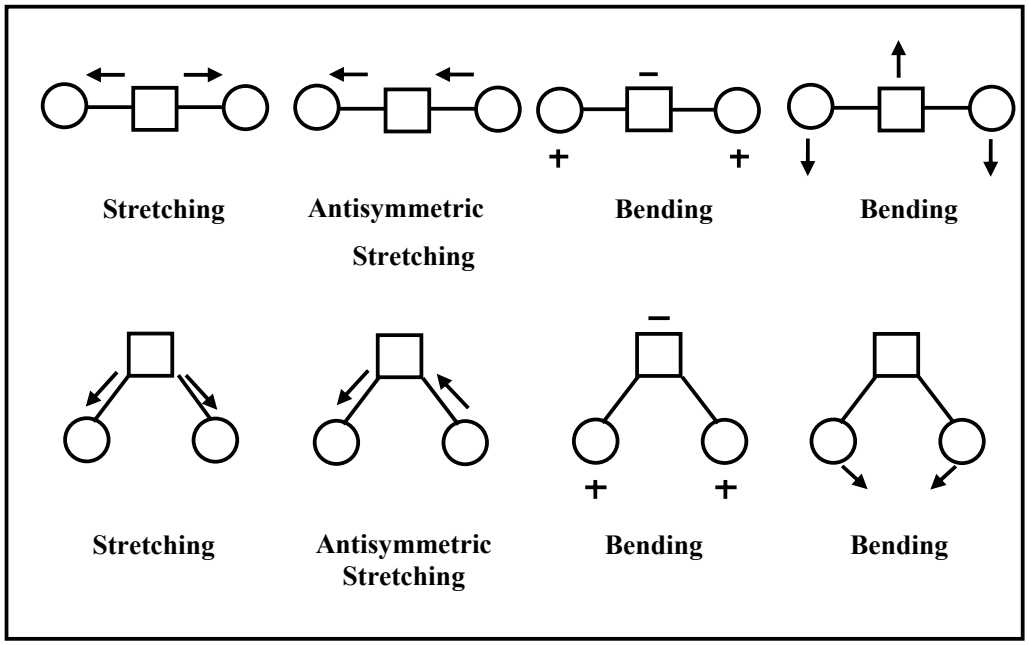

Figure 1. 4. Types of normal vibration in a linear and non-linear triatomic molecule. Atomic displacements are represented by arrows (in plane of page) and by + and – symbols (out of page plane) (Arrondo *et al*., 1993).

1.5.2. Advantages and Applications of Fourier Transform Infrared Spectroscopy

1. The instrumentation is inexpensive; the operation of the equipment is simple.
2. FTIR method is a rapid and sensitive technique and data processing is simple with computer programs (Ci *et al.,* 1999; Manoharan *et al.,* 1993; Rigas *et al.,* 1990).
3. The FTIR technique yields high quality infrared spectra from sample amounts as low as few micrograms (Dighton *et al.,* 2001).
4. It gives information that may have diagnostic value.
5. The system permits manipulation of data, permanent data storage and quantitative calculations (Ci *et al*., 1999; Yano *et al*., 1996).
6. It can be applied to the analysis of solids, liquids and gases.

7. It is a non-invasive physical method (Yano *et al*., 1996).

IR-spectroscopy has been applied to microorganisms for over 40 years, based on the observation that different microorganisms display different IR spectra. However, a cell or a biofilm represent an extremely complex system. Many different signals arise from vibrations of molecules in the extracellular polymeric components, the cell wall, the membrane and the cytoplasm. Nevertheless, the region between 4000 and 500 cm^{-1} holds characteristic bands and is suitable for the characterization of microorganisms. The functional groups of peptide and protein structures, of polysaccharides, of phospholipids and of nucleic acids can be observed and a spectrum with a high content of information is obtained. This is already exploited for the classification of clinical microorganisms (Naumann, 2000). Mathematical and statistical methods allow further analysis of spectral information (Schmitt and Flemming, 1998). In recent years, various studies in biosorption have been made in order to analyze the biosorption mechanism on the surfaces of bacteria, algae, yeast and fungi. Localization of the adsorbed metal on cell surface is subject to interest, so the functional groups and the changes upon interaction with heavy metals have been monitored at molecular level by using FTIR spectroscopy (Kuyucak and Volesky, 1988, Kapoor and Viraraghavan, 1997, Bai and Abraham, 2002, Vasudevan *et al.,* 2003).

1. 6. Aim of the present study

The white-rot fungus *Phanerochaete chrysosporium,* the biomass of which is very promising for applications in paper and pulp industry, has a potential for use as a metal biosorbent in growing bioengineering technologies for treating industrial effluents and contaminated resources.

The aim of the present study was to investigate the main biosorption mechanisms for lead [Pb(II)] in *Phanerochaete chrysosporium.* Our approach involved the determination of the active binding sites on the biomass surface, by selectively blocking the known functional groups via subjecting *P.chrysosporium* biomass to

chemical modifications. Relative concentrations of the Pb (II) sorbed from the aqueous environment to all types of biomass as well as Mg and Ca release into the aqueous phase were measured and compared by using Atomic Absorption Spectroscopy. Fourier-Transform Infrared (FT-IR) Specroscopy technique was used to monitor and analyze the changes at molecular-level on *P.chrysosporium* cell surfaces upon chemical modifications to figure out the interactions of lead with non-modified and modified biomass surface.

CHAPTER II

MATERIALS AND METHODS

2.1.1. Microorganism and Its Maintenance

The white-rot fungus, *Phanerochaete chrysosporium* (ATTC 24725) kindly provided by Prof. Dr. Filiz B. Dilek (Environ. Eng. Dept., METU) was used in this study. The fungus was inoculated to Sabaroud Dextrose agar slants and incubated for 4 days at 35 ^{o}C for spore formation. The organism was then transferred monthly and stored at 4 ^{o}C.

2.1.2. Chemicals

The chemicals used and their suppliers are listed in Appendix A.

2.2.1. Preparation of Biomass

Biomass was prepared as reported by Yetiş *et al.* (2000). *P. chrysosporium* spores were separated from Sabaroud Dextrose agar slant surfaces by scrapping. They were homogenized with glass tube homogenizer and suspended in sterile distilled water. The suspension was then transferred into 1 L Erlenmayer flasks each containing 500 ml of growth medium and incubated for 41 h at 160 rpm in a rotary shaker at 35 ^{o}C. The growth medium used to grow *P. chrysosporium* was that described by Prouty (1990). The medium composed of (in g/L) 10 glucose, 2 KH_2PO_4, 0.5 $MgSO_4$, 0.1 CaCl, 0.12 NH_4Cl, and 0.001 Thiamine. The biomass was

separated from the culture medium through Whatman No 1 filter paper and washed several times with distilled deionized water. The biomass was then dried overnight at 60 ºC. Dried biomass was ground and powdered by using mortar and pestle. Consquently, the biomass was obtained as uniform particles with sizes between 90-125 μm by sieving the ground biomass through test sieves with pore sizes 90 μm and 125 μm.

2.2.2. Chemical Modification of Biomass

Portions of the raw biomass were chemically treated in different ways to understand the role of functional groups in biosorption of lead, as described by Kapoor and Viraraghavan (1997).

Two grams of dry raw biomass were suspended in 130 mL of anhydrous methanol, and 1.2 mL of concentrated HCl was added to the suspension. The reaction mixture was shaken on a rotary shaker for 6 h at 125 rpm. The metal binding ability of carboxyl groups should be significantly reduced as a result of esterification. (Kapoor and Viraraghavan (1997)).The reactions occur as follows:

$$RCOOH + CH_3OH \xrightarrow{H^+} RCOOCH_3 + H_2O$$

The methylation of amino groups were performed by contacting one gram of biomass with 20 mL formaldehyde (HCHO) and 40 mL of formic acid (HCOOH), and then the reaction mixture was shaken on a rotary shaker for 6 h at 125 rpm. This treatment is expected to result in methylation of amines by the following general reaction:

$$RCH_2NH_2 \xrightarrow{HCHO,HCOOH} RCH_2N(CH_3)_2 + CO_2 + H_2O$$

One gram of raw biomass was heated under reflux conditions with 40 mL triethyl phosphite and 30 mL of nitromethane described by Tobin et al. (1990), to chemically modify the phosphate groups on the biomass surface by esterification.

One gram of raw biomass was heated separately with 75 mL benzene and acetone under reflux conditions. Both of these chemical treatments are expected to extract the lipid fraction of biomass (Tobin et al., 1990).

2.3.1. Heavy Metal Biosorption

All the glassware used in biosorption experiments were washed in the following sequence, to avoid metal contamination that can arise from the environment: detergent, tap water, 1:1 nitric acid (65%):water, tap water, 1:1 hydrochloric acid (37%):water, tap water and reagent water which is ultra pure of at least 16 Mega Ohm Quality, defined by EPA. Furthermore, all the aqueous solutions were prepared with ultra pure water. Aqueous lead (Pb) solution were prepared by dilutions from previously prepared stock solutions of 10.000 ppm. Pb solutions were prepared by using the nitrate salt, $Pb(NO_3)_2$, of this heavy metal. Aqueous Pb solutions were prepared as 50 mL solutions of 100 ppm concentration in 100 mL Erlenmayer flasks. The pH of the solution was initially adjusted to 5.0 with 0.01 N HCl and 0.01 N NaOH.

100 mg dry weight of raw or chemically modified biomass was subjected to above defined Pb solution by shaking on a rotary shaker at 200 rpm for 6 h at 25ºC. For each type of modified biomass, incubation experiments were run in triplicates. Furthermore, for each modified biomass incubation set, control groups of raw biomass with no heavy metal treatment, were run as triplicates. The changes in pH of the biosorption medium were observed for all biomass types by measuring the pH of the solutions with pH meter (Hanna Instruments) within regular intervals.

2.3.2. Sample Preparation for Metal Analysis

After heavy metal treatment, biomass was harvested by filtration on 0.40 µm-Millipore Isopore polycarbonate filter membrane. Filtered biomass were then taken into crucibles and air dried at 105 ^{o}C. Recovered amount of biomass was calculated by measuring the weight of the biomass in crucibles until constant weight was achieved. After then, some portion of each biomass was separated for FTIR studies

and the remaining portion was used for acid digestion process to analyze the amount of Pb adsorbed onto cell surfaces. Dried samples of chemically modified and non-modified biomasses of *P. chrysosporium* were burned at 625 ^{o}C for 3 h (since Pb showed the tendency to get evaporated upon a longer exposure) in a muffle oven. Digestion of biomass samples was started in the crucibles and performed on a hot plate and then continued in 50 mL Griffin beakers by dissolving the ash formed from the samplings in 68 % of nitric and 70 % perchloric acid with a ratio of 2:1 by successive additions of 5 mL and 2.5 mL, respectively (Kuyucak and Volesky, 1988; EPA). Heating and acid addition was continued until the digestion is complete which is generally indicated when the digestate and vapor is light in color; and not changed with continued heating and acid addition. Initial volume of solution was vaporized to a few milliliters. This solution was again filtered through Whatman No 1 filter paper and the obtained metal solution was made up to 50 mL by washing down the beaker walls with water and then refiltrating the solution. Measurement of concentration of heavy metals was performed as described in the next section.

2.3.3 Measurement of Metal Ion Concentration

Heavy metal analyses were performed by using a Unicam Model 929 Atomic Absorption Spectrophotometer. Before every metal analysis, calibration curve was prepared by spectrophotometric determinations with standard solutions. For the metal analyses with high metal concentrations exceeding the range of Pb lamp, samples were diluted (1/10 and 1/25) with ultra pure water. The pH of the solutions was adjusted to below pH 2 with 68 % nitric acid.

Filtered solutions obtained right after Pb treatment as well as acid-digested samples were analyzed to obtain more precise data. Moreover atomic absorption measurements were conducted with the same Atomic Absorption Spectrophotometer for calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions in the filtrates to quantify the release of these ions from the biomass surface during heavy metal biosorption.

2.4 FTIR Studies

2.4.1. Sample Preparation

The specimens for FTIR studies were prepared using potassium bromide (KBr) pellet technique. KBr is the most commonly used alkali halide disk which is used as a beam condensing system. It is completely transparent in the mid-infrared region (Stuart, 1997).

1 mg dry weight of raw biomass, modified biomass types, and the samples obtained after Pb exposure for each biomass type were mixed with 100 mg dry weight of KBr (at the ratio of 1/100). Then, the mixture was grounded in agate mortar to obtain a homogenous powder and dried overnight in Labconco freeze dryer. The mixture was then subjected to a pressure of 1200 kg/ cm^2 for 10 minutes in an evacuated die to produce a thin transparent KBr disk or pellet. Figure 2.1 shows the FTIR spectrum of 100% pure KBr pellet.

2.4.2. FTIR Instrumentation

Infrared spectra were obtained by scanning the prepared pellets with a Spectrum One Spectrometer (PerkinElmer, Norwalk, CT, U.S.A.). Prior to sample scan, spectrum of air was recorded as background and subtracted automatically to eliminate the disturbances on the FTIR spectra that would result from atmospheric water vapor. Figure 2.2 shows the infrared spectrum of air used as background in FTIR studies. The FTIR spectra of samples were recorded in the region between 4000 cm^{-1} and 400 cm^{-1} wavenumber at room temperature. 100 scans were taken for each interferogram at resolution of 4 cm^{-1}. Atmospheric vapor was automatically subtracted from the sample spectrum via Spectrum One software of PerkinElmer. Spectrum One software was used for digital data processing.

2.4.3. Statistical Analysis

The results were expressed as mean±standard deviation values. The differences in the means (average of 5 spectra) of the modified *P.chrysosporium* biomass, raw

P.chrysosporium biomass and Pb-incubated raw *P.chrysosporium* biomass (control group) were compared using Mann-Whitney U-test. A p value of the less than 0.05 was considered as significant ($p<0.05$*, $p<0.01$**, $p<0.001$***).

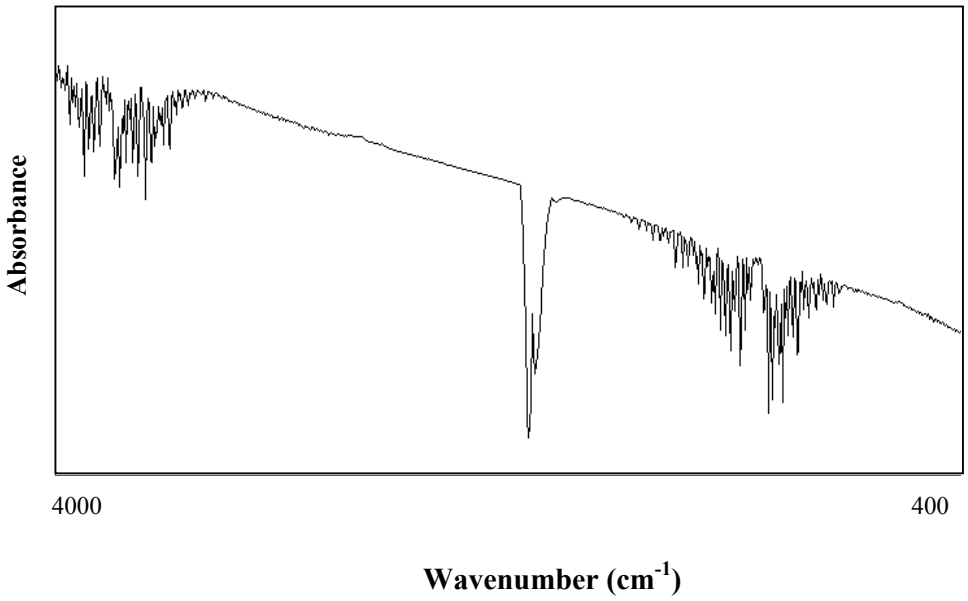

Figure 2.1. The infrared spectrum of 100 % pure KBr pellet

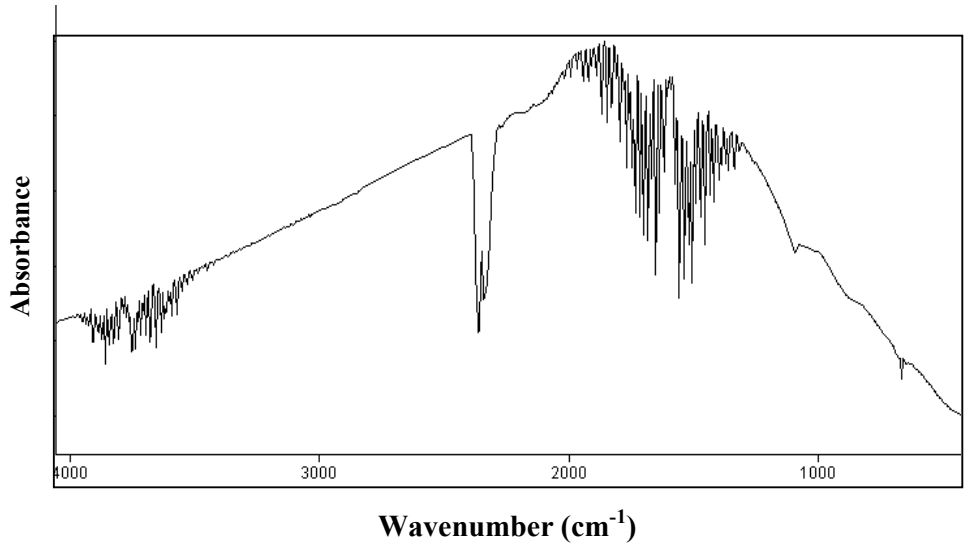

Figure 2.2. The infrared spectrum of air

CHAPTER III

RESULTS AND DISCUSSION

3. 1. FTIR Spectrum of the Raw *P. chrysosporium* Biomass

Interactions of heavy metals with biomass surfaces are often reflected to FTIR spectra as changes in wavenumber, absorbance intensity, peak area and bandwidth of a signal for a particular functional group acting as a binding site for a given metal (Gardea-Torresdey *et al.*, 2002; Bai and Abraham, 2002). Therefore, the changes at a molecular level in *P. chrysosporium* biomass upon Pb (II) exposure were detected in this study by monitoring the changes in the spectra. For all FTIR studies, including raw, chemically modified and Pb (II)-exposed biomass types, average of five spectrum was used for analysis (n=5).

Figure 3.1 demonstrates the absorption bands of an average infrared spectrum obtained from powdered dry *P. chrysosporium* biomass in the 4000-400 cm^{-1} (mid-IR) wavenumber range. The major absorption bands are labeled in this figure and defined in detail in Table 3.1. The band assignments were made by consulting to the information available in the previously published reports (Guibal and Roulph,1995; Bai and Abraham, 2002; Gardea-Torresdey et al., 2003; Schmitt and Flemming, 1998; Natarajan *et al*., 2001, Kacurakova *et al*., 2000, and Severcan *et al.,* 2000; 2003).

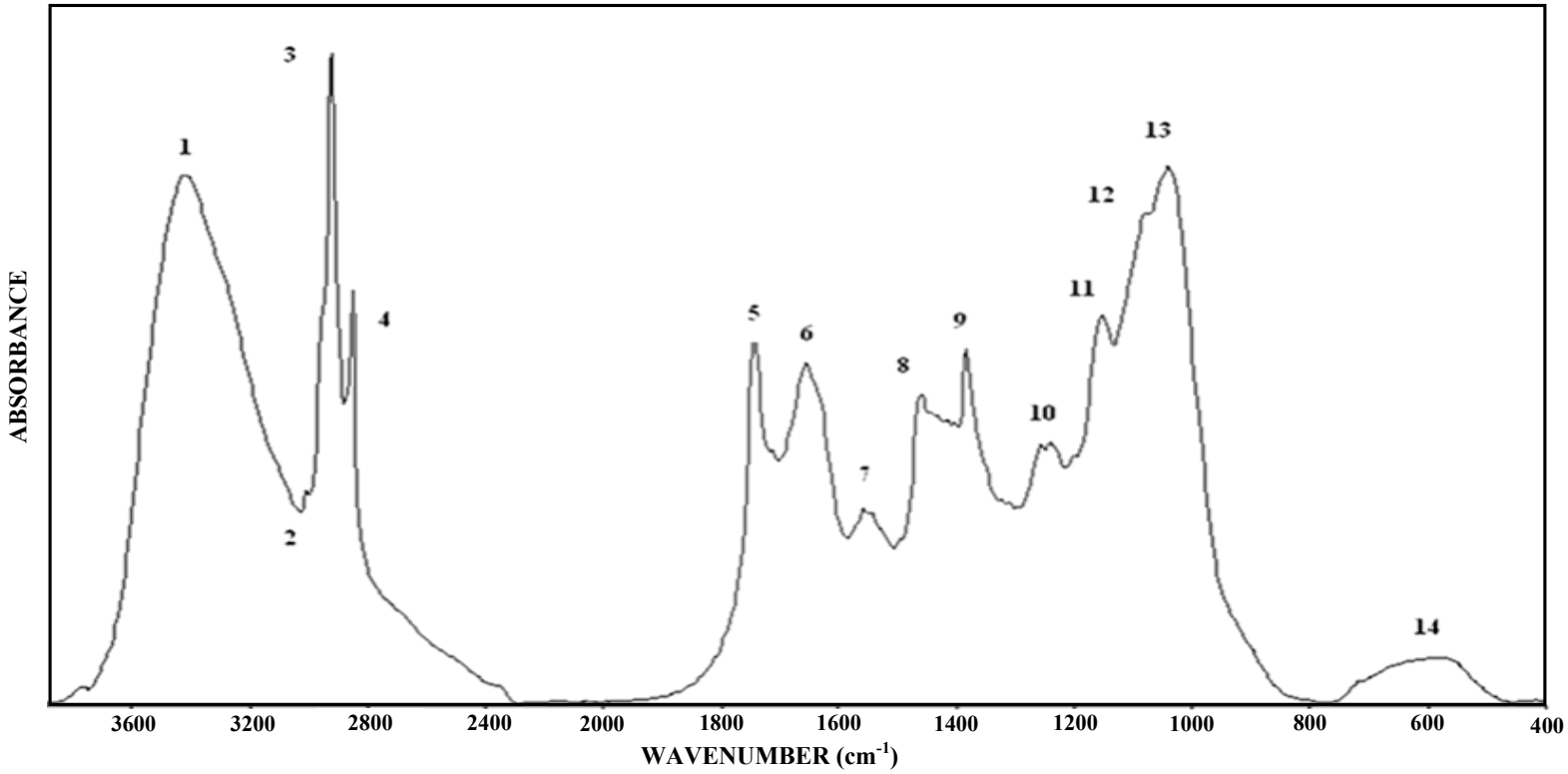

Figure 3.1. Mean FTIR spectrum of the raw *P. chrysosprium* biomass in 4000 – 400 cm^{-1} region (n=5).

Table 3.1. Band assignment to raw *P. chrysosporium* biomass.

PEAK NO	WAVENUMBER (cm^{-1})	DEFINITION
1	**3400** **3300**	N-H stretch: protein, polysaccharides (chitin) Intermolecular H bonding.
2	**3008.56**	Olefinic HC=CH stretch: lipids
3	**2924.91**	CH_2 antisymmetric stretch: mainly lipids
4	**2854.00**	CH_2 symmetric stretch: mainly lipids
5	**1744.79**	C=O stretch: chitin
6	**1655.59**	Amide I: protein, polysaccharide (amide in chitin)-(C=O stretch, N-H bending, C-N stretch)
7	**1549.23**	Amide II: protein, polysaccharide (N-H bend, C-N stretch)
8	**1460.49**	CH_3 asymmetric bending (acetyl, protein, lipid, sugar)
9	**1377.11**	In plane O-H deformation (sugars)
10	**1241.66**	Amide III: C-H stretch: polysaccharide, nucleic acids, PO_2^- asymmetric stretching: phospholipids
11	**1151.35**	C-N stretch (chitin), C-OH (glucan),
12	**1081.68**	C-C stretch, C-O-C stretch: (glucan), PO_2^- symmetric stretching: phospholipids
13	**1040.66**	Glucose ring
14	**580.40**	P=O vibrations

3.2. General Changes in the FTIR Spectra of *P. chrysosporium* Upon Pb (II) Biosorption

Figure 3.2 compares the NH symmetric region of the raw *P. chrysopsorium* biomass and *P. chrysosporium* biomass exposed to 100 ppm Pb (II) in aqueous solution. The NH symmetric band, or Amide A region signal arises from NH moieties of proteins and chitin content of the cell wall, as in the case of all fungi (Kapoor and Viraraghavan, 1997). Functionality of amine and amide groups in heavy metal biosorption for several fungi were previously reported (Bai and Abraham, 2002; Guibal and Ruolph, 1995). Biosorption of Pb (II) ions by *P. chrysosporium* biomass upon exposure to this metal resulted in an increase in the wavenumber of NH stretching band and the peak became narrower. This phenomenon could be due to the interaction of Pb (II) with NH groups as well as intermolecular –H molecules, whose peak signal is conjugated to the NH stretch, so that the peak intensity dramatically decreases upon interaction with Pb (II). This interaction might have resulted in a decrease in absorption level in –H bonding, arising from the water found bound in the system.

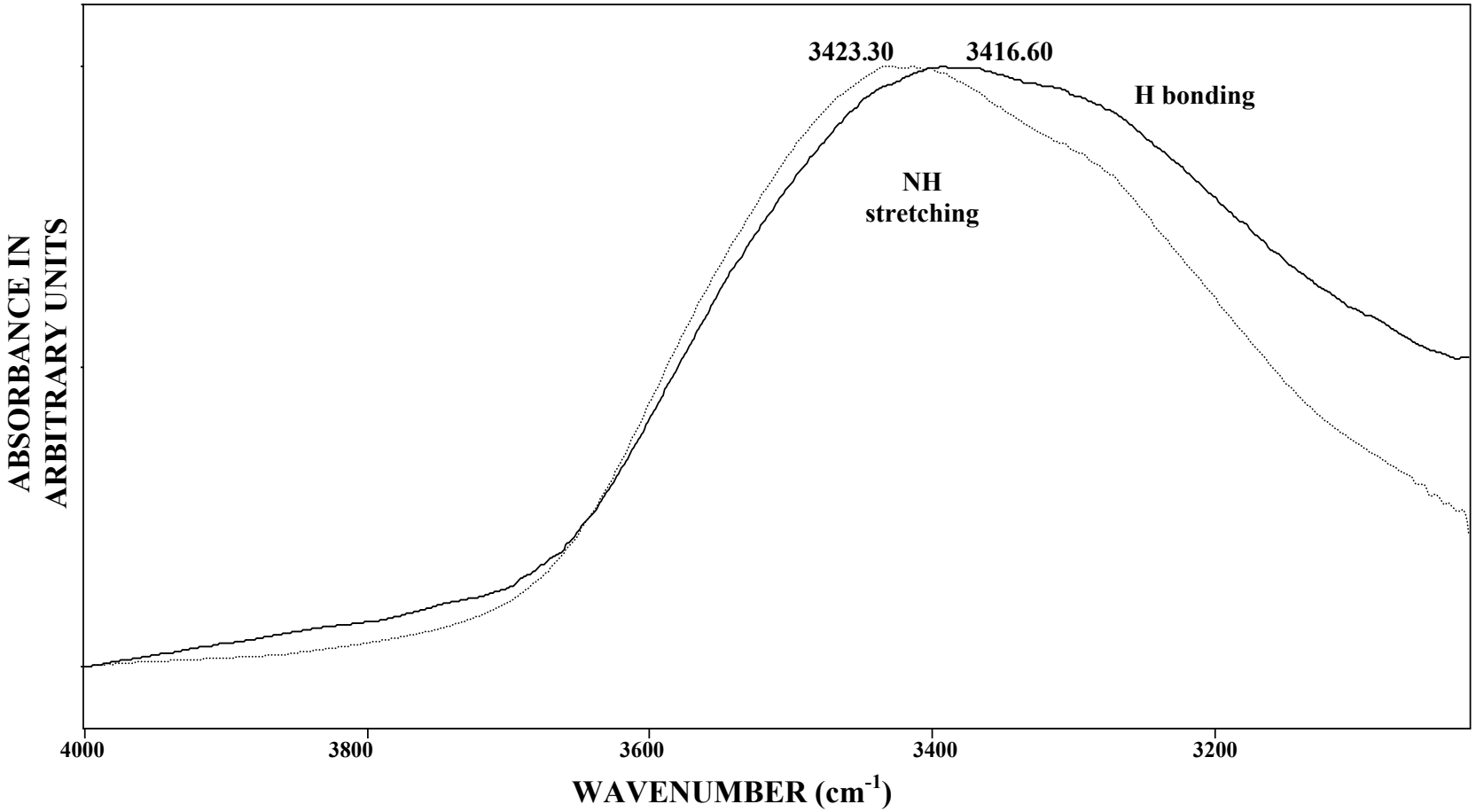

Figure 3.2. Mean normalized FTIR spectra of the raw (———) and Pb (II)-exposed (------) *P. chrysosporium* biomass at RT in the region between 4000-3030 cm^{-1}.

Figure 3.3. compares the C=O stretching (1730 cm^{-1}) and Amide I region (1650 cm^{-1}) of the raw *P. chrysopsorium* biomass and *P. chrysosporium* biomass exposed to Pb (II) in aqueous solution. A substantial decrease in the relative absorption of C=O stretching mode upon metal exposure was observed, with an increase in its wavenumber. This influence of Pb (II) exposure on the intensity of C=O stretching band indicated a possible complexation type of interaction between Pb (II) and carbonyl and carboxyl groups found in chitin and glucan matrix on the cell wall. Supporting our previous finding (Figure 3.2), the shift at the peak position of the C=O band pointed to an increase in dehydration of the carbonyl group. In other words, intermolecular –OH molecules seemed to be liberated from the system as ligands upon interaction with Pb (II) ions. An increase in wavenumber in Amide I band upon Pb (II) exposure accorded well with the wavenumber shift in NH stretching band (Figure 3.2), implying that secondary amine (NH) groups might have involved in Pb (II) biosorption. In previous studies, the shifts in wavenumber and decrease in absorption level of these bands have been evaluated as the consequences of the interaction of heavy metals with amino, carbonyl, and carboxyl groups (Kapoor and Viraraghavan, 1997; Bai and Abraham, 2002; Mashitah *et al*., 1999; Guibal and Roux, 1995; Vasudevan *et al*., 2003). There are conflicting studies in the literature related to the behaviour of this peak regarding its wavenumber. Some of these studies reported a decrease in the peak positions, however, in agreement with our findings, the others reported an increase.

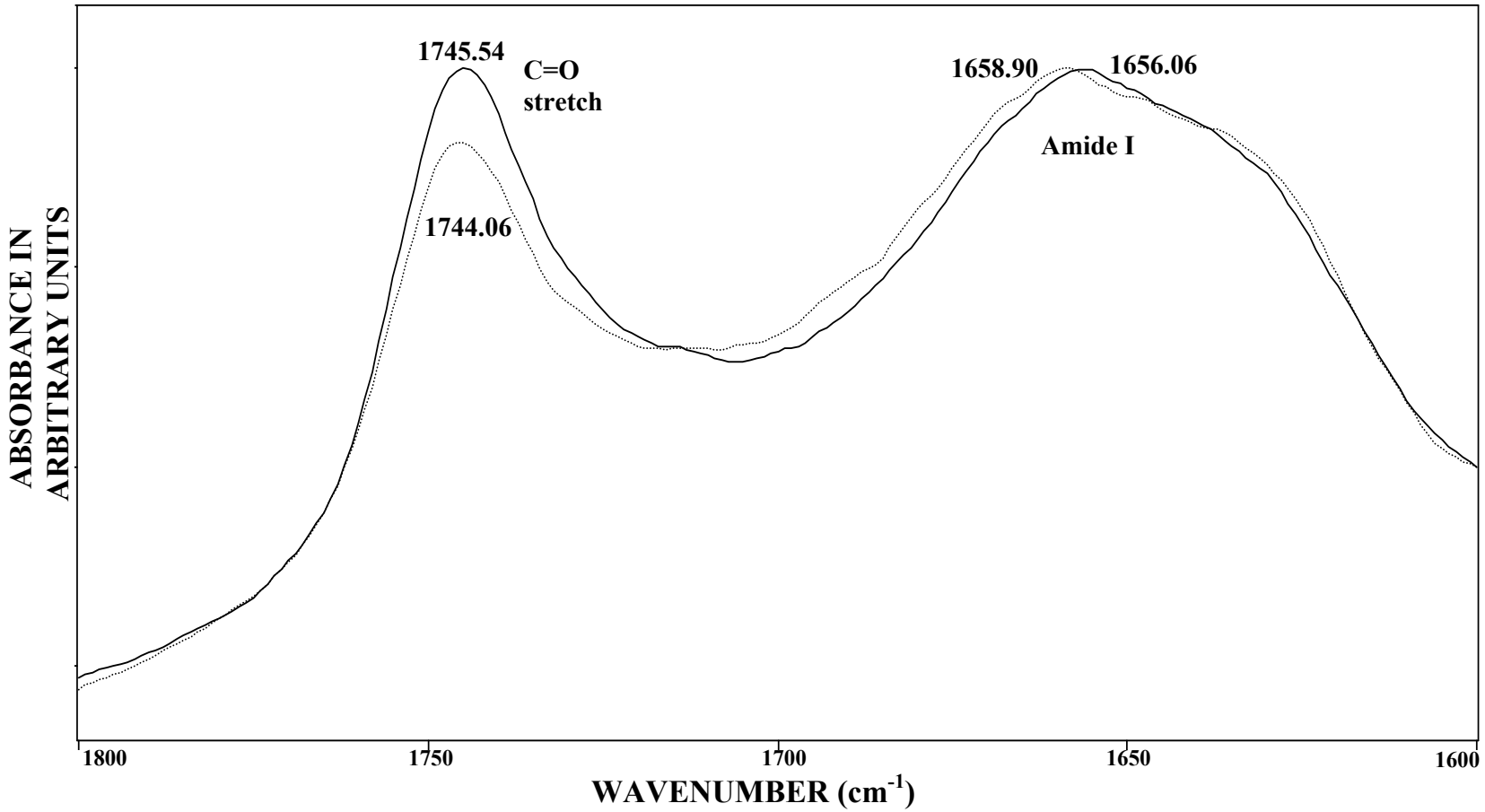

Fig. 3.3. Mean normalized FTIR spectra of the raw (——) and Pb (II)-exposed (------) *P. chrysosporium* biomass at RT in the 1800-1600 cm^{-1} range

Figures 3.4 and 3.5 show the difference observed in *P. chrysosporium* FTIR spectra in the OH deformation, C-O-C stretch, C-N stretch and glucose ring bands upon Pb (II) biosorption. A shift to higher wavenumbers in OH deformation mode was detected. The band was thought to arise from OH groups of glucose monomers abundantly found in glucan and chitin polymers. OH groups of glucan must have acted via complexation rather than ion exchange since these groups are ionizable only at high pH values such as 13, a pH which was never employed in our study (Schiewer and Volesky, 2000)

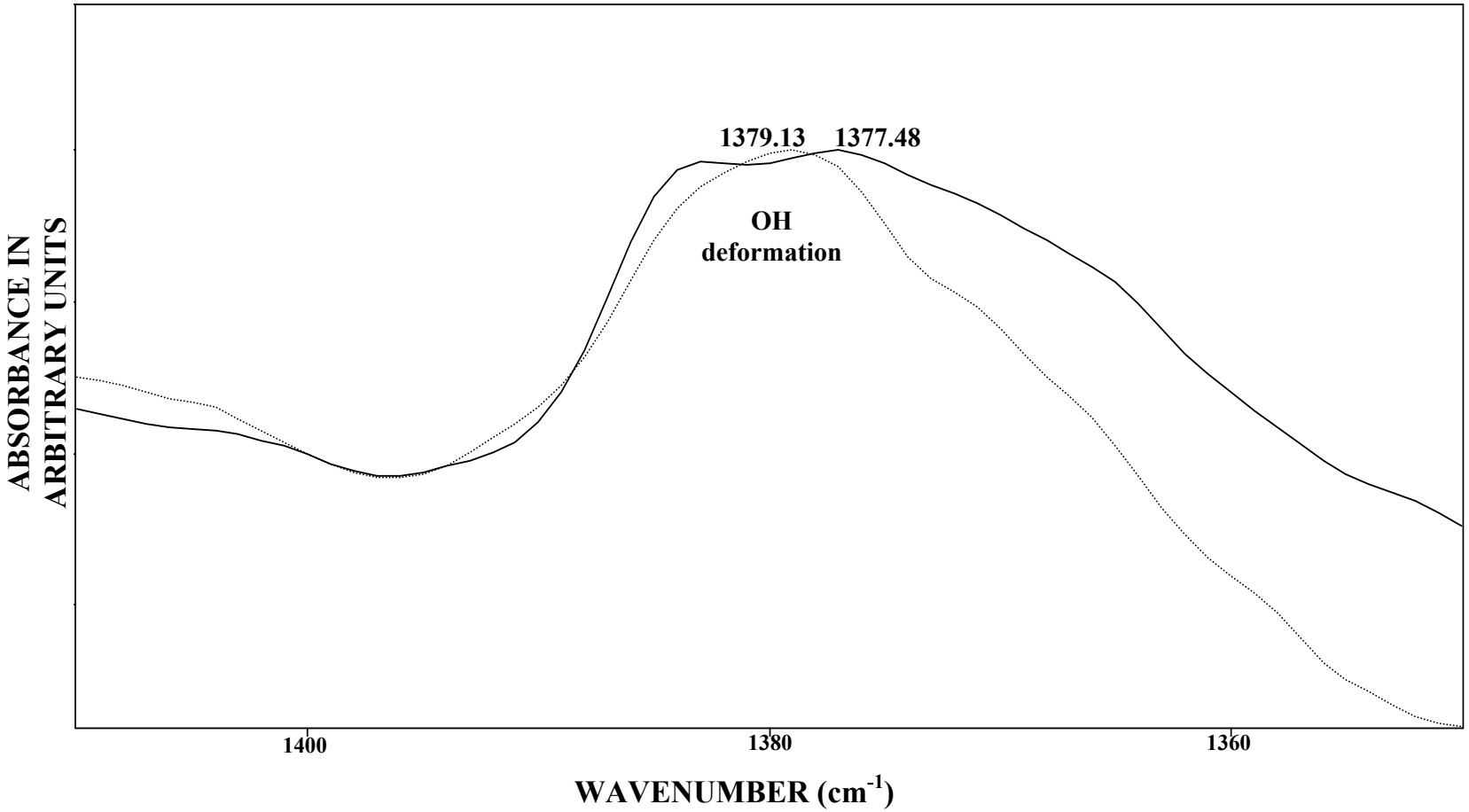

Figure 3.4. Mean normalized FTIR spectrum of the raw (——) and Pb (II)-exposed (------) *P. chrysosporium* biomass at RT in the 1400-1300 cm^{-1} range.

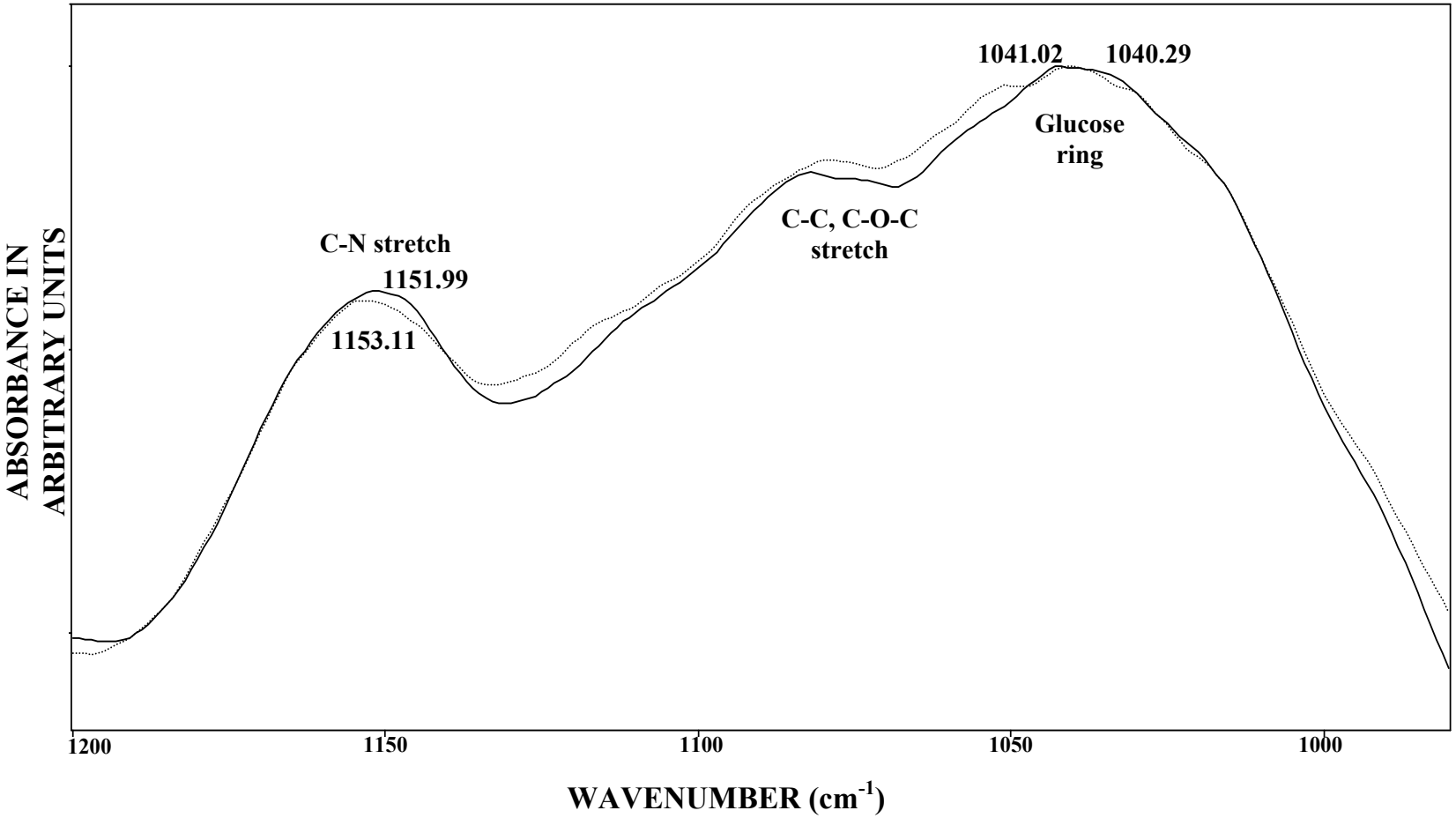

Fig. 3.5. Mean normalized FTIR spectra of the raw (——) and Pb (II)-exposed (------) *P. chrysosporium* biomass at RT in the 1200-950 cm^{-1} range.

Statistical analysis of the above-discussed data was performed by using Mann-Whitney U-test and p values were obtained for the wavenumbers of the control and Pb (II)-exposed *P. chrysosporium* biomasses. The p values presented in Table 3.2 clearly showed that the changes in C=O stretch and Amide I band wavenumber average values were statistically significant.

Table 3.2. Numerical summary of the differences in wavenumbers of the selected absorption bands between the raw and Pb (II)-exposed *P. chrysosporium* biomasses. P values less than or equal to 0.05 was accepted as statistically different with respect to the control group.

PEAK AND WAVELENGTH (cm^{-1}) / BIOMASS TYPE	N-H stretch	Amide I	C=O stretch	-OH deformation	CN stretch	Glucose ring
Raw	**3416.60 ± 14.76**	**1656.06 ± 1.19**	**1744.06 ± 0.69**	**1377.48 ± 5.13**	**1151.99 ± 1.06**	**1041.02 ± 2.29**
Raw+ Pb (II)	**3423.30 ± 12.86**	**1658.90 ± 0.83**	**1745.54 ± 0.41**	**1379.13 ± 0.84**	**1153.11 ± 1.17**	**1040.29 ± 1.64**
p value (<0,05)	**0.12**	**0.02**	**0.04**	**0.27**	**0.27**	**0.71**

3.3. Modification and Specific Blocking of Major Functional Groups on Biomass Surface and Their Effects on FTIR Spectra and Pb (II) Biosorption

For convenience, the raw biomass is referred in the text as M0. Accordingly acidic methanol-treated biomass, formaldehyde plus formic acid-treated biomass, nitromethane with triethyl phosphite treated-biomass, acetone-treated biomass and benzene-treated biomass are referred as M1, M2, M3, M4 and M5, respectively.

In this study, after each modification experiment, changes in the FTIR spectrum was determined in order to verify the modification made. The level of Pb (II) biosorption by *P. chrysosporium* biomass was also studied to reveal the extent of surface adsorption after modification. Release of magnesium and calcium in response to Pb (II) biosorption was also investigated. The biosorption experiments were conducted in triplicates in 50 mL of 100 ppm lead nitrate ($PbNO_3^-$ salt) aqueous solution with an initial pH of 5. For each group, 100 mg of raw or chemically-modified biomass was contacted with the solution for 6 hours, during which the change of pH of the solution was monitored. The biosorption results were expressed as mg Pb (II) biosorbed per g of dry biomass as well as mmoles of Pb (II) biosorbed per g dry biomass.

Each set of experiment (a particular biomass modification) had its own control which was unmodified, raw biomass also run in triplicates. This was of special importance since atomic absorption spectrophotometry is a very sensitive technique affected by temperature and humidity as well as the conditions of the device components of at the time of measurement. In the forthcoming sections, the results of Pb (II) biosorption, Ca^{2+} and Mg^{2+} release and change in pH during biosorption will be presented as the averages of three measurements.

3.3.1. Effects of Specific Blocking of Carboxyl (-COOH) Groups

3.3.1.1. FTIR Spectrum of *P. chrysosporium* Upon Specific Blocking of Carboxyl (-COOH) Groups

The raw biomass was treated with acidic methanol to cause methylation of the carboxylic groups on the biomass surface (Kapoor and Viraraghavan, 1997). Methylation ($-COOCH_3$) of the biomass is expected to result in a decrease in the absorption of the related bands such as the C=O stretch and an increase in absorption band of the CH_3 groups.

Figure 3.6 illustrates the mean normalized FTIR spectra of the raw and acidic methanol-treated (-COOH specifically blocked) *P. chrysosporium* biomass in the 1800-1600 cm^{-1} range. A drastic decrease in the absorption band of C=O

stretch was observed, indicating the decrease in the population of the carboxyl groups. This result verified the successful methylation of the COOH groups on biomass surface.

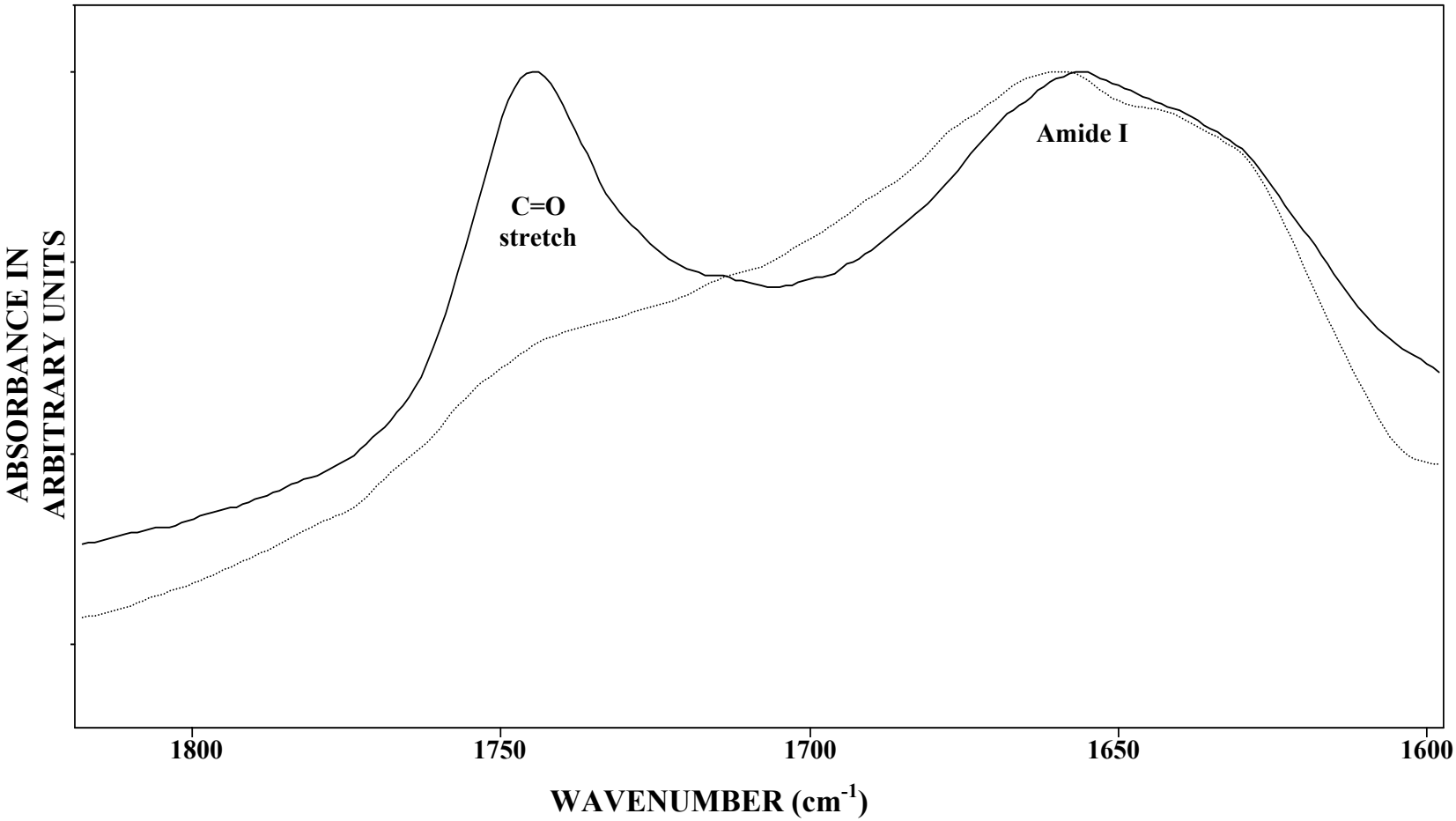

Fig. 3.6. Mean normalized FTIR spectra of the raw (——) and acidic methanol-treated (-COOH groups specifically blocked) (------) *P. chrysosporium* biomass with respect to the Amide I band at RT in the 1800-1600 cm^{-1} range.

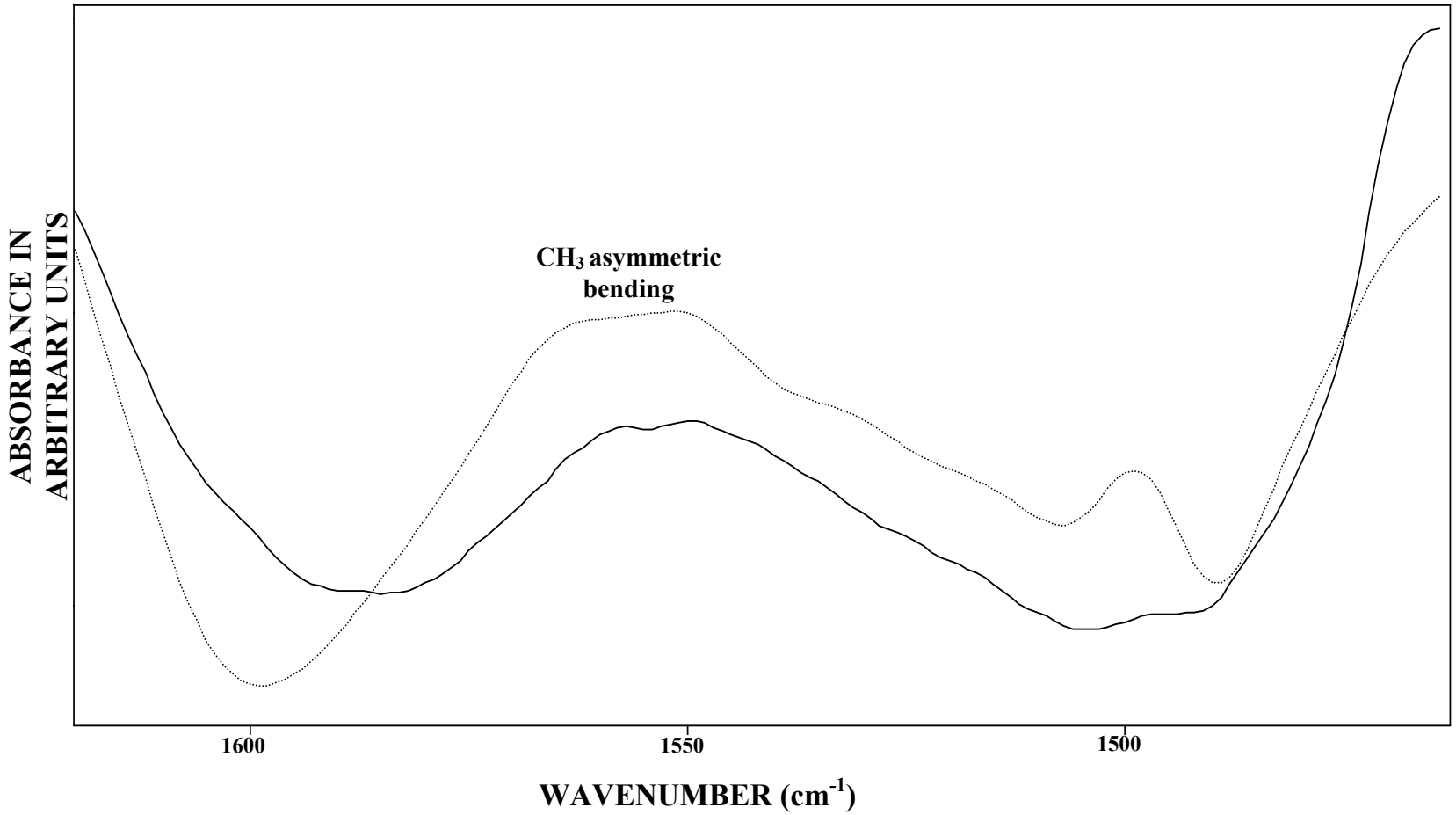

Fig. 3.7. Mean normalized FTIR spectra of the raw (▬▬) and acidic methanol-treated (-COOH groups specifically blocked) (▪▪▪▪▪▪) *P. chrysosporium* biomass with respect to OH deformation band in the 1600-1400 cm^{-1} range.

On the other hand, a significant increase in the CH_3 asymmetric bending absorption peak (Figure 3.7) pointed to a relative increase in the amount of methyl groups after modification of the raw biomass. This provided a direct proof about the methylation of the biomass.

Moreover, an increase in the bandwidth and elongation of the peak area in lipid region of the modified biomass was recorded (Figure 3.8). The unlabeled peaks indicated by the arrows represent the CH_3 stretching of the spectra and were of higher absorbance than the raw biomass as the control. Table 3.3 summarizes the comparisons of the relative peak intensities of C=O stretching band and CH_3 asymmetric bending band for the raw and carboxyl groups-blocked biomasses. Reduction of peak intensities for these bands were found to be statistically significant and further supports the successive modification of the biomass as well as the changes observed in FTIR spectra upon modification of the biomass.

Table 3.3. Numerical summary of the differences in peak intensities of the C=O stretching and CH_3 asymmetric bending absorption bands between the raw (M0) and carboxyl groups-blocked (M1) biomasses. P values less than or equal to 0.05 was accepted as statistically different with respect to the control group.

PEAK AND SIGNAL INTENSITY / BIOMASS TYPE	C=O stretch	CH_3 asymmetric bending
Raw biomass (M0)	**0.98±0.03**	**0.25±0.11**
Carboxyl-groups blocked biomass (M1)	**0.72±0.03**	**0.50±0.05**
p value	**0.03**	**0.03**

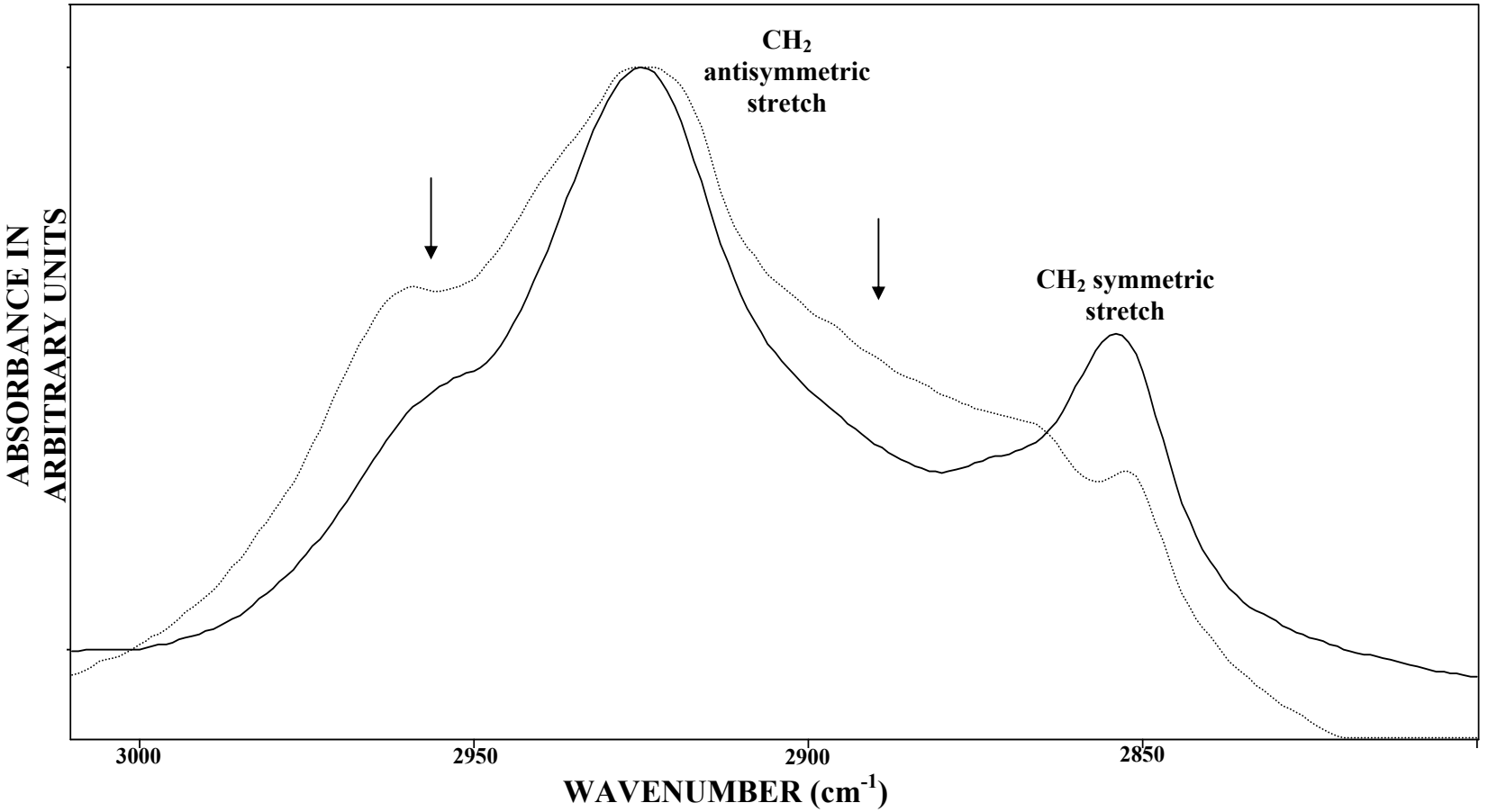

Fig. 3.8. Mean normalized FTIR spectra of the raw (——) and acidic methanol-treated (-COOH groups specifically blocked) (------) *P. chrysosporium* biomass with respect to olefinic band in the 3000-2800 cm^{-1} range.

3.3.1.2. Changes in Pb (II) Biosorption by *P. chrysosporium* Upon Specific Blocking of Carboxyl (-COOH) Groups

Carboxyl groups are the major binding sites for a variety of heavy metal biosorbents. This fact has the following reasons: The carboxyl group is relatively more abundant in cell surfaces of biosorbents like algae, bacteria and fungi. Secondly, at the pH ranges optimum for heavy metal biosorption by above-mentioned biosorbents, carboxyl groups are ionizable, thus are available for electrostatic attraction and ion exchange as well as complexation and physical adsorption (Kratochvil and Volesky, 1998) (Table 1.1). Firstly, changes in Ca^{2+} and Mg^{2+} release patterns in *P. chrysosporium* raw biomass in response to presence of Pb (II) in biosorption solution were investigated. The results for the changes in Mg^{2+} and Ca^{2+} release patterns in biosorption solution which does not contain Pb (II) and the biosorption solution that does contain Pb (II) were compared in Table 3.4 and 3.5.

Table 3.4. Changes in Mg^{2+} and Ca^{2+} release patterns in *P. chrysosporium* raw biomass in response to presence of Pb (II) in biosorption solution.

Biomass type	mg Mg released / g biomass	mmoles Mg^{2+} released / g biomass	mg Ca^{2+} released / g biomass	mmoles Ca^{2+} released / g biomass
Pb-non-exposed raw biomass	0.62±0.05	0.026±0.002	0.36±0.08	0.009±0.002
Pb-exposed raw biomass	0.95±0.03	0.039±0.001	0.41±0.04	0.010±0.001

Table 3.5. Changes in Mg^{2+} and Ca^{2+} releases in *P. chrysosporium* raw biomass in response to presence of Pb (II) in biosorption solution.

Biomass type	Decrease in Mg^{2+} release (%)	Decrease in Ca^{2+} release (%)
Pb-non-exposed-Pb-exposed	**53.0**	**14.6**

Moreover, changes in the pH of the biosorption solution when raw biomass was introduced to solution in which Pb (II) is absent and present were investigated. The results were shown in Figure 3.9.

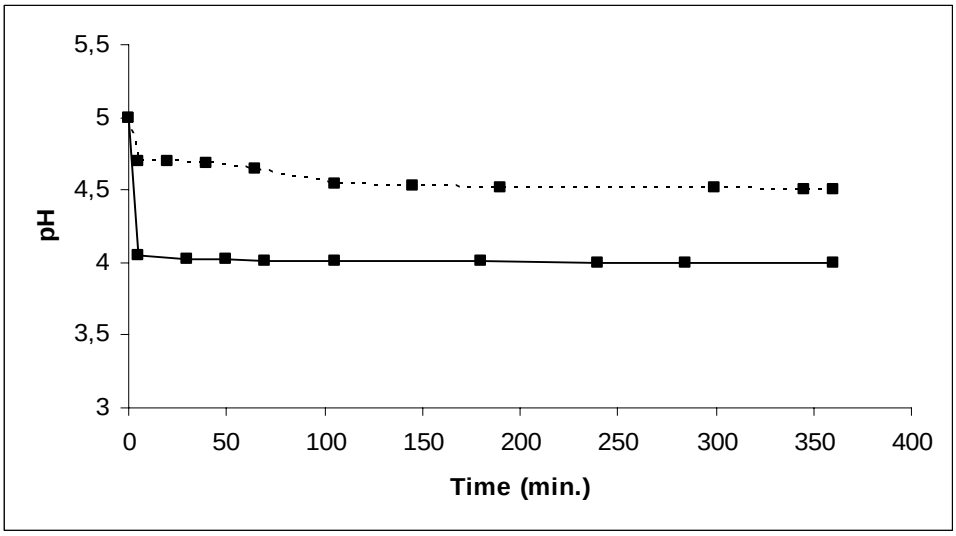

Figure 3.9. Changes in the pH of the biosorption solution as a function of time when raw biomass introduced to solution Pb (II) present (——) and absent (·····).

Relative biosorption capacities of the raw (M0) and the carboxyl groups blocked-biomass (M1) are presented in Table 3.6.

Table 3.6. Pb (II) biosorption capacities of the M0 and M1 type biomasses.

Biomass type	mg Pb (II) biosorbed / g biomass	mmoles Pb (II) biosorbed / g biomass	Decrease in Pb biosorption (%)
M0	9.25±0.09	0.05±0.0004	**92.8**
M1	0.66±0.01	0,003±0,0004	

The biosorption capacity drastically decreased (by 92.8 %) in acidic methanol-treated *P. chrysosporium* biomass in which carboxyl groups were modified via esterification, indicating that carboxyl groups dominate in Pb (II) biosorption in this organism.

The role of carboxyl groups in biosorption has been intensively studied in different organisms. In alfalfa, chromium biosorption was reduced by 92 to 93 % when carboxyl groups were blocked by using the technique used in the present study (Gardea-Torresdey *et al.*, 2002). Kuyucak and Volesky (1988) stated for *Ascophyllum nodosum* that carboxyl groups on the alginates of the cell wall play an important role in cobalt binding. The blocking of carboxyl group was shown to diminish lanthanum, uranium and zinc uptake to a small degree (7.4-10 %), and this did not affect lead uptake in *Rhizopus arrhizus*. Alteration of carboxyl groups was reported to severely limit the deposition of the metals such as Mn, Fe (III), Ni, Cu, Au (III) ranging from 10 % to 60 % in *Bacillus subtilis* (Beveridge et al., 1980). In *Chlamydomonas reinhardtii*, Cd binding was dominated by complexation to carboxylic functional groups (Adhiya *et al.,* 2002). Regarding *R. nigricans*, chromium uptake was shown to decrease by 42 % upon blocking of carboxyl groups (Bai and Abraham, 2002). Kapoor and Viraraghavan (1997) reported that carboxyl group modification in *Aspergillus niger* decreased the uptake values of lead, cadmium and copper by approximately 84 %, 86 % and 80 %, respectively. Lead uptake was reduced by

50 % in *Pycnoporus sanguineus* after carboxylic acid modification (Mashitah et al., 1999). Copper biosorption in *S. cerevisiae* decreased from 6mg/g dry biomass to 4mg/g due to the esterification of carboxyl groups by the method used in the present research. Our results, overall, indicated that carboxyl groups are the major sites of Pb (II) deposition on *P. chrysosporium* surfaces.

Table 3.7 shows a comparison of Mg^{2+} and Ca^{2+} release in *P. chrysosporium* biomass type M0 and the biomass type M1. For the raw, untreated biomass (control), 0.02 mmoles of Mg^{2+} and 0.006 mmoles of Ca^{2+} (a total of 0.026 mmoles of these metal ions) were found to be released per g dry biomass in response to 0.045 mmoles of Pb (II) biosorbed per g dry biomass. The major role of ion exchange in biosorption was previously emphasized. For seaweed *Ascophyllum nodosum*, it was reported that the amount of Mg^{2+}, Ca^{2+} and H^{+} release into the solution increased in the presence of cobalt (Kuyucak and Volesky, 1988). In *Mucor rouxii*, the exchange of Pb (II) with K^{+} and Ca^{2+} was confirmed with EDAX. Upon biosorption, the peaks corresponding to these light metals on *Mucor* biomass disappeared, pointing to the dominant role of ion exchange (Lo *et al*., 1999). Kapoor and Viraraghavan (1997) have shown that for 55 μmoles of Pb (II) biosorbed, approximately 32 μmoles of Ca^{2+} and 15 μmoles of Mg^{2+} was released. Likewise, 35 μmoles of Ca^{2+} and 15 μmoles of Mg^{2+} were released in response to 60 μmoles of Cd biosorbed. Our results showed that Mg^{2+} release is much more than Ca^{2+} release during Pb (II) biosorption in *P. chrysosporium*. This finding might imply that ion-exchange via Mg^{2+} and Ca^{2+} release occurs nonstoichiometrically in Pb (II) biosorption.

Table 3.7. Changes in Mg^{2+} and Ca^{2+} release patterns in response to Pb (II) biosorption in *P. chrysosporium* upon blocking the carboxyl groups.

Biomass type	mg Mg released / g biomass	mmoles Mg^{2+} released / g biomass	mg Ca^{2+} released / g biomass	mmoles Ca^{2+} released / g biomass
M0	0.48±0.04	0.020±0.002	0.23±0.02	0.006±0.0004
M1	0.10 ± 0.01	0.004±0.0003	0.13 ± 0.03	0.003 ± 0.001

The data tabulated in Table 3.8 are summarized in Table 3.9 to show the consequences of the present treatment systematically. A remarkable decrease in Mg^{2+} and Ca^{2+} release after blocking the carboxyl groups was evident. This event seemed to be resulted from the modification during which the H^{+} ions present in the acidic methanol might have replaced the Mg^{2+} and Ca^{2+} ions on the cell wall. Therefore, ion exchange capacity of the modified *P. chrysosporium* biomass was significantly reduced, as Mg^{2+} and Ca^{2+} release was reduced by 80 % and 40.5 % with respect to the control group, respectively.

Table 3.8. Changes in Mg^{2+} and Ca^{2+} releases and Pb (II) biosorption upon acidic methanol treatment of the biomass.

Biomass type	Decrease in Mg^{2+} release (%)	Decrease in Ca^{2+} release (%)	Decrease in Pb (II) biosorption (%)
M0-M1	**80.0**	**40.5**	**92.8**

The change in the pH of the biosorption solution as a function of time is demonstrated in Figure 3.10.

We had chosen an initial pH of 5 for two reasons:First, for dry biomass, pH 5 was found to be optimal for Pb (II) biosorption (Yetiş *et al.,* 1998, 2000). Secondly, Pb (II) is known to precipitate at pH values higher than 5.5 becoming inavailable to the sorbent. On the other hand, at pH values lower than 4.0, concentrated H^+ ions compete with heavy metals including Pb (II) for the possible binding sites on the biomass surface, decreasing uptake values for this metal. For the raw biomass (M0), the pH of the solution decreased sharply to a value of 3.9 within 5 minutes and did not show any further change. The rapid pH decline observed strongly suggested that biosorption phenomenon took place mainly via ion exchange which is known to occur in very short period of time (Crist *et al.*, 1988). Other major mechanisms like complexation and chelation can occur in relatively longer time periods when compared to ion exchange. When compared to the M0, the M1 type biomass caused more decrease in pH in the same period of time. This result could be interpreted as a consequence of non-stoichiometric ion exchange between H^+ ions introduced to the biomass during modification and Pb (II) ions in the solution, since Pb (II) uptake in the M1 type biomass was severely impaired upon modification. Alternatively, some H^+ ions may be located residually on treated biomass, although several washings of the biomass had been made with sterile distilled water after the modification. In various research studies, H^+ ions were shown to be involved in heavy metal biosorption by being released to the aqueous media. In their study on Pb (II) biosorption by the macrofungi *Pycnoporus sanguineus*, Mashitah *et al*. (1999) have detected a decrease in pH of the Pb (II) solution from 5 to 3.9 within 2 minutes after which it remained constant. Contribution of H^+ ion exchange to the Pb (II) uptake was shown to be at the extent of 10 % in *Phellinus badius* (Yu et al., 1997). The pH was observed to drop to a value of 3.4. Moreover, H^+ ions were also shown to play a role in biosorptive cobalt uptake in *A. nodosum* (Kuyucak and Volesky, 1988).

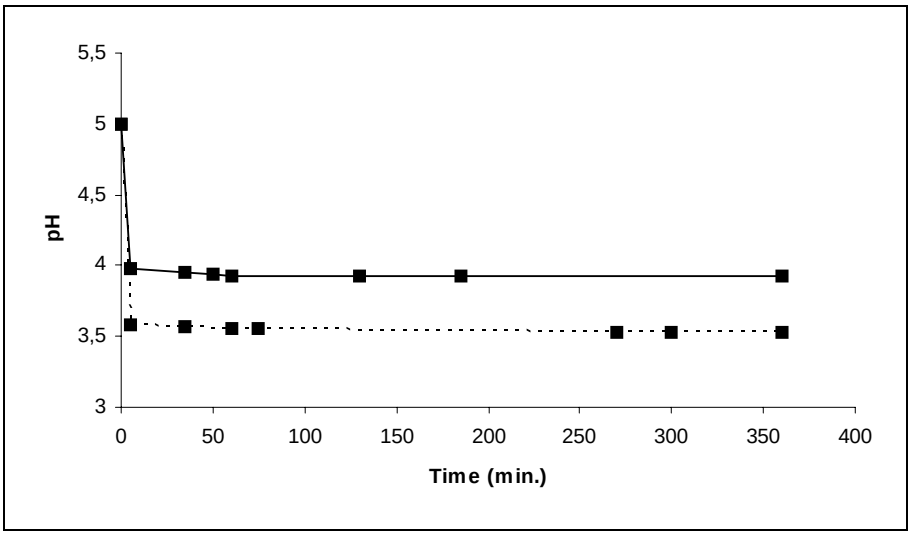

Figure 3.10. Changes in the pH of biosorption solution as a function of time when the raw (——) and acidic methanol-treated (------) biomasses were used.

3.3.2. Effects of Specific Blocking of Amine (NH) Groups

3.3.2.1. FTIR Spectrum of *P. chrysosporium* Upon Specific Blocking of Amine (NH) Groups

P.chrysosporium biomass was reacted with formic acid and formaldehyde (HCOOH, HCHO) to specifically block amino and amine groups of the biomass in order to eliminate Pb (II) biosorption capacity due to the these functional groups.

Figure 3.11 compares the mean normalized FTIR spectra of the raw (n=5) and formaldehyde-formic acid-treated (amines specifically blocked) *P. chrysosporium* biomass in the 3600-2800 cm^{-1} range. A remarkable decrease in the NH stretch band of the modified biomass as compared to the raw biomass was detected. This phenomenon was most likely due to the reduction of NH groups upon their conversion to NCH_3. Moreover, there was an abrupt decrease in the absorption intensity of the Amide I band (Figure 3.12). This reduction of the signals of NH bending vibrations upon the selective modification of the biomass was expected. An elongation in the C=O stretch band was apparent in

the spectra and the broadening of the C=O stretching band appeared to be due to the introduction of carboxyl (-COOH) groups in the system during formic acid treatment (HCOOH). Table 3.9 summarizes the comparisons of the relative peak intensities of N-H stretching band and Amide I band for the raw and amine groups-blocked biomasses. Reductions in peak intensities for these bands were found to be statistically significant. Obtained data further support the successive modification of the biomass as well as the changes observed in FTIR spectra upon modification of the biomass.

Table 3.9. Numerical summary of the differences in peak intensities of the N-H stretching and Amide I absorption bands between the raw (M0) and amine groups-blocked (M2) biomasses. P values less than or equal to 0.05 was accepted as statistically different with respect to the control group.

PEAK AND SIGNAL INTENSITY / BIOMASS TYPE	N-H stretch	Amide I
Raw biomass (M0)	**0.72±0.09**	**0.88±0.1**
Amine groups blocked biomass (M2)	**0.12±0.08**	**0.52±0.05**
p value	**0.03**	**0.03**

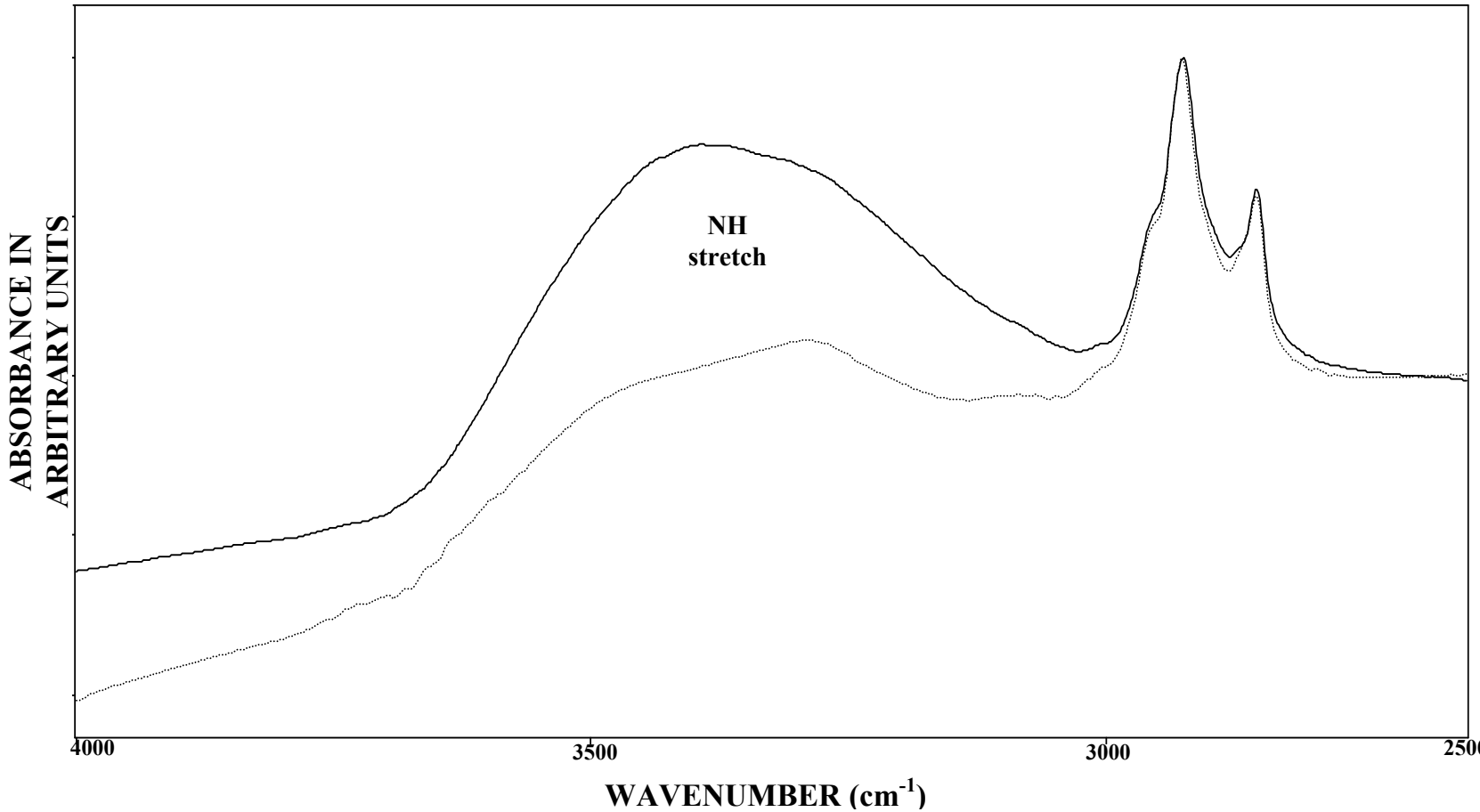

Fig. 3.11. Mean normalized FTIR spectra of the raw (———) and HCOOH+HCHO-treated (amines specifically blocked) (▪▪▪▪▪▪) *P. chrysosporium* biomass with respect to the lipid region in the 3600-2800 cm^{-1} range.

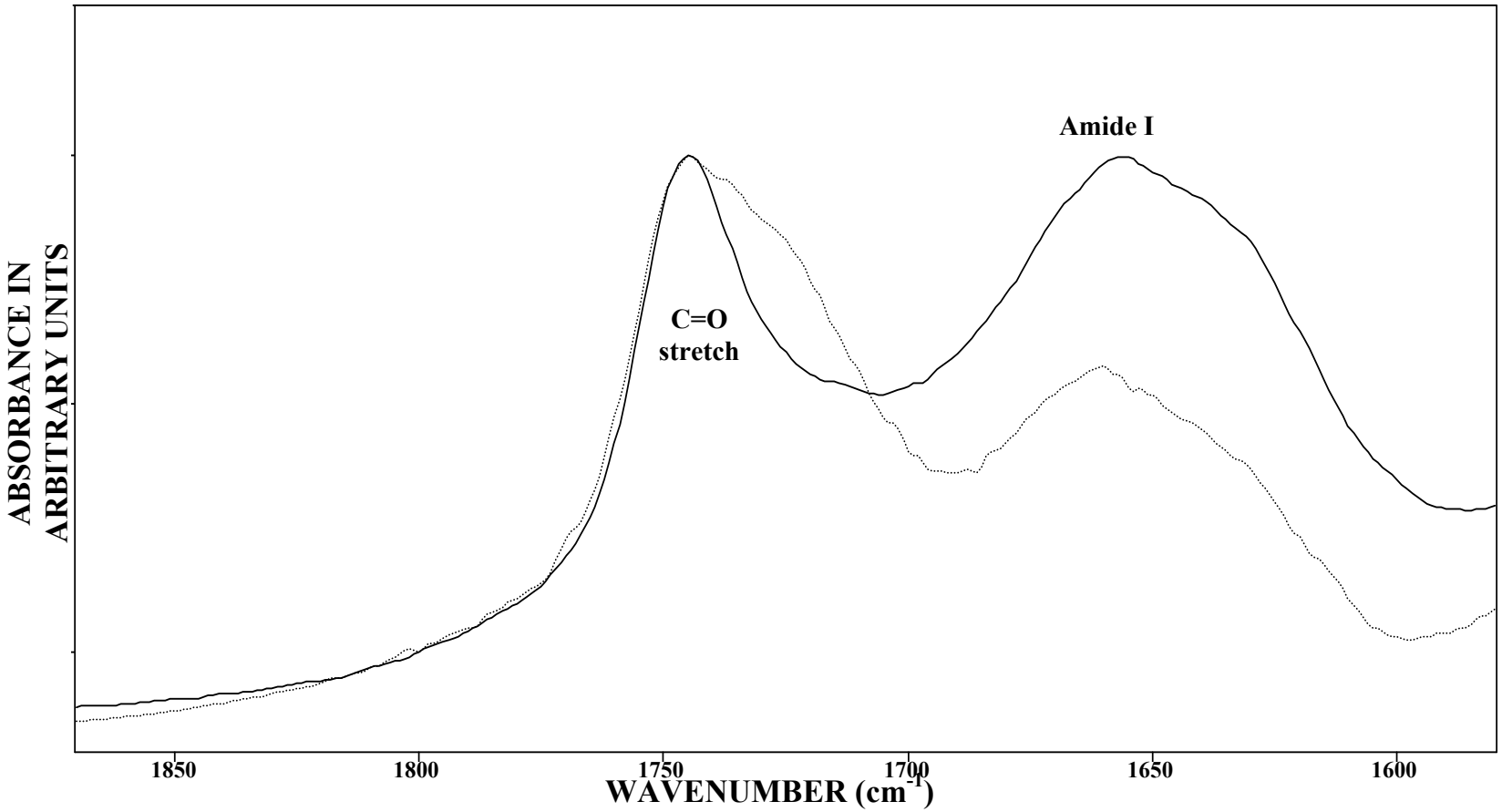

Fig. 3.12. Mean normalized FTIR spectra of the raw (▬▬) and HCOOH+HCHO-treated (amines specifically blocked) (▪▪▪▪▪▪) *P. chrysosporium* biomass with respect to the C=O stretching band in the 1800-1600 cm^{-1} range.

3.3.2.2. Changes in Pb (II) Biosorption by *P. chrysosporium* Upon Specific Blocking of Amine (-NH) Groups

Amine groups constitute one of the major binding sites for heavy metals in biosorption. Amine groups are found in chitin, the major polysaccharide polymer together with glucan matrix in the cell wall of fungi. Various reports implying the role of amine groups in heavy metal biosorption have been published. Mashitah *et al.* (1999) showed that methylation of amino groups significantly decreased copper and cadmium uptake while not affecting the capacity of lead adsorption in *P. sanguineus.* Vasudevan *et al*. (2003) reported that amino groups on the polysaccharides of baker's yeast were the important binding sites of nickel. Chemical modification of *A. niger* revealed the role of amine groups in lead, cadmium and copper biosorption. Amine groups were the most important binding sites, dominating the biosorption capacity of *A. niger* for copper and cadmium constituting up to 97 % and 98 % of the uptake, where contribution of other sites were negligible. For lead, amine groups appeared as the secondary binding sites after carboxyl groups (Kapoor and Viraraghavan, 1997). Acetylation of amine groups of *R. nigricans* resulted in a decline in Cr uptake of 80 %. Bai *et al* (2002) postulated that Cr binding in *R. nigricans* is possible by a complexation mechanism, amine groups having a great functionality. On the other hand, Beveridge and Murray (1980) emphasized that amine groups could hardly play a crucial role in metal deposition in *B. subtilis.* Contribution of amine groups in uranyl, lanthanum and zinc uptake in *R. arrhizus* is considerably high, just blocking 23.8 % of the zinc and 33 % of the lanthanum uptake (Tobin *et al.*, 1990). Guibal and Roux (1995) suggested that uranyl biosorption of the cell wall of *P. chrysogenum, Mucor miehei,* and *A. niger* occurs via complexation of the ligand onto the amino groups found on the chitin and chitosan polymer of these fungal species.

Table 3.10. shows the percent decrease in Pb (II) biosorption after specific blocking of amine groups.

Table 3.10. Pb (II) biosorption capacities of the M0 and M2 type biomasses.

Biomass type	mg Pb (II) biosorbed / g biomass	mmoles Pb (II) biosorbed / g biomass	Decrease in Pb biosorption (%)
M0	10.25±0.78	0.049±0.004	**6.0**
M2	9.63±0.35	0.047±0.002	

The results presented above indicated that amine groups play a minor role in Pb (II) biosorption by *P.chrysosporium*. Indeed, amine groups do not contribute to heavy metal biosorption at pH 5.0 by ion-exchange since these groups are not ionizable at this pH (Schiewer and Volesky, 2000) The main functions of amine groups involve physical adsorption, electrostatic attraction and complexation. A decrease of only 6 % in Pb (II) biosorption by amine groups-blocked biomass clearly revealed that the contribution of above-mentioned mechanisms regarding amine groups.

Table 3.11 and 3.12 compare the release of Mg^{2+} and Ca^{2+} ions during Pb (II) uptake for M0 and M2.

Table 3.11. Changes in Mg^{2+} and Ca^{2+} release patterns in response to Pb (II) biosorption in *P. chrysosporium* after blocking the amine groups.

Biomass type	mg Mg^{2+} released / g biomass	mmoles Mg^{2+} released / g biomass	mg Ca^{2+} released / g biomass	mmoles Ca^{2+} released / g biomass
M0	0.47± 0.07	0.019±0.003	0.14±0.03	0.003±0.001
M2	0.13±0.01	0.005± 0.001	0.17± 0.01	0.0042± 0.0002

Table 3.12. Changes in Mg^{2+} and Ca^{2+} releases and Pb (II) biosorption upon formaldehyde and formic acid treatment of the biomass.

Biomass type	Decrease in Mg^{2+} release (%)	Increase in Ca^{2+} release (%)	Decrease in Pb (II) biosorption (%)
M0-M2	**71.9**	**23.4**	**6.0**

Magnesium and calcium release was found to be drastically reduced when compared to that from the control group. Formic acid in the chemical modification mixture liberates H^+ atoms to the biosorption medium, which must have replaced the vast majority of the Mg^{2+} and some amount of Ca^{2+} found on the cell surfaces. Competition and affinity of H^+ ions was expected to be relatively high, due to pure formic acid and formaldehyde used in the treatment. Yet, the picture of pH change in biosorption solution was not much different for raw and modified biomasses (Figure 3.13)

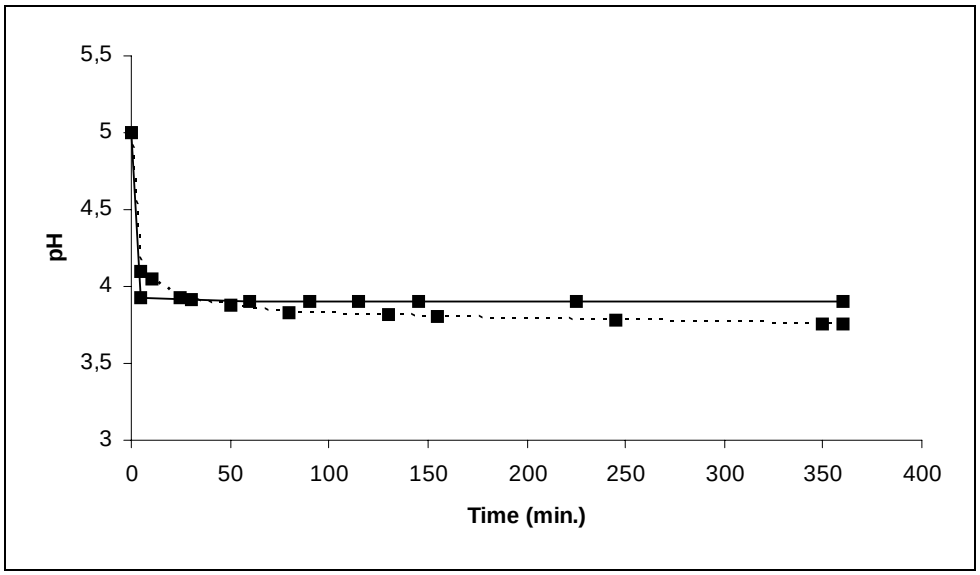

Figure 3.13. Changes in the pH of the biosorption solution as a function of time when the raw (——) and the formic acid plus formaldehyde-treated (------) biomasses were used.

3.3.3. Effects of Specific Blocking of Phosphate Groups

3.3.3.1. FTIR Spectrum of *P. chrysosporium* Upon Specific Blocking of Phosphate Groups

Figure 3.14 shows the mean normalized FTIR spectrum of the raw and nitromethane plus triethyl phosphite-treated (phosphates specifically blocked) *P. chrysosporium* biomasses in the 1300-950 cm^{-1} range (n=5). A slight decrease observed in the PO^{-}_{2} symmetric stretch was most probably due to the esterification of phosphate groups of phospholipids and phosphated amino acids found on the cell surfaces. Table 3.13 summarizes the comparisons of the relative peak intensities of PO_2^- symmetric stretching band for the raw and phosphate groups-blocked biomass.

Table 3.13. Numerical summary of the difference in peak intensity of the PO_2^- symmetric stretching band between the raw (M0) and phosphate groups-blocked (M3) biomass. P values less than or equal to 0.05 was accepted as statistically different with respect to the control group.

PEAK AND SIGNAL INTENSITY / BIOMASS TYPE	**PO_2^- symmetric stretch**
Raw biomass (M0)	**0.91±0.03**
Phosphate-groups blocked biomass (M3)	**0.87±0.01**
p value	**0.16**

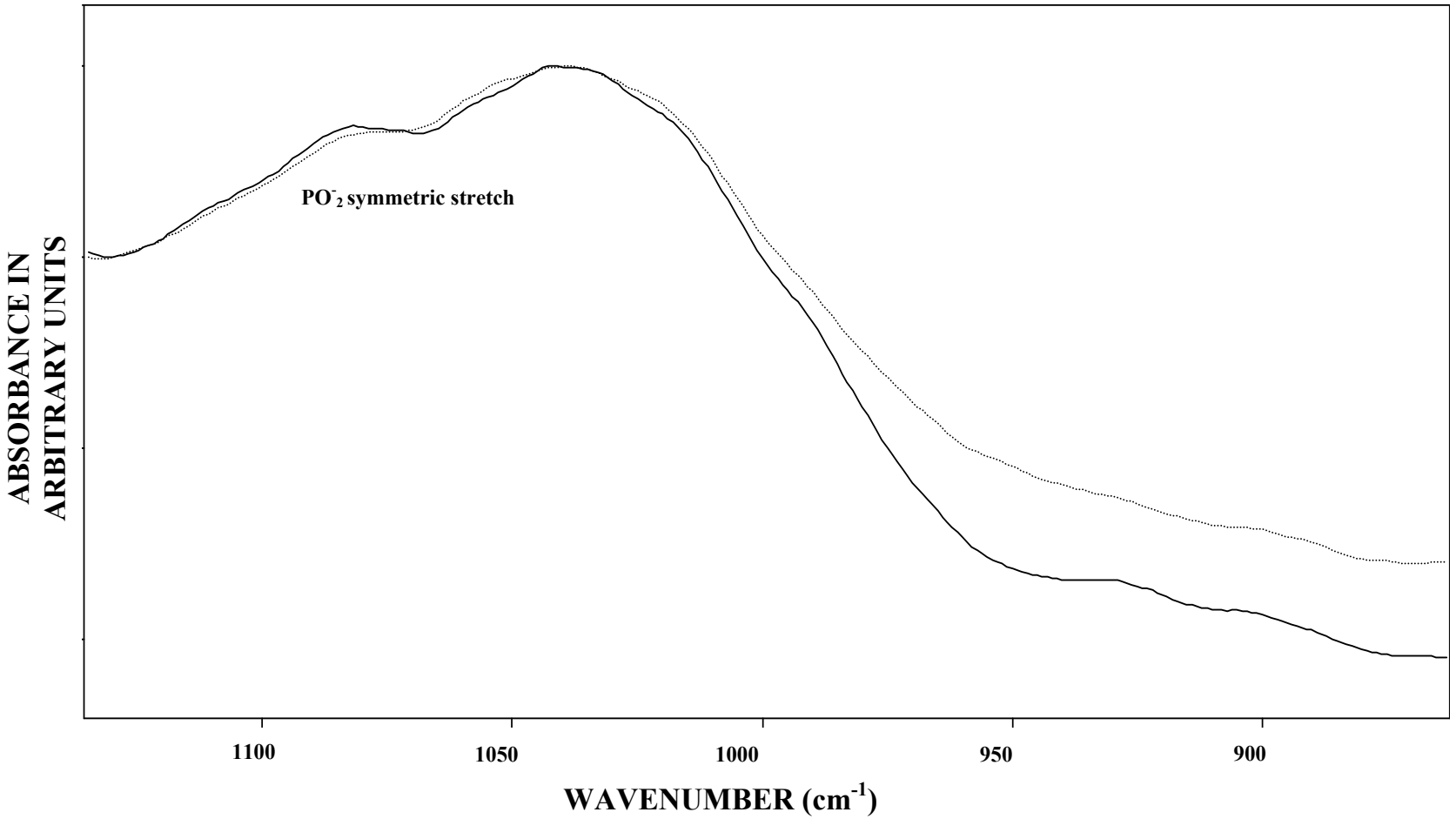

Fig. 3.14. Mean normalized FTIR spectra of the raw (——) and the nitromethane plus triethyl phosphite-treated (phosphates specifically blocked*)* (.....) *P. chrysosporium* biomass with respect to the glucose ring band in the 1130-950 cm^{-1} range.

3.3.3.2. Changes in Pb (II) Biosorption of *P.chrysosporium* Upon Specific Blocking of Phosphate Groups

Phosphate groups are among the functional groups of interest in biosorption research. Phosphate group abundance shows a great variation in different types of biosorbents and phosphate groups were found to be considerably important in heavy metal biosorption in only a few biosorbent types. In this respect, the most striking finding came from Tobin *et al*. (1990) who found that zinc biosorption reduced by 60 % in *R. arrhizus* when phosphate groups were blocked. The extents of decrease under the same conditions for uranium, lead and lanthanum were 31.1 %, 13 %, and 38 %, respectively. For *A. niger*, phosphate groups were of minor contribution to lead, cadmium and copper biosorption since uptake values of the modified biomass showed slight decrease with respect to the control groups, being less than 10% (Kapoor and Viraraghavan, 1997).

Table 3.14 tabulates the relative difference in Pb (II) biosorption recorded in phosphate groups-blocked biomass type (M3) with respect to control group (M0).

Table 3.14. Biosorption capacities of M0 and M3 type of biomasses.

Biomass type	mg Pb biosorbed / g biomass	mmoles Pb (II) biosorbed / g biomass	Increase in Pb biosorption (%)
M0	18.91±0.59	0.091±0.003	**4.2**
M3	19.70±1.07	0.10±0.01	

This modification did not have an adverse effect on Pb (II) biosorption, instead a slight increase in biosorptive capacity was observed. Therefore, we

concluded that phosphate groups do not have a significant role in Pb (II) biosorption by *P. chrysosporium*.

The Mg^{2+} and Ca^{2+} release data presented in Table 3.15 and 3.16 accorded well with the Pb (II) biosorption data. For the M3 type biomass, Mg^{2+} and Ca^{2+} release values also showed a slight increase with respect to those obtained from the control groups.

Table 3.15 Changes in Mg^{2+} and Ca^{2+} release patterns in response to Pb (II) biosorption in *P. chrysosporium* upon blocking phosphate groups.

Biomass type	**mg Mg^{2+} released / g biomass**	**mmoles Mg^{2+} released / g biomass**	**mg Ca^{2+} released / g biomass**	**Moles Ca^{2+} released / g dry biomass**
M0	0.55±0.02	0.022±0.001	0.25±0.03	0.006±0.001
M3	0.55±0.003	0.023±0.0001	0.26±0.03	0.006±0.001

Table 3.16. Changes in Mg^{2+} and Ca^{2+} release and Pb (II) biosorption upon blocking phosphate groups.

Biomass type	**Increase in Mg^{2+} release (%)**	**Increase in Ca^{2+} release (%)**	**Increase in Pb (II) biosorption (%)**
M0-M3	**0.6**	**3.7**	**4.2**

Figure 3.15 shows the pH changes occurred during Pb (II) biosorption with the raw and the phosphate groups-blocked biomasses.

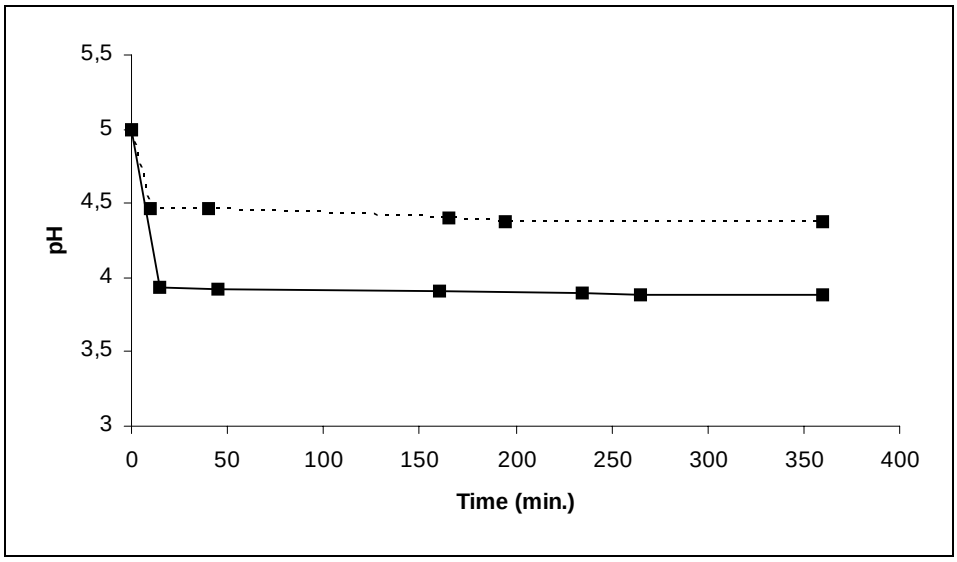

Figure 3.15. Changes in the pH of the biosorption solution as a funcion of time when the raw (——) and phosphate group-blocked (------) biomasses were used.

During Pb (II) biosorption with the modified biomass, the drop in pH was not as much as that found with the raw biomass. This might suggest an elimination of H^+ ions from the surface of the biomass as a result of the chemical treatment.

3.3.4. Effects of Extraction of Lipid Fraction of Biomass

3.3.4.1 FTIR Spectra of *P. chrysosporium* Upon Extraction of Lipid Fraction of the Biomass

Figures 3.16 and 3.17 show the comparative normalized FTIR spectra of the raw versus the acetone-treated and benzene-treated biomasses in the 3000-2800 cm^{-1} range. A reduction in the absorption of the CH_2 symmetric and CH_2 antisymmetric bands was observed as a result of lipid extraction in both modifications. Moreover, in the spectra of the lipid-extracted samples, the olefinic band seemed to be completely omitted from the system. Nonetheless, it has been recently reported that olefinic band is specific only for lipids (Severcan *et al*., 2000; 2003). The reason for the relatively limited presence, but not complete absence of the CH_2 bands could be the occurrence of these functional groups in polysaccharides as well as in lipids (Volesky *et al*.,2003). As could be seen in figure 3.15 and 3.16, FTIR data clearly revealed the lipid extraction process, further supporting the successful occurrence of the chemical modifications performed with acetone and benzene treatments. Table 3.17 summarizes the comparisons of the relative peak intensities of CH_2 symmetric stretching band for the raw and acetone and benzene treated biomasses. Reductions in peak intensities for these bands were found to be statistically significant. Obtained data further support the successful lipid extraction of the biomass as well as the changes observed in FTIR spectra upon modification of the biomass (Figures 3.16 and 3.17).

Table 3.17. Numerical summary of the differences in peak intensities of the C=O stretching and CH_3 asymmetric bending absorption bands between the raw (M0) and lipid fraction extracted (M4 and M5) biomasses. P values less than or equal to 0.05 was accepted as statistically different with respect to the control group.

PEAK AND SIGNAL INTENSITY / BIOMASS TYPE	CH_2 symmetric stretch
Raw biomass (M0)	**0.54±0.06**
Acetone-treated biomass (M4)	**0.22±0.05**
p value	**0.049**
Raw biomass (M0)	**0.54±0.06**
Benzene-treated biomass (M5)	**0.27±0.09**
p value	**0.029**

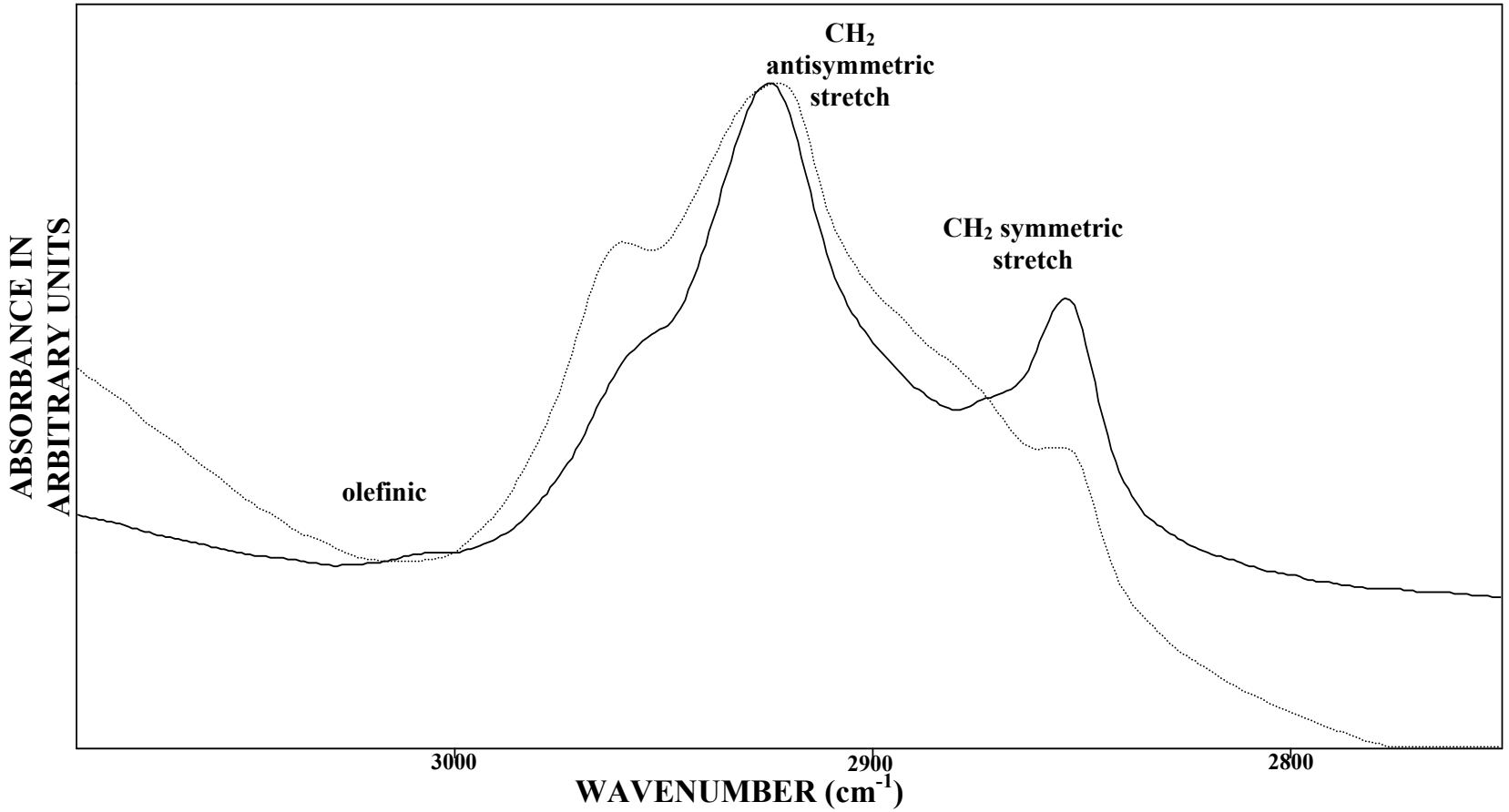

Fig. 3.16. Mean normalized FTIR spectra of the raw (——) and the acetone-treated (lipid fraction of the biomass extracted) (------) *P. chrysosporium* biomasses with respect to olefinic band in the 3000-2800 cm^{-1} range.

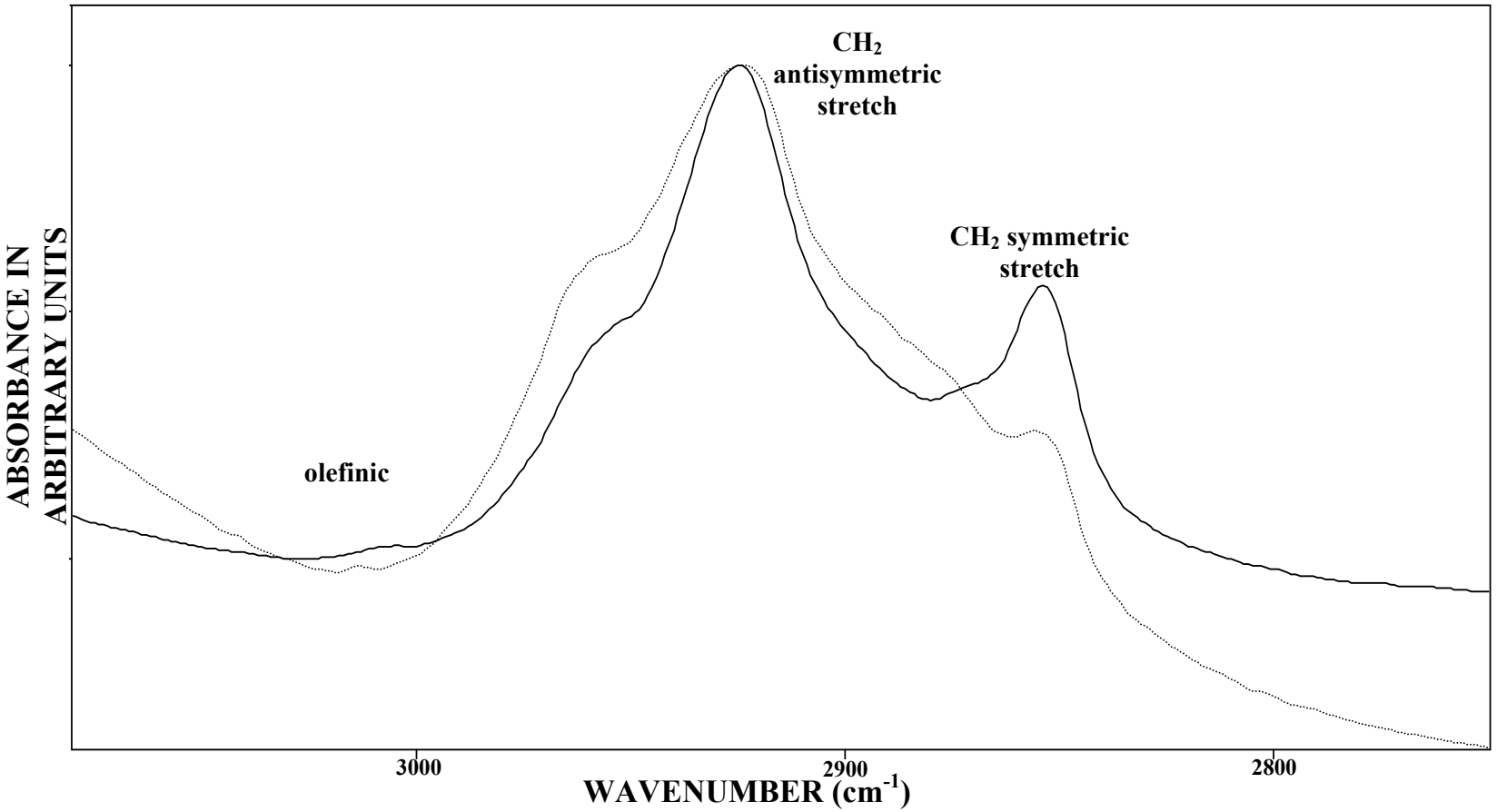

Fig. 3.17. Mean normalized FTIR spectra of the raw (———) and benzene-treated (lipid fraction of the biomass extracted) (·····) *P chrysosporium* biomass with respect to olefinic band in the 3000-2800 cm^{-1} range

3.3.4.2. Pb (II) Biosorption of *P. chrysosporium* Biomass Upon Extraction of Lipid Fraction

Lipid fractions on the biomass surface have been shown to play a minor role in biosorption. Kapoor and Viraraghavan (1997) observed no significant change in biosorptive capacity of the lipid extracted-*A. niger* biomass for copper, cadmium and lead when compared to the raw biomass. Mashitah *et al.* (1999) recorded a slight decrease in lead biosorption by *P. sanguineus* upon extraction of lipids. Tobin *et al*. (1990) stated that benzene and acetone treatments did not remarkably affect the metal uptake capacity in *R. arrhizus*.

In the present study, two types of methods, benzene and acetone treatment were applied for lipid extraction. Our data, however, did not fit to those reported in the studies mentioned above. Especially, upon benzene and acetone treatment, 20.3 % increase in Pb (II) biosorptive capacity was determined (Tables 3.18 and 3.19) One possibility for this finding is that the major binding sites like carboxyl and amine groups have become more exposed to the metal solution upon lipid extraction. We also observed that there was more Pb (II) binding to the acetone-treated biomass as compared to the benzene-treated biomass, indicating that the acetone treatment is more efficient to improve metal biosorption. Unfortunately, there is a scarcity of relevant literature reports that would help us explain the different outcomes of two different lipid extraction techniques.

Table 3.18. Biosorption capacities of the M0 and M4 type of biomasses.

Biomass type	mg Pb (II) biosorbed per g biomass	mmoles Pb (II) biosorbed per g biomass	Increase in Pb (II) biosorption (%)
M0	9.39±0.71	0.045±0.003	
M4	11.29±0.82	0.054±0.004	**20.31**

Table 3.19. Biosorption capacities of the M0 and M5 type of biomasses.

Biomass type	mg Pb (II) biosorbed / g biomass	mmoles Pb (II) biosorbed / g biomass	Increase in Pb biosorption (%)
M0	11.22±0.49	0.054±0.002	
M5	12.16±0.90	0.059±0.004	**8.4**

Mg^{2+} and Ca^{2+} release data tabulated in Tables 3.20, 3.21, 3.22 and 3.23 indicate a nonstoichiometric relation between the increases in Mg^{2+} and Ca^{2+} release into the aqueous media and enhanced Pb (II) biosorption by *P. chrysosporium* biomass.

Regarding biotechnological applications after which the waste biomass of *P. chrysosporium* was obtained as a by-product, the lipid extracted biomass types, especially the acetone treated biomass (Type M4) could be considered as a promising and an alternative candidate for removal of Pb (II) from waste waters, with an increased capacity of Pb (II) biosorption (by 20.3 %) with respect to the untreated raw biomass. Since the conditions and parameters of

these modifications are open to be optimized, enhanced levels of biosorptive capacity could be expected to be established.

Table 3.20 Mg^{2+} and Ca^{2+} release patterns in response to Pb (II) biosorption in *P. chrysosporium* after acetone treatment to extract lipids.

Biomass type	mg Mg^{2+} released / g dry biomass	mmoles Mg^{2+} released / g dry biomass	mg Ca^{2+} released / g dry biomass	mmoles Ca^{2+} released / g dry biomass
M0	0.63±0.01	0.026±0.001	0.34±0.014	0.0084±0.0004
M4	0.73±0.03	0.03±0.001	0.38±0.10	0.009±0.003

Table 3.21. Changes in Mg^{2+} and Ca^{2+} release in response to Pb (II) biosorption in *P. chrysosporium* after benzene treatment to extract lipids.

Biomass type	mg Mg^{2+} released / g dry biomass	mmoles Mg^{2+} released / g biomass	mg Ca^{2+} released / g biomass	mmoles Ca^{2+} released / g dry biomass
M0	0.56±0.04	0.023±0.002	0.22±0.02	0.0060±0.0004
M5	0.69± 0.02	0.028±0.001	0.240±0.002	0.0060±0.0001

It was thought that the lipid fraction present on the cell wall might have blocked a portion of the active binding sites including Mg^{2+} and Ca^{2+} residing on the cell surfaces. After the treatment, the release of these ions showed an increase paralleling the increase detected in Pb (II) biosorption. (Tables 3.22 and 3.23). Again for this treatment, the percentage increase in the Mg^{2+} and Ca^{2+} release were comparable to the increase in the Pb (II) biosorption observed.

Table 3.22. Changes in Mg^{2+} and Ca^{2+} release and Pb (II) biosorption after acetone treatment to extract lipid fraction.

Biomass type	Increase in Mg^{2+} release (%)	Increase in Ca^{2+} release (%)	Increase in Pb (II) biosorption (%)
Raw-M4	**16.8**	**11.9**	**20.3**

Table 3.23. Changes in Mg^{2+} and Ca^{2+} release and Pb (II) biosorption after benzene treatment to extract lipids.

Biomass type	Increase in Mg^{2+} release (%)	Increase in Ca2+ release (%)	Increase in Pb biosorption (%)
Raw-M5	**22.9**	**9.7**	**8.4**

Figures 3.18 and 3.19 show the pH changes observed within the course of biosorption with above-described biomass types. The final pH values of M4 and M5 solutions were similar, hence indicating the presence of no difference in the amounts of ionizable H^+ ions found on the lipid fraction of the biomass after the modifications. Yet, it is to be noted that the pH drop was slower for benzene-treated biomass, possibly accounting for a lower level of biosorption after this modification.

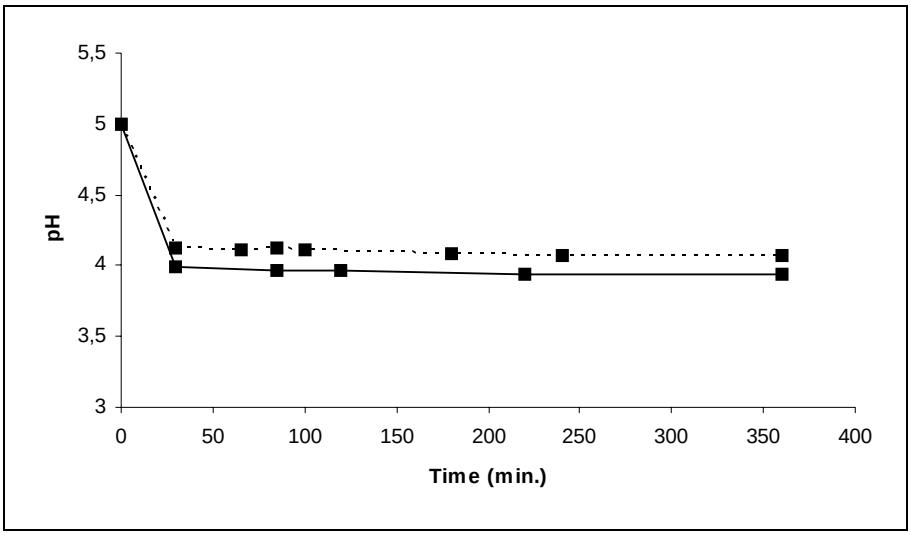

Figure 3.18. Changes in the pH of the biosorption solution as a function of time when the raw (——) and the acetone-treated (▪▪▪▪▪▪) biomasses were used.

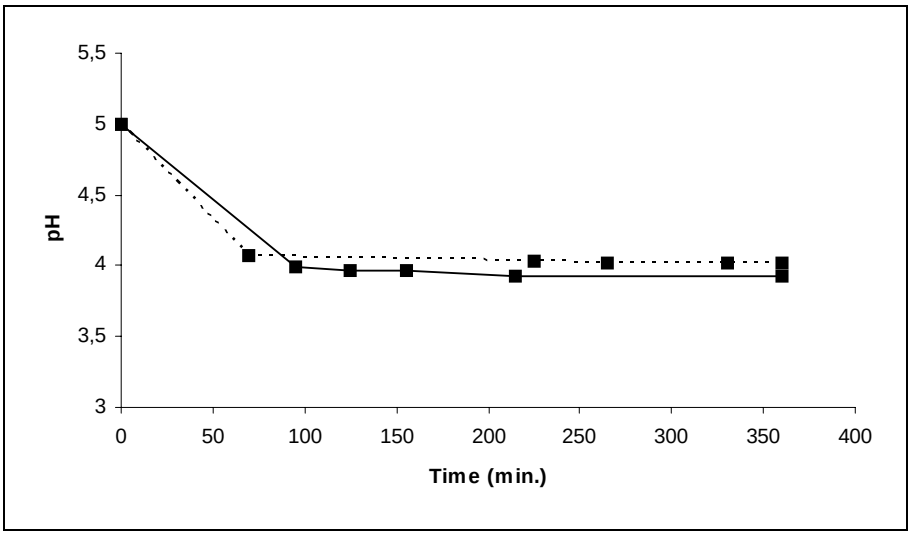

Figure 3.19 Changes in the pH of the biosorption solution as a function of time when the raw (——) and the benzene-treated (▪▪▪▪▪▪) biomasses were used.

The use of waste biomass from pharmaceutical or food industries is one way to minimize the costs of the bioprocesses for additional costs associated with biomass generation and/or pretreatment are avoided. (Niu *et al.,* 1993).

P. chrysosporium has extensively been studied for the treatment of pulp bleaching effluents and bioremediation of a wide range of organic pollutants. In this regard, the organism might be considered as a potential candidate for heavy metal biosorption. For a possible future use as a metal biosorbent for removal of toxic heavy metals from polluted waters, it now appears that pretreatment of biomass with acetone will be useful to increase the biosorptive capacity. The acetone treatment can be further optimized in terms of its conditions such as the duration of the treatment, acetone concentration, amount of the biomass, etc. with the aim of further increasing the biosorptive capacity of the biomass.

CHAPTER IV

CONCLUSION

- FTIR studies demonstrated a substantial decrease in relative absorption of C=O stretching mode, with an increase in its wavenumber upon Pb (II) exposure. This influence of Pb (II) exposure on the intensity of C=O stretching band indicated a possible complexation interaction between Pb (II) and carbonyl and carboxyl groups found in chitin and glucan matrix on the cell wall. An increase in wavenumber in Amide I band upon lead exposure supports the wavenumber shift in NH stretching band implying that secondary amine (NH) groups might involve in Pb (II) biosorption. The changes in C=O stretch and Amide I band wavenumber average values were statistically significant.

- In the FTIR spectra of the acidic methanol-treated *P. chrysosporium* biomass, a drastic decrease in the absorption band of C=O stretch was observed, indicating the decrease in the population of the carboxyl groups. This result verified the successful methylation of COOH groups on biomass surface. Carboxyl groups seemed to be the most important functional groups for Pb (II) biosorption in *P. chrysosporium*, since the biosorption capacity dramatically decreased (by 92,.8 %) in acidic methanol-treated biomass in which carboxyl groups were modified via esterification. Ion exchange capacity of modified *P.chrysosporium* biomass was significantly reduced, as reflected by co-reduction of Mg^{2+} and Ca^{2+} release (by 80 % and 40.5 %, respectively).

- A remarkable decrease in the NH stretch band was detected in FTIR spectrum of amine groups-blocked biomass. This phenomenon was most likely due to

the reduction of NH groups upon their conversion to NCH_3. Only a slight decrease (6 %) in Pb (II) biosorption was detected in amine groups-blocked biomass, thus, amine groups seem to play a secondary and minor role in Pb (II) biosorption in *P.chrysosporium*.

- Modification and blocking of phosphate groups were verified by the comparison of the phosphate groups-modified biomass and raw biomass FTIR spectra. A slight decrease observed in the PO^{-}_2 symmetric stretch was most probably due to the esterification of phosphate groups. Phosphate groups were not found to have much a role in Pb (II) biosorption by *P. chrysosporium* since a slight increase, but no decrease in biosorptive capacity (4.2 %) was detected. For the phosphate groups-blocked biomass, Mg^{2+} and Ca^{2+} release values slightly increased in parallel to that slight increase in Pb (II) biosorption.
- FTIR spectra of acetone and benzene-treated biomass types showed a reduction in the absorption of the CH_2 symmetric and CH_2 antisymmetric bands. Upon benzene and acetone treatment to extract lipids, increase in some extents in the Pb (II) biosorptive capacity was detected. We also observed that there was more Pb (II) binding (20.3 % increase) to the acetone-treated biomass when compared to the benzene-treated biomass, indicating the acetone treatment is more efficient to improve metal biosorption in this organism. There was a nonstiochiometric relationship between the increase in Mg^{2+} and Ca^{2+} release into the aqueous media and Pb (II) uptake by the biomass.
- From biotechnological point view, acetone pretreatment of biomass is useful to increase its metal biosorptive capacity in related applications.

REFERENCES

- **Aksu, Z., Acikel, U. and Kutsal, T. (1997).** Application of multicomponet adsorption-isotherms to simultaneous biosorption of iron (III) and chromium (VI) on *Chlorella vulgaris. J. Chem. Technol. Biot.* 70(4): 368-378.

- **Adhiya, J.**, **Cai, X., Sayre, R.T., Traina, S.J. (2002).** Binding of aqueous cadmium by the lyophilized biomass of *Chlamydomonas reinhardtii. Colloid. Surface.* Article in Press.

- **Ahuja, P.**, **Gupta, R., Saxena, R.K., (1999).** Zn^{2+} biosorption by *Oscillatoria anguistissima. Process. Biochem.* 34: 77-85.

- **Akthar, N. M., Sastry, K. S. and Mohan, P. M. (1996).** Mechanism of metal ion biosorption by fungal biomass. *Biometals.* 9: 21-28.

- **Ali, T. A. and Wainwright, M. (1994).** Growth of *Phanerochaete chrysosporiuın* in soil and its ability to degrade the fungicide benomyl. *Bioresource Technol.* 49: 197-201.

- **Arrondo, J.R., Muga, A., Castresa, J., and Goni, R. M. (1993).** "Quantative Studies of the Structure of Proteins in Solution by Fourier Transfrom Infrared Spectroscopy". *Progress. Biophys. Acta.* 468:63-72.

- **Bai, S.R. , Abraham, E.T.,** Studies on enhancement of Cr (VI) biosorption by chemically modified biomass of *Rhizopus nigricans. Wat. Res.* 36 (2002) 1224-123.

- **Baik, W.Y., Bae, J.H., Cho, K.M., Hartmeier, W. (2002)** Biosorption of heacy metals using whole mold mycelia and parts thereof. *Bioresour. Technol.* 81: 167-170.

- **Baldrian, P., (2003).** Interactions of heavy metals with white-rot fungi. *Enzyme Microb. Technol.* 32: 78-91.

- **Beveridge, T.J., and Murray, R.G.E., (1980).** Sites of Metal Deposition in the Cell Wall of *Bacillus subtilis. J. Bacteriol.* 141: 876-887.

- **Brierley, C.L. (1990).** Metal immobilization by using bacteria, p. 303-324. In *Microbial Mineral Recovery.* McGraw-Hill Book Co., New York, N.Y.

- **Bumpus, J. A., Tien M., Wright D. and Aust S. D. (1985).** Oxidation of persistent environmental pollutants by a white rod fungus. *Science.* 228: 1434-1436.

- **Campbell, J.D., and Dwek, R.A. (1984),** "Biological Spectroscopy" Chapters 3 and 4. Edited by: Elias, P. The Benjamin/Cummings Publishing Company, Inc.

- **Ci, X.Y., Gao, T.Y., Feng, J., and Guo, Z.Q. (1999),** " Fourier Transform Infrared Spectroscopic Characterizaiton of Human Breast Tissues: Implications for Breast Cancer Diagnosis", *Applied Spectroscopy*, 53 (3): 312-315.

- **Cotoras, D., Viedma, P., Cifuentes, L., Mestre, A., (1992)** Sorption of Metal ions by whole cells of *Bacillus* and *Micrococcus. Environ. Technol.* 13: 551-559.

- **Crist, R. H., Oberholser, K., Schwartz, D., Marzoff, J. and Ryder, D. (1988).** Interactions of metals and proteins with algae. *Environ. Sci. Technol.* 22(7): 75-760.

- **Dhawale, S. S., Lane, A. C. and Dhawale, S. W. (1996).** Effects of mercury on the white rot fungus *Phanerochaete chrysosporium*. *Bull. Environ. Contam. Toxicol*. 56**:** 825-832.

- **Dighton, J., Mascarenhas, M., and Arbuckle-Keil, G. (2001),** " Changing Resources: Assesment of Leaf Litter Carbohydrate Resource Change at A Microbial Scale of Resolution", *Soil Biology and BioChemistry*., 33: 1429-1432.

- **Falih, A. M**. **(1997).** Influence of heavy metals toxicity on growth of *Phanerochaete chrysosporium*. *Bioresource Technol.* 60: 87-90.

- **Fischer, E. H. and Davie, E. W. (1998).** Recent excitement regarding metallothionein. *Proc. Natl. Acad. Sci.* USA. 95: 3333-3334.

- **Fourest, E., Canal, C., and Roux, J.C., (1994)** Improvement of heavy metal biosorption by mycelial dead biomasses (*Rhizopus arrhizus, Mucor miehei,* and *Penicillium chrysogenum)*: pH control and cationic activation. *FEMS Microbiol. Rev.* 14: 325-332

- **Freifelder, D. 1982.** Applications to Biochemistry and Molecular Biology In: Physical Chemistry, Chapter 14. Freeman, W.H. and Company, New York.

- **Gadd, G. M. (2000).** Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. *Curr. Opin. Biotech.* 11: 271-279.

- **Gadd, G. M. (1990).** Heavy metal accumulation by bacteria and other microorganism. *Experianta.* 46: 285-286.

- **Gadd, G.M., and Sayer, J.A.,** Influence of fungi on the environmental mobility of metals and metalloids. In Environmental Microbe-Metal Interactions ASM Press 2000, Chapter 11, 237-256.

- **Gardea-Torresdey, J.L., Tiemann, K.J., Peralta-Videa, J.R., Parsons, J.G., Delgado, M. (2004).** Binding of erbium (III) and holmium (III) to native and chemically modified alfalfa biomass: a spectroscopic investigation. *Microchem. J.* 76: 65-76.

- **Gardea-Torresdey, J.L., Dokken, K., Tiemann, K.J., Parsons, J.G., Ramos, J., Pingitore, N.E., Gamez, G. (2002).** Infrared and X-ray absorption spectroscopic studies on the mechanism of chromium (III) binding to alfalfa biomass. *Microchem. J.* 71: 157-166.

- **Godwin, H. A. (2001).** The biological chemistry of lead. *Curr. Opin. Chem. Biol.* 5:223-227.

- **Guibal, E., Roulph C. and Cloirec, P.L. (1995).** Infrared spectroscopic study of uranyl biosorption by fungal biomass and materials of biological origin. *Environ. Sci. Technol.* 29: 2496-2502.

- **Hamdy, A. A. (2000).** Biosorption of heavy metals by marine algae. *Curr. Microbiol.* 41(4): 232-238.

- **Holan, Z. R., and Volesky, B. (1994).** Biosorption of Pb (II) and Ni (II) by biomass of marine algae. *Biotechnol. Bioeng.* 43: 1001-1009.

- **Holan, Z. R., Volesky, B. and Prasetyo, I. (1993).** Biosorption by biomass of marine algae. *Biotechnol. Bioeng.* 41: 819-825.

- **Huang, C. and Huang, C. P. (1996).** Application of *Aspergillus oryzae* and *Rhizopus oryzae* for Cu (II) removal. *Water. Res.* 30(9): 1985-1990.

- **Jalali, R., Ghafourian, H., Asef, Y., Davarpanah, S.J., and Sepehr, S., (2002)** removal and recovery of lead using nonliving biomass of marine algae. *J. Hazard. Mater.* 92: 253-262.

- **Kacurakova, M., Capek, P., Sasinkova, V., Wellner, N., Ebringerova, A. (2000).** FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. *Carbohydrate Polymers* 43: 195-203.

- **Kapoor, A. and Viraraghavan, T. (1997).** Heavy metal biosorption sites in *Aspergillus niger. Bioresource Technol.* 61: 221-227.

- **Kefala, M. I., Zouboulis, A. I. and Matis, K. A. (1999).** Biosorption of cadmium ions by *Actinomycetes* and separation by flotation. *Environ. Pollut.* 104: 283-293.

- **Kille, P., Hemmings, A. and Lınney, E. A. (1994).** Memories of metallothionein. *Biochim. Biophys. Acta.* 1205: 151-161.
- **Kratochvil D. and Volesky B. (1998).** Advances in the biosorption of heavy metals. *TIBTECH.* 16: 291-300.

- **Kuyucak, N. and Volesky, B. (1989a).** Accumulation of cobalt by marine alga. *Biotechnol. Bioeng.* 33: 809-814.

- **Kuyucak, N. and Volesky, B. (1989b).** The mechanism of cobalt biosorption. *Biotechnol. Bioeng.* 33: 823-831.

- **Kuyucak, N. and Volesky, B. (1990).** Biosorption by algal biomass In *Biosorption of Heavy Metals*; Volesky, B., Ed., CRC Press: Boca Raton, FL. pp. 173-198.

- **Lanphear, B. P. (1998).** The paradox of lead poisoning and prevention. *Science*. 281: 1617-1618.

- **Leusch, A. and Volesky, B. (1995).** The influence of film diffusion on cadmium biosorption by marine biomass. *J. Biotechnol.* 43: 1-10.

- **Macaskie, L. E., Bonthrone, K. M., Yong, P. and Goddard, D. T. (2000).** Enzymically mediated bioprecipitation of uranium by a Citrobacter sp.: a concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation. *Microbiol.* 146(8): 1855-1867.

- **Macaskie, L.E., Empson, R.M., Cheetham, A.K., Grey, C.P., and Skarnulis, A.J. (1992).** Uranium bioaccumulation by a *Citrobacter* sp. as a result of enzymatically mediated growth of polycrystalline HUO_2PO_4. *Science.* 257: 782-784.

- **Manoharan, R., Bagara, J.J., Rava, R.P., Dasari, R.R., Fitzmavrice, M., and Feld, M.S. (1993),** " Biochemical Analysis and mapping of Atherosclerostic Human Atery Using FTIR Microspectroscopy", *Atherosclerosis*, 103: 181-193.

- **Margoshes, M. and Vallee, B. L. (1957).** A Cadmium protein from equine kidney cortex. *J. Am. Chem. Soc*. 79: 4813-4814.

- **Martinez, D.**, Larrondo, L.F., Putnam, N., Gelpke, M.D.S., Huang, K., Chapman, J., Helfenbein, K.G., Ramaiya, P., Detter, J.C., Larimer, F., Pedro, M.C., Henrissat, B., Berka, R., Cullen, D. and Rokhsar, D. (2004). Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium strain RP78. Nature Biotechnol.* 22: 695-700.

- **Mashitah, M.D., Zulfadhly, Z., and Bhatia, S. (1999)** Binding mechanism of heavy metals biosorption by *Pycnoporus sanguineus. Art. Cells, Blood Subs., and Immob. Biotech.* 27: 441-445.

- **Matheickal, J.T. and Yu, Q. (1997),** Biosorption of lead (II) from aqueous solutions by *Phellinıs badius. Minerals Engineering.* 10: 947-957.

- **Mc Donalds, S.R. (1986),** " Review: Infrared Spectroscopy", *Analytical Chemistry*, 58: 1906-1925.

- **Natarajan, K.A., Santhiya, D., and Subramanian, S., (2001).** Surface chemical studies on Sphalerite and Galena using *Bacillus polymyxa. J Coll. Inter. Sci.* 235: 298-309.

- **Naumann, D. (2000).** Infrared spectroscopy in Microbiology, in *Encyclopedia of Analytical Chemistry,* R.A. Meyers (Ed.) p. 102-131. John Wiley & Sons Ltd., Chichester, 2000.

- **Niu, H.**, **Xu, X. S., Wang, J.H. and Volesky, B. (1993).** Removal of Pb (II) from aqueous solutions by *Penicillium* biomass. *Biotechnol. Bioeng.* 42: 785-787.

- **Omar, N. B., Merroun, M. L., Gonzales-Munoz, M. T. and Arias, J. M. (1996).** Brewery yeast as a biosorbent for uranium. *J. Appl. Bacteriol.* 81: 283-287.

- **Otvos, J. D., Liu, X., Li, H., Shen, G. and Basti, M. (1993).** *Melallothionenin II*-Basel. metallothionein genes are conspicuously expressed during embryogenesis (Suziki KT, Imura N- Kimura M. eds.), Birkhauser Verlag,. pp. 57.

- **Özer, A., Ekiz, H. I., Özer, D., Kutsal, T. and Çağlar, A. (1997).** A staged purification process to remove heavy metal ions from waste water using *Rhizopıs arrhizus. Process Biochem.* 32: 319-326.

- **Pethkar, A.V., Kulkarni, S.K., Paknikar, K.M., (2001).** Comparative studies on metal biosorption by two strains of *Cladosporium cladosporioides. Bioresour. Technol.* 80: 211-215.

- **Pighi, L., Pumpel, T. and Schinner, F. (1989).** Selective accumulation of silver by fungi. *Biotechnol. Lett.* 11: 275-280.

- **Prouty, A. L. (1990).** Bench-scale development and evaluation of a fungal bioreactor for color removal from bleach effluence. *Appl. Microbiol. Biotechnol.* 32: 490-493.

- **Remacle, J. (1990).** The cell wall and metal binding, p. 83-92 In *Biosorption of Heavy Metals.* CRC Press, Inc.

- **Rigas, B., Morgella, S., Goldman, I.S., and Wang, P.T.T. (1990),** " Human Colorectal Cancers Display Abnormal Fourier Transform Infrared Spectra", *Proc. Natl. Acad. Sci.* USA., 87: 8140-8144.

- **Sağ, Y. and Kutsal, T. (1997).** The simultaneous biosorption process of lead (II) and nickel (II) on *Rhizopus arrhizus. Process Biochem.* 32: 591-597.

- **Schiewer, S.**, **and Volesky, B., (1995).** Modeling of the proton-metal ion exchange in biosorption. *Environ. Sci. Technol.* 29: 3049-3058.

- **Schiewer, S.**, **and Volesky, B., (1997).** Ionic strength and electrostatic effects in biosorption of divalent metal ions and protons. *Environ. Sci. Technol.* 31: 2478-2485.

- **Scheiwer, S. and Volesky, B. (2000).** Biosoprtion processes for heavy metal removal. In Environmental Microbe-Metal Interactions ASM Press 2000, Chapter 14, 329-362.

- **Schmitt, J.**, **Flemming, H.C., (1998).** FTIR spectroscopy in microbial and material analysis. *Int. Biodeterior. Biodegrad.*. 41: 1-11.

- **Severcan, F.**, **Kaptan, N., and Turan, B. (2003).** Fourier transform infrared spectroscopic studies of diabetic rat heart crude membranes. *Spectroscopy* 17: 569-577.

- **Severcan, F.**, **Toyran, N., Kaptan ,N., and Turan, B. (2000).** Fourier transform infrared study of the effect of diabetes on rat liver and heart tissues in the C-H region. *Talanta*. 53:55-59.

- **Severcan, F., Durmus, H.O., Eker, F., Akinoglu, B.G., and Haris P.I. (2000).** Vitamin D_2 modulates mellitin-membrane interactions. *Talanta.* 53:205-211.

- **Severcan, F.**, Boyar, H., Zorlu, F., and Mut, M. (2004). The effects of chronic hypoperfusion on rat cranial bone mineral and organic matrix. *Anal. Bioanal. Chem.* 379: 433-438.

- **Shuttleworth, K.L., and Unz, R.F., (1993)** Sorption of Heavy to the Filamentous Bacterium *Thiothrix* Strain A1. *Appl. Environ. Microbiol.* 59: 1274-1282.

- **Silbergeld, E. K. (2003).** Facilitative mechanisms of lead as a carcinogen. *Mutat Res.* 533: 121-133.

- **Sing, C. and Yu, J. (1998).** Copper adsorption and removal from water by living mycelium of white-rot fungus Phanerochaete chrysosporium. *Water. Res.* 32: 2746-2752.

- **Smith, B. (1999):** "Infrared Spectral. Interpretation Systematic Approach", USA: CRC Press. LLC.

- **Strandberg, G. W., Shumate, S. E. and Parrot, J. R. (1981).** Microbial cells as biosorbents for heavy metals: accumulation of uranium by *Saccharomyes cerevisiae* and *Pseudomonas aeruginosa Appl. Environ. Microbiol.* 41(1): 237-245.
- **Texier, A. C., Andres, Y., and Cloirec P. L. (1999).** Selective Biosorption of Lanthanide (La, Eu, Yb) ions by *Pseudomonas aeruginosa. Environ. Sci. Technol.* 33: 489-495.

- **Tobin J.M., Cooper D. G. and Neufeld R. J. (1990).** Investigation of the mechanism of metal uptake by denatured *Rhizopus arrhizus* biomass. *Enzyme Microb.Technol.* 12: 591-595.

- **Tsezos, M. and McCready, R. G. L. (1991).** The pilot plant testing of the continuous extraction of radionuclides using immobilized biomass. *Environmenlal Biotechnology for Waste Treatment. G. S.* Sayler, R. Fox, and J. Blackbum (eds.). Plenum, New York. pp. 249-260.

- **Tsezos, M.**, **Remoudaki, E., and Angelatou, V. (1997).** Biosorption sites of selected metals using electron microscopy. *Comp. Biochem. Physiol.* 118: 481-487.

- **Unz, R.F., Shuttleworth, K.L., (1996).** Microbial mobilization and immobilization of heavy metals. *Curr. Opin. Biotechnol.* 7: 307-310.

- **Vasudevan, P., Padmavathy, V., and Dhingra, S.C., (2003).** Thermal and spectroscopic studies on sorption of nickel (II) ion on protonated baker's yeast. *Chemosphere.* 52: 1807-1817.

- **Veglio, F., Beolchini, F., (1997).** Removal of metals by biosorption: a review. *Hydrometallurgy* 44:301-316.

- **Volesky, B., Davis, T.A. and Mucci, A. (2003).** A review of the biochemistry of heavy metal biosorption by brown algae. *Wat. Res.* 37: 4311-4330.

- **Volland, W. (1999).** Organic Compound Identification Using Infrared Spectroscopy. USA: Bellewue Community College.

- **White, C., Sayer, J. A. and Gadd, G. M. (1997).** Microbial solubilization and immobilization of toxic metals: key biogeochemical processes for treatment of contamination. *FEMS Microbiol. Rev.* 20: 503-516.

- **Yetiş, Ü., Dölek, A., Dilek, F. B. and Özcengiz, G. (2000).** The removal of Pb (II) by *Phanerochaete chrysosporium. Wat. Res.* 34(16): 4090-4100.

- **Yetiş, Ü., Özcengiz, G., Dilek, F. B., Ergen, N. Erbay, A. and Dölek, A. (1998).** Heavy metal biosorption by *Polyporous versicolor* and *Phanerorochaete chrysosporium*. IAWQ 19th Biennial Conference, Water Quality Internanatinal 98, June 21-26, Vancouver, pp. 324-331.

- **Yetiş, Ü., Özcengiz, G., Dilek, F. B., Ergen, N. Erbay, A. and Dölek, A. (1998).** Heavy metal biosorption by white-rot fungi. *Water. Sci. Technol*. 38(4-5): 323-330.

- **Yano, K., Ohoshima, S., Shimizu, Y., Moriguchi, T., and Katayama, H. (1996),** "Evaluation of Glycogen Level in Human Lung Carcinoma Tissues by an Infrared Spectroscopic Method", *Cancer Letters*, 110: 29-34.

- **Yin, P., Yu, Q., Jin B. and Ling Z. (1999).**Biosorption removal of cadmium from aqueous solution by using pretreated fungal biomass cultured from starch wastewater. *Wat. Res.* 33(8): 1960-1963.

- **Yu, Q., Matheickal, J. T., Yin, P. and Kaewsarn, P. (1999).** Heavy metal uptake capacities of common marine macro algal biomass. *Wat. Res.* 33(6): 1534-1537.

- **Zhou, J. L. (1999).** Zn biosorption by *Rhizapus arrhizus* and other fungi. *Appl. Microbiol. Biotechnol.* 51: 686-693.

APPENDIX A

Chemicals and Their Suppliers

Chemicals	**Supplier**
Acetone	Merck
Benzene	Merck
$CaCl_2\,H_2O$	Aldrich
Formaldehyde	Merck
Formic acid	Merck
Glucose	Merck
HCI	Fluka
$MgSO_4\ 7H_2O$	Sigma
NaOH	Merck
NH_4Cl	Sigma
KH_2PO_4	Sigma
$Pb(NO_3)_2$	Sigma
Methanol	Merck
Nitric acid	Merck
Nitromethane	Aldrich
Perchloric acid	Merck
Thiamine	Sigma
Triethyl phosphite	Fluka