COMPARISON OF OPTICAL MEASUREMENTS FOR BETTER NI TRACKING IN BACTERIA

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ABSTRACT

COMPARISON OF OPTICAL MEASUREMENTS FOR BETTER NI TRACKING IN BACTERIA

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Metal pollution is a common problem in industrial areas. Microorganisms, as well as macroorganisms are affected from metal pollution. In order to cope with this problem, microorganisms develop resistance mechanisms.

Traditional microbiological techniques alone are not sufficient in resistance studies. Additional data acquisition by using other measurement techniques and devices are also required. However, there are differences in sentitivities of the measurement devices. A given device may work under a set of conditions and concentrations but may not be suitable when the conditions and concentrations change. Therefore, testing the efficiency of measurement device that is suitable to produce meaningful data is essential.

In this study, the possibility and feasibility of using spectroscopy based measurements to quantitate Ni in the spent media of bacterial cultures were explored. The UV-Vis Spectroscope and ATR-FTIR spectroscope were the two optical devices that were compared in this study.

Two strains of *Microbacterium oxydans*, another undefined *Microbacterium* freshwater isolate, and *E. coli* were grown at their corresponding minimum inhibitory concentrations of Ni. Then the Ni concentrations left in the culture supernatants following the removal of bacteria were measured by UV-Vis and ATR-FTIR spectroscopes. The data obtained from the two devices were compared. The results indicated that ATR-FTIR spectroscopy had a higher sensitivity and the band intensities provided reasonable approximation for better estimations of Ni concentrations. The data were discussed in terms of detection of metals while the metal sorption capacities of bacteria being evaluated.

Keywords: nickel, freshwater bacteria, UV-Vis Spectroscopy, ATR-FTIR Spectroscopy

ÖZ

BAKTERİLERDE DAHA VERİMLİ NİKEL TAKİBİ İÇİN OPTİK ÖLÇÜM YÖNTEMLERİNİN KARŞILAŞTIRILMASI

Sungur, Sıla
Yüksek Lisans, Biyoloji Bölümü
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Aralık 2015, 55 sayfa

Metal kirliliği, sanayi bölgelerinde sık görülen bir problemdir. Metal kirliliğinden, diğer canlılar kadar mikroorganizmalar da etkilenir. Bu nedenle, mikroorganizmalar direnç mekanizmaları geliştirmiştir.

Geleneksel mikrobiyolojik uygulamalar, metal dirençliliği çalışmalarında tek başına yeterli olmamaktadır. Başka ölçüm teknikleri ve cihazların kullanımı yoluyla elde edilebilecek ek veriler gereklidir. Ancak, ölçüm yapan cihazların hassasiyetleri farklılık göstermektedir. Bir cihaz, belli koşullarda ve konsantrasyonlarda çalışabilir; fakat bu koşullar ve konsantrasyonlar değiştiğinde hassasiyeti düşük olabilir. Bu nedenle, uygun verileri sağlayabilecek olan ölçüm yöntemlerinin verimliliğinin belirlenmesi gereklidir.

Bu çalışmada, bakteri kültürlerinin besiyerlerindeki nikel miktarının ölçümünde kullanılan spektroskopi temelli yöntemlerin uygunluğu ve verimliliği denenmiştir. Çalışmada karşılaştırılan iki optik cihaz, UV-Vis spektroskobu ve ATR-FTIR spektroskobudur.

İki *Microbacterium oxydans* suşu, türü tanımlanmamış başka bir *Microbacterium* izolatı ve *E. coli* suşu, üreyebildikleri en yüksek nikel konsantrasyonunda üretilmiş ve bakterilerin ayrıştırılmasından sonra kültür sıvılarında kalan nikel konsantrasyonu, UV-Vis ve ATR-FTIR spektroskoplarıyla ölçülmüştür. Ölçümlerin ardından, iki farklı cihazdan elde edilen veriler karşılaştırılmıştır. Bulunan sonuçlar, ATR-FTIR cihazının ölçüm hassasiyetinin daha yüksek olduğunu ve nikelin yaklaşık konsantrasyonunun hesaplanmasında bant yoğunluğu değerlerinin kullanılmasının kabul edilebilir bir yöntem olduğunu göstermektedir. Bakterilerin metal emilim kapasiteleri kültür sıvılarında kalan metal miktarı üzerinden değerlendirilmiştir.

Anahtar Kelimeler: nikel, tatlısu bakterileri, UV-Vis Spektroskopisi, ATR-FTIR Spektroskopisi

To My Family and Friends

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LIST OF ABBREVIATIONS

Ni: Nickel

RND: Resistance-Nodulation-Cell Division Protein Family

Co: Cobalt

V: Vanadium

MRL: Microbial Resistance Level

LS: Lab Scale

GM: Growth Media

UV-Vis: Ultra Violet-Visible

ATR-FTIR: Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy

ATCC: American Type Culture Collection

FS: Fish Surface

Rpm: Revolution per minute

MIC: Minimum Inhibitory Concentration

CFU: Colony Forming Unit

RCF: Relative Centrifugal Force

A.U.: Arbitrary Units

SA: Samil

CHAPTER 1

INTRODUCTION

1.1 Resistance to Heavy Metals

Metal pollution is a common problem in industrial areas. In such areas, wastes are deposited in soil and/or water, which affects the natural microorganisms in these areas. In order to cope with high metal ion concentrations, microorganisms have developed resistance mechanisms. According to Bruins *et. al* (2000), the key mechanisms for heavy metal resistance are exclusion of the metal by the restriction of permeability, intra/extracellular sequestration of the metals, enzymatic detoxification, decrease of metal sensitivity of cellular targets, and active transport of the metal away from the cell or the organism.

Genes for resistance to heavy metals are present in either bacterial chromosomes or plasmids (Silver, 1996; Bruins *et. al.*, 2000). Chromosomal resistance mechanisms are more complicated than plasmid-mediated resistance mechanisms. Chromosomal mechanisms are for essential metals, while plasmid-mediated resistance mechanisms are for toxic metals (Silver, 1998; Bruins *et. al.*, 2000).

Table 1.1 Gene families for resistance to heavy metals in bacteria (Nies,1999).

Family	Direction of transport	Energy	Metal ions	Composition
ABC	Uptake	ATP	Mn^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}	2 membrane-integral parts[a] + 2 ATPase parts = ABC core + periplasmic binding protein
	Efflux	ATP	–	ABC core + membrane fusion protein and outer membrane factor
P-type[b]	Both	ATP	Mg^{2+}, Mn^{2+}, Ca^{2+}, K^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Ag^{+}	1 membrane-bound protein as core
A-type[c]	Efflux	ATP	Arsenite	1 membrane-integral protein + a dimeric ATPase subunit
RND	Efflux	Proton gradient	Co^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}?, Ag^{+}?	1 CPM proton/cation antiporter + membrane fusion protein (dimer?) + outer membrane factor: CBA transport systems
HoxN	Uptake	Chemiosmotic	Co^{2+}, Ni^{2+}	Membrane-Integral protein
CHR	Antiport?	Chemiosmotic	Chromate	Membrane-integral protein (ChrA)
MIT	Uptake	Chemiosmotic	Most cations	Membrane-integral protein (CorA)
CDF	Efflux	Chemiosmotic	Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}?	Membrane-integral protein (CzcD, ZRC1p, ZnT1)

[a] "Parts" are proteins or protein domains, depending on the specific transporter
[b] Fagan and Saier 1994
[c] Saier 1994

1.2 Ni As A Heavy Metal

Ni is a transition metal with the atomic number 28 and atomic weight 58.7. In the form of Ni chloride hexahydrite ($NiCl_2.6H_2O$), it is green and has an absorption spectrum in between 400 nm and 800 nm; with the peak absorption around 400 nm (Liu *et. al.*, 2012) (Figure 1. 1).

In cells, Ni is an important part of the enzymes such as ureases, hydrogenases, and CO dehydrogenases (Nies, 1999). However, Ni is toxic in high concentrations like most of the heavy metals. In order to cope with high Ni concentrations, eukaryotic cells use sequestration and/or transport mechanisms (Nies, 1999). The most well-known Ni detoxification mechanism in bacteria is metal efflux by RND transporter (Nies, 1999; Kim *et. al.*, 2011). Most common operons used for Ni resistance in bacteria are *ncc* and *ncr* operons (Nies, 1999; Tibazarwa *et. al.*, 2000; Liesegang *et. al.*, 1993; Schmidt & Schlegel, 1994).

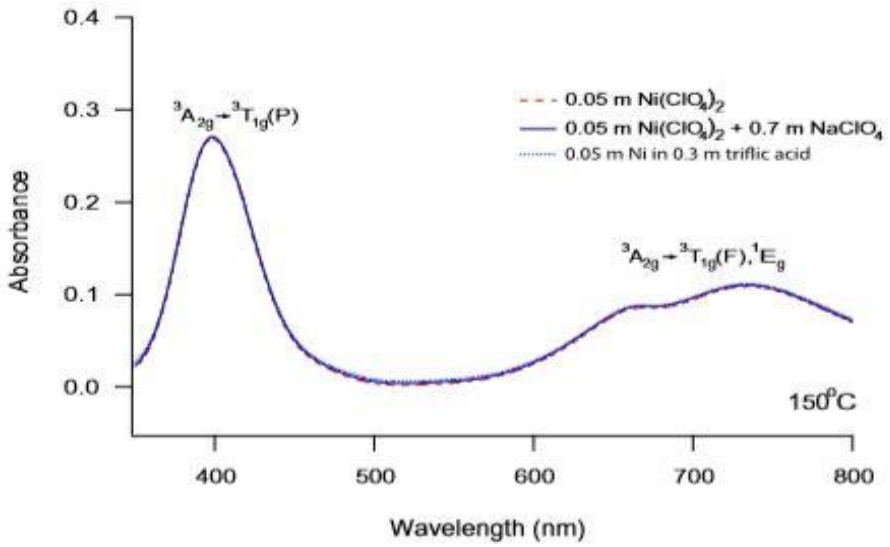

Figure 1.1 Molar absorption spectra of Ni^{2+} in three different aqueous solutions (Liu *et.al.*, 2012). The labelled peaks are d-d transition bands, and Ni^{2+} did not form complexes with the solutions (Liu *et. al.*, 2012).

1.3 Resistance against Ni

The well-known resistance mechanism for Ni is the efflux by RND transporter (Nies, 1999; Kim *et. al.*, 2011). In order to pump substrates out, RND proteins use proton motive force (Goldberg *et. al.*,1999; Nies, 1995; Kim *et. al.*, 2011). By using two different RND polypeptides, they form homotrimers (Murakami *et. al.*, 2002; Kim *et. al.*, 2011) or heterotrimers in a ratio of 2:1 (Kim *et. al.*, 2010; Kim *et. al.*, 2011). With 12 transmembrane alpha helices, each of the monomers of RND spans the inner membrane (Murakami *et. al.*, 2006; Seger *et. al.*, 2006; Kim *et. al.*, 2011). RND proteins work in two ways: either by periplasmic efflux (binding and exportation) or transenvelope efflux (the substrate is not released in periplasm) (Nies, 2003; Kim *et. al.*, 2011) (Figure 1.2).

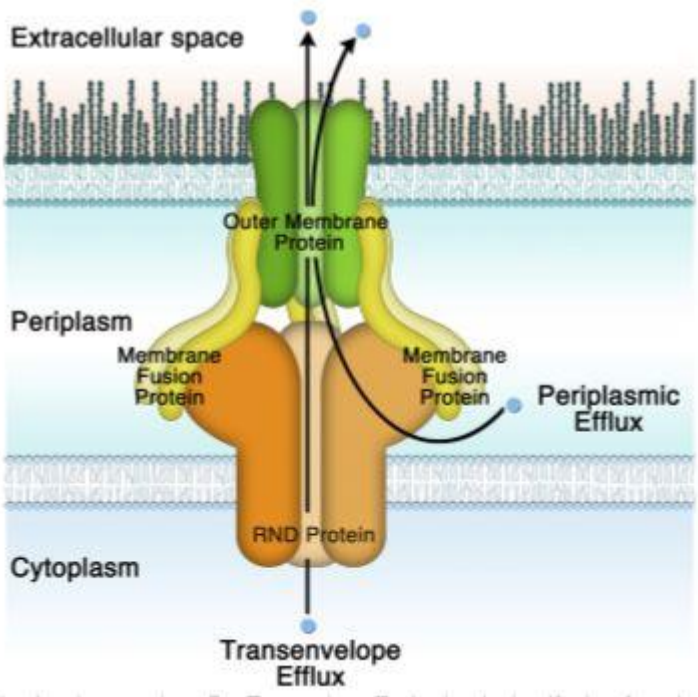

Figure 1.2 Model for a RND transporter protein, which shows both periplasmic and transenvelope efflux (Kim *et. al.*, 2011).

Some other less-known Ni resistance mechanisms include *nrf* gene which is included in a *Enterobacter cloacae* strain (Pickup *et. al.*, 1997), CznABC metal efflux pump which operates in *Helicobacter pylori* (Stähler *et. al.*, 2006), and biofilm formation (Harrison *et. al.*, 2007).

1.4 Biosorption

The definition of biosorption can be stated as the capability of nonliving or surviving cells to accumulate toxic metals (Hassan *et. al.*, 2010; Volesky, 2003; Fourest & Roux, 1992). Most potent cells for biosorption belong to bacteria, fungi, algaea, and yeast (Ahalya *et. al.*, 2003; Volesky, 1986). Table 1.2 indicates microbial resistance and bioaccumulation of several metals (Malik, 2004).

There are several biosorption mechanisms. For example, biosorption can be either dependent or non-dependent of the cell's metabolism. Moreover, biosorption can be extracellular accumulation/precipitation, cell surface sorption/precipitation and intracellular accumulation (Ahalya *et. al.*, 2003).

Biosorption has many biotechnological applications. For example, the product AMT-Bioclaim™, which has *Bacillus subtilis* inside, has the ability to accumulate gold, cadmium, and zinc (Atkinson *et. al.*, 1998) (Vijayaraghavan & Yun, 2008). Another example is AlgaSORB™, which contains *C. vulgaris* and some other algae, has been shown to have excellent heavy-metal ion affinities and used in cleaning US nuclear sites (Eccles, 1995) (Vijayaraghavan & Yun, 2008).

Many publications about biosorption applications are available, but there are very few attempts to apply these in industrial scales (Gadd, 2008). The commercialization of biosorption has several limitations, some of which are the requirement of a reliable supply of waste microbial biomass, the cost of transforming the biomass into biosorbents, logistic problems for biomass distribution and re-use, and the presence of solution matrix co-ions on the biomass which makes the recycling more difficult (Tsezos, 2001).

For the removal of Ni, one mechanism is bacterial sulfate reduction as a reactor-based process. In an example, a reactor which included a spent mushroom compost removed 75% of 16.9 mM of Ni; even 95% when sodium lactate was added (Hammack & Edenborn, 1992) (White *et. al.*, 1997). In another study, the phantom midge *Chaoborus* was found to be very effective for Ni biomonitoring (Ponton & Hare, 2009).

Table 1.2 Resistance and biosorption of Ni and some other metals by isolated microbial strains (Malik,

Microorganism (isolation source)	Name of organism	Metal	MRL/uptake efficiency	Removal (%)	Scale of experiment/source of metal (concentration)	Comment	Reference
Algae (acquired)	*Chlorella vulgaris*	Ni	0.6 mg/g	33–41 (24 h)	LS/metal solution (10–40 mg/l)	Cell viable after Ni accumulation but growth rate reduced	Wong et al. (2000)
Waste water treatment plant	*Chlorella miniata* (WW1)	Ni	1.4 mg/g	>99 (24 h)			
Cyanobacteria	*Anabaena cylindrica*	Ni	2 mg/g		LS/metal solution or effluent (<20 mg/l)	Low Ni biosorption in actual effluents	Corder and Reeves (1994)
	Anabaena flos aquae	Ni	6 mg/g				
	Nostoc spp.	Ni	3 mg/g				
Yeast (sewage)	*Candida* spp. (nonadapted)	Ni	10.3–46.8 mg/g	29–57 (5–15 days)	LS (100 ml)/GM (66–514 mg/l)	Metal concentration and pH-dependent uptake	Donmez and Aksu (2001)
	Candida spp. (adapted)	Ni	10.9–30.8 mg/g	44–71 (5–15 days)			
Fungi (metal-contaminated soil)	*Aspergillus niger*	Ni	0.38 mg/ml (23.5 mg/g)	98 (4 days)	LS/GM (381 mg/l)	Ni uptake requires energy metabolism	Magyarosy et al. (2002)
Bacteria (local isolate)	*Pseudomonas* spp.	Ni	74.9 mg/g		LS/metal solution	Concentration-dependent uptake	Ghozlan et al. (1999)
Bacteria (oil refinery soil)	*Escherichia hermannii* CNB50	Ni	2 mg/ml (10.2 mg/g)		LS (10 ml)/metal solution 100 mg/l)	Outer membrane profile altered in presence of metals	Hernandez et al. (1998)
		V	1 mg/ml (53.8 mg/g)				
	Enterobacter cloacae CNB60	Ni	2 mg/ml (6.9 mg/g)				
		V	1 mg/ml (39.3 mg/g)				
Fungi	*Neurospora crassa*	Co	–	90 (24 h)	LS/GM or metal solution (500 mg/l)	Growth medium affects removal	Karna et al. (1996)

LS: lab-scale. GM: growth media.

1.5 UV-Vis Spectroscopy

UV-Vis spectroscopy is an optical measurement technique that measures absorbance in both visible (400-800 nm) and ultraviolet spectrum (200-400 nm) (UV-Visible Spectroscopy, http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/UV-vis/uvspec.htm#uv1). This device can measure the absorbance of the sample in only one wavelength at once.

UV-Visible spectroscopic measurement technique is used in photometric determination of many compounds, water analyses, enzymatic analyses, multicomponent analyses, chemometrics, and identification and structure determination (Perkampus, 1992).

In microbiology-related applications, absorbance is a measure of bacterial cell growth. However, absorbance measurement of a compound alone is not a very reliable source for the detection of impurities or contamination; hence further analyses may be needed.

1.6 ATR-FTIR Spectroscopy

ATR-FTIR (Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy) is principally different from UV-Vis Spectroscopy. This device uses an infrared light source; and collects data from the sample in different wavenumbers simultaneously. With the help of a special mathematical technique called Fourier Transformation, the data is turned into transmittance or absorbance vs. wavenumber graphics on a computer.

The difference of ATR-FTIR from traditional FTIR is that traditional FTIR samples require special preparation before measurement. This preparation includes diluting the sample with an IR transparent salt and then pressing the diluted sample into a thin pellet or pressing to a thin film. Such preparation is made in order to get rid of totally absorbing bands. However, ATR-FTIR does not require any sample

preparation; dropping the sample on the ATR crystal and drying it with gaseous nitrogen is sufficient (Application Note - ATR-Theory and Applications, 2011).

The power of this spectroscopic measurement technique comes from the ability to process very tiny amounts of sample as little as 0.5 μL (Carter, 2010) in different wavenumbers simultaneously and quickly. Moreover, every material shows a unique spectrum when measured under infrared exposure (fingerprint). For this reason, FTIR spectroscopy in general can be used in quality control analyses of newly produced materials; so if any contaminants are present, they can be determined (Thermo Nicolet Corporation, 2001).

FTIR spectroscopy takes measurements mainly in mid-infrared (MIR) range; namely in between 400 – 4000 cm^{-1} [Near-Infrared Region Measurement and Related Considerations Part 1 : SHIMADZU (Shimadzu Corporation), 2015] . However, there are also FTIR devices which can take measurements in near-infrared (NIR) range; namely in between 12.500 – 4000 cm^{-1}. FTIR devices which enables both mid-infrared and near-infrared measurements are also present. NIR-FTIR enables the measurement of unstable and/or hazardous materials which are kept in a container during measurement (Application Note - Infrared Spectroscopy – A Combined Mid-IR/Near-IR Spectrometer, 2010).

1.7 Aim and Scope of The Study

The aim of this study is to compare optics based measurement techniques for tracking and quantitation of Ni in bacterial cells.

The accurate measurement technique can then be confidently used in Ni biosorption or accumulation determinations.

CHAPTER 2

MATERIALS AND METHODS

2.1 Equipments

Table 2.1 Equipments and suppliers.

Equipments	Suppliers
Autoclave	Nuve, Turkey
Incubator	Binder, Germany
Shaker Incubator	Medline, Korea
Magnetic Stirrer	Velp Scientifica, Italy
Centrifuge	Sigma, Germany
Vortex	Velp Scientifica, Italy
UV-Vis Spectrophotometer	SOIF, China
Class II Biological Safety Cabinet	ESCO, USA
Multiscan UV-Vis Spectrophotometer	Thermo Scientific, USA
FTIR Spectrometer	Perkin Elmer, USA

2.2 Bacterial Isolates

The bacterial strains which were used in this study were previously isolated by Tugba Ozaktas (Ozaktas *et. al.*, 2012). A total of sixty bacteria were isolated. However, only three of these species were used in this study. *Escherichia coli* (ATCC 8739) was used as reference.

Table 2.2 Abbreviations and names of the studied bacteria.

Isolate Abbreviation	Name
FS45*	*Microbacterium oxydans*
FS42	*Microbacterium oxydans*
FS10	*Microbacterium spp.*
Reference	*Escherichia coli* (ATCC 8739)

*FS: Fish Surface

2.3 Growth Conditions of the Isolates

Since all of the three strains used in this study were aerobic (Ozaktas *et. al.*, 2012), they were grown under aerobic conditions and incubated at 28^{o} C. Liquid cultures were aerated at 200 rpm.

2.4 Media

Nutrient broth (Merck, USA) and nutrient agar (Becton-Dickinson, USA) were used as growth media for all experiments. In order to prepare Ni stock solutions, 2.52 g of the Ni salt $NiCl_2.6H_2O$ (AppliChem) was dissolved in 20 ml distilled H_2O. The resulting Ni stock solution was 0.315M. Filter sterilization of the stock solutions was made by using 0.2-µm filters (Minisart, Germany). The solutions were kept in +4^{o} C, in the dark. After the preparation and autoclaving of the broth and agar, Ni solution from the stock solution was added to both in laminar flow chamber. All media were stored in +4^{o} C.

2.5 Minimum Inhibitory Concentration Determination of the Cultures by Plate Assay Method

For MIC determination, bacteria were tested in concentrations of 3mM, 2mM, 1.5mM and 1mM. Testing concentrations were determined according to

Nedelkova et. al. (2007). The same concentrations were also applied to the reference. For the respective concentrations, 476 µl, 317 µl, 238 µl, and 159 µl of Ni solution from the stock and 1 ml of bacteria from an overnight culture were added to 50 ml nutrient broth. Spectrophotometric measurements and agar inoculations were taken in 1st and 7th days. The cultures were inoculated to both nutrient agar and nutrient agar with Ni in the studied concentration. CFU counts were taken 4 days after inoculations.

2.6 Ni Biosorption

All bacterial isolates were grown in 1mM Ni. At the first day, absorbances of control groups were recorded, and then the control groups were inoculated on to nutrient agar. Controls were centrifuged at 20.000 RCF for 20 minutes. The supernatants were filtered through 0.2-µm filters (Minisart, Germany) twice. At the second day, the experimental cultures were inoculated on to Ni containing agar and then the same centrifugation and filtering procedures were applied to the experimental groups. Absorbance values of the experimental groups were recorded both in the first and the second day.

For the construction of standard curves (absorbance vs. concentration), UV-Vis absorbance values for 0.25mM, 0.50mM, 0.75mM, and 1.00mM of Ni were used. Since *Escherichia coli* was the reference, supernatants of *Escherichia coli* control groups were used for the standard preparation related to culture supernatants. The other group of standards were prepared from fresh nutrient broth. Absorbance values for multiple wavelengths (400 nm, 550 nm, 660 nm and 750 nm) were taken for both standards and experimental groups. Wavelengths were determined according to Liu *et. al.* (2012).

After that, absorbance spectra of all Ni solutions and bacterial supernatants (both control and experimental groups) were measured in ATR-FTIR spectrometer in triplicates of 5µL in between 4000-650 cm^{-1}. For controls and band intensity measurements for percent biosorption calculations, single subsamples were

measured. Since our compound contained intramolecular water, the infrared spectrum of water was also measured and subtracted from the original data. Each 5 μL sample was dropped on the ATR crystal and dried with gaseous nitrogen, then the spectra were recorded. The ATR crystal was cleaned with 70% ethanol and ethanol was wiped before and after sample measurement. This cleaning procedure was applied before and after every sample in order to avoid sample contamination. The second derivative, vector-normalized spectra were used to determine band positions and relative intensities. Second derivative spectra were used to get better resolution of bands. The bands are assigned and illustrated in Table 3.1.

Percent biosorption calculations were performed by using 100-multiplied 1mM Ni-containing control group supernatants' intensities divided by experimental culture supernatants' intensities; then subtracting the result from 100.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Minimum Inhibitory Concentration For Ni

Optical density measurements and CFU counts were recorded in the 1st and 7th day of the experiments. Growth of bacteria at 1mM and 3mM Ni were shown in terms of optical density and CFU in Figures 3.1 and 3.2, respectively. Growth in Ni containing nutrient agar was observed in all four bacteria at 1mM and no growth was observed at 3mM. No growth was observed in 3mM Ni, therefore this was the minimum growth inbiting concentration for the bacteria.

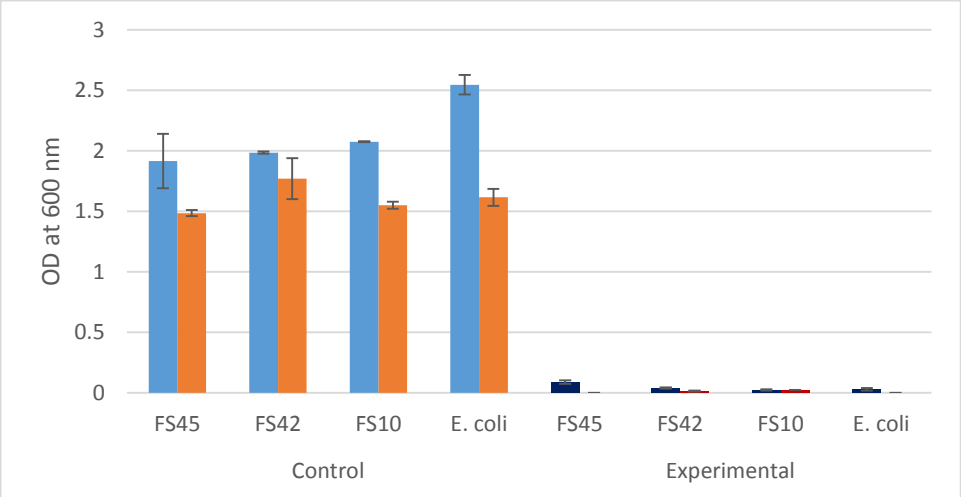

Figure 3.1 Cell densities at 3mM Ni concentration. Experimental indicates the groups in which Ni was added. Light blue and dark blue bars indicate the results for Day 1; orange and red bars indicate the results for Day 7.

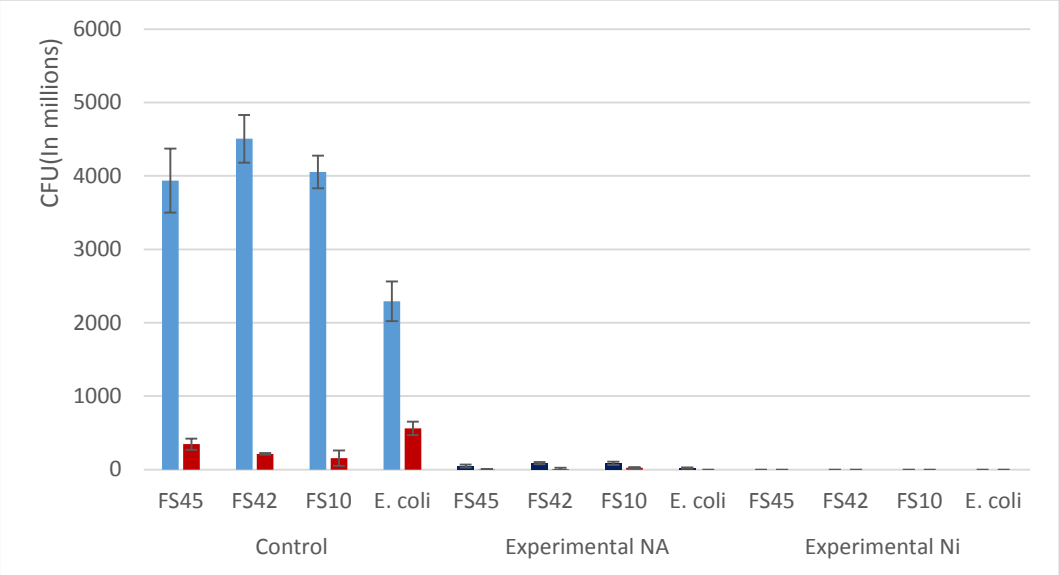

Figure 3.2 CFU values for 3mM Ni concentration. Experimental NA indicates the inoculations performed on plain nutrient agar and Experimental Ni indicates the inoculations performed on 3mM Ni-containing agar, whose innoculents were taken from 3mM Ni-containing experimental groups. Light and dark blue bars indicate results for Day 1; light and dark red bars indicate results for Day 7.

The growth of bacteria at 1mM Ni and in nutrient agar without Ni followed by the liquid culturing at 1mM Ni shown in Figure 3.4. The highest CFU counts were recorded on the 7th day for both groups. The highest growth was observed in FS10. FS10 is an unidentified species belonging to *Microbacterium* genera (Ozaktas *et. al.*, 2012). Although FS45 and FS42 belonged to the same species, they showed significant difference in growth. This indicates the strain differences in the species. These two freshwater fish surface mucous dwelling *Microbacterium* isolates also exhibits differences in their antibiotic resistances (Ozaktas *et. al.*, 2012).

Becerra-Castro *et. al.* (2011) also studied with a different *Microbacterium oxydans* strain and reported the same resistance value to Ni (1mM). Furthermore,

Spain & Alm. (2003) cited from Meargeay *et. al.* (1985) the same resistance to Ni in a different strain of *E. coli*.

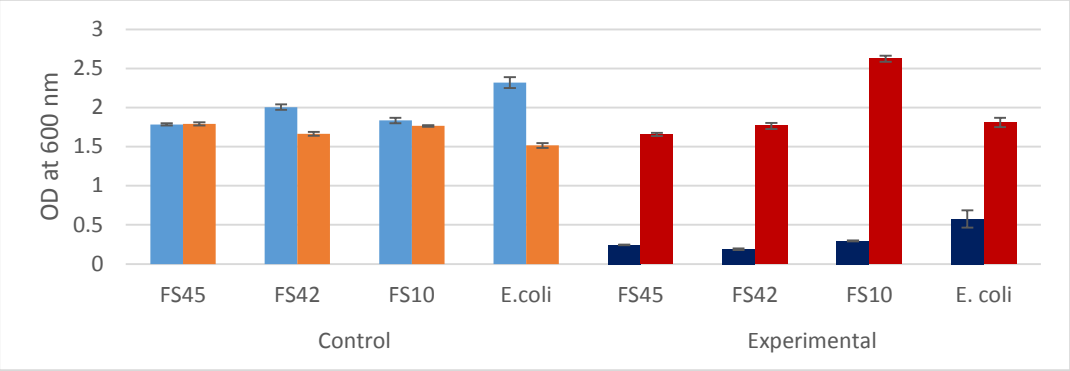

Figure 3.3 Cell densities at 1mM Ni concentration. Experimental indicates the groups in which Ni was added. Light and dark blue bars indicate results for Day 1; orange and red bars indicate results for Day 7.

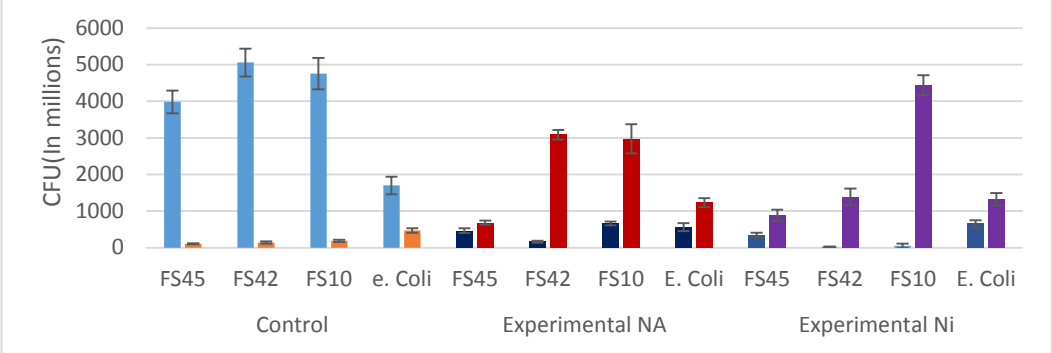

Figure 3.4 CFU values for 1mM Ni concentration. Experimental NA indicates the inoculations performed on plain nutrient agar and Experimental Ni indicates the inoculations performed on 1mM Ni-containing agar, whose innoculents were taken from 1mM Ni-containing experimental groups. Light, medium, and dark blue bars indicate results for Day 1; orange, red, and purple bars indicate results for Day 7.

No bacteria grew at 3 mM Ni-containing solid media. However, when 3mM experimental groups were inoculated on solid media without Ni, growth was resumed at a certain low rate. This indicates that some bacteria are inactivated but not killed by Ni at 3 mM. Upon lifting off the pressure these inactivated alive bacteria started to grow again. However, there are *Microbacterium oxydans* strains that have had the reported MIC value of even 15 mM (Abou-Shanab *et. al.*, 2007), so our strains have comparably low resistance.

Since 1mM was the concentration at which all the bacteria had been growing, we decided to work with that sublethal concentration for the biosorption experiments.

3.2 Biosorption

3.2.1 Multiple UV-Vis Absorbance Measurements

As seen at Figure 3.5, the peak absorbance for pure Ni was at 400 nm and the weakest absorbance was at 550 nm. The absorbance data for 660 nm and 750 nm was in between. This was consistent with the data published by Liu *et. al.* (2012) (see Figure 1.1). However, the same report states that the absorbance at 750 nm is slightly greater than the absorbance at 660 nm. However, in our case the absorbance values for the given wavelengths were similar to each other. This might be because 750 nm is close to infrared region, and our device does not have enough sensitivity for this region but sensitive enough as it does for the UV-visible.

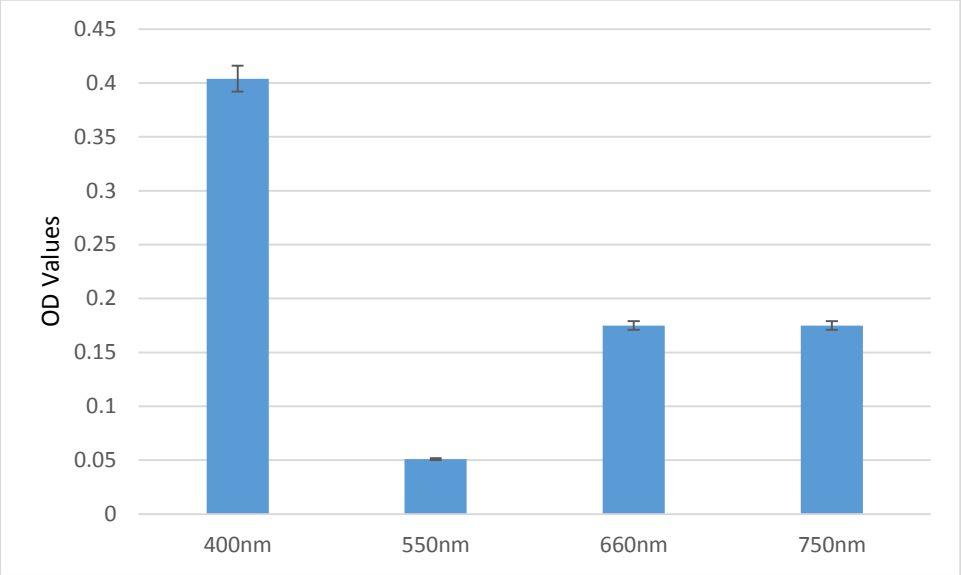

Figure 3.5 Absorbance of pure Ni in multiple wavelengths.

We recorded the peak absorbance values at 400 nm (Figure 3.6). Absorbance values that were recorded at 550 nm, 660 nm, and 750 nm did not show significant variation among themselves, and they were much lower compared to absorbance values that were recorded at 400 nm (Figure 3.6).

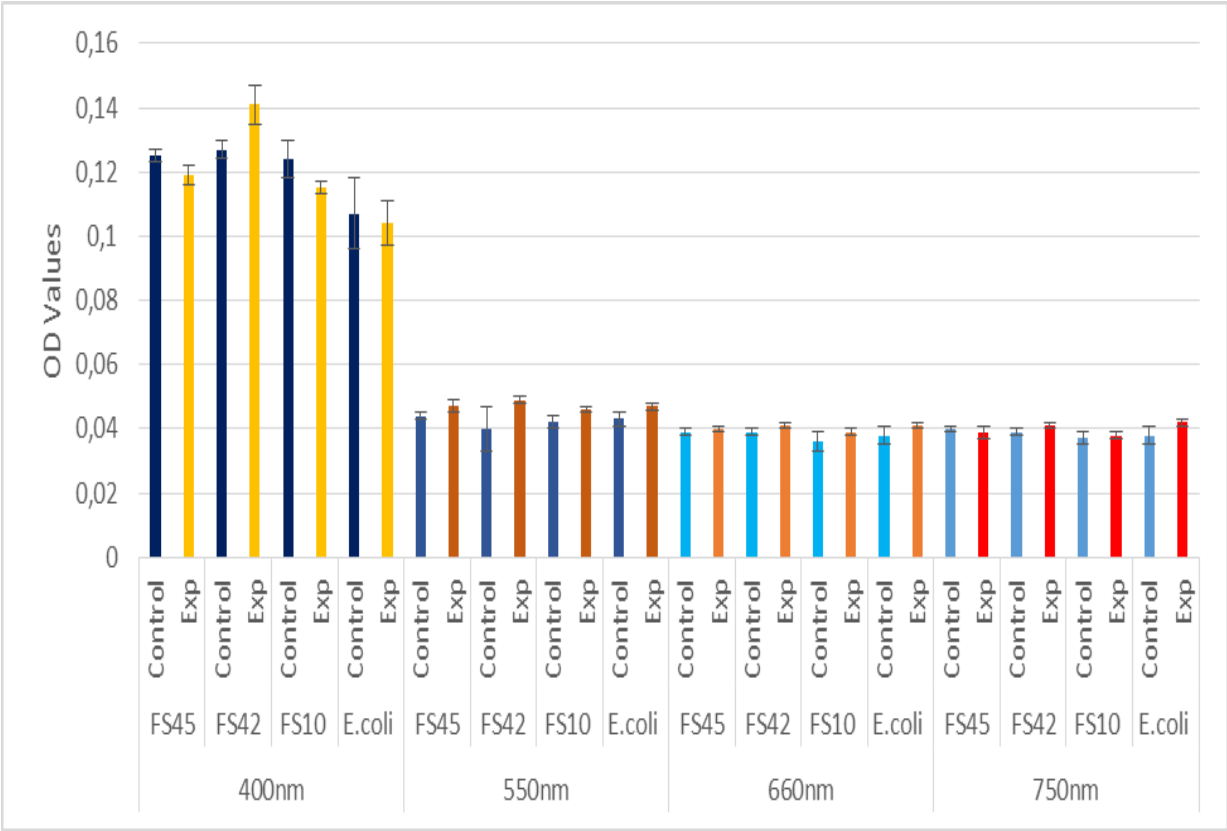

Figure 3.6 Absorbance values for control and experimental groups for multiple wavelengths. Dark to light blue bars indicate OD values for control groups for different wavelengths and yellow, dark orange, light orange and red bars indicate OD values for experimental groups for different wavelengths.

3.2.2 Determination of Ni Concentration By Using Standard Curves

In order to determine the amount of Ni internalized by bacteria, the remaining amount of Ni in culture supernatants were measured by using UV-Vis and ATR-FTIR spectrometers.

According to proportionality approach of Beer's Law, light absorbances of the solutions which includes same compound in varying concentrations are related (Beer's Law Tutorial, http://www.chem.ucla.edu/~gchemlab/colorimetric_web.htm).

The absorbance values of Ni concentrations to generate standard curves prepared by using the supernatants of control cultures, spent medium and as well as in water (Figure 3.7). Absorbance values for the three types of standards were different from each other. The standards prepared by using the fresh broth had the highest absorbance readings. When UV-Vis were to be used as a measurement tool, the readings in experimental groups were higher than the nickel dissolved in water but either the lower or similar compared to the standards prepared in spent medium. The small organic molecules in the spent medium appears to change the absorbance values towards a decrease compared to fresh broth. Obviously fresh broth absorbs more than water only as solvent due to the presence of many additional molecules absorbing the light at the same wavelengths.

The highest absorbance among experimental groups belongs to FS42, which may indicate that it has the lowest biosorbance. Likewise, the lowest absorbance among experimental groups belongs to *E. coli*, which may indicate that it has the highest biosorbance. Although the data might not have provided us information about the actual concentration, it might have provided us visible information about the differences in biosorption.

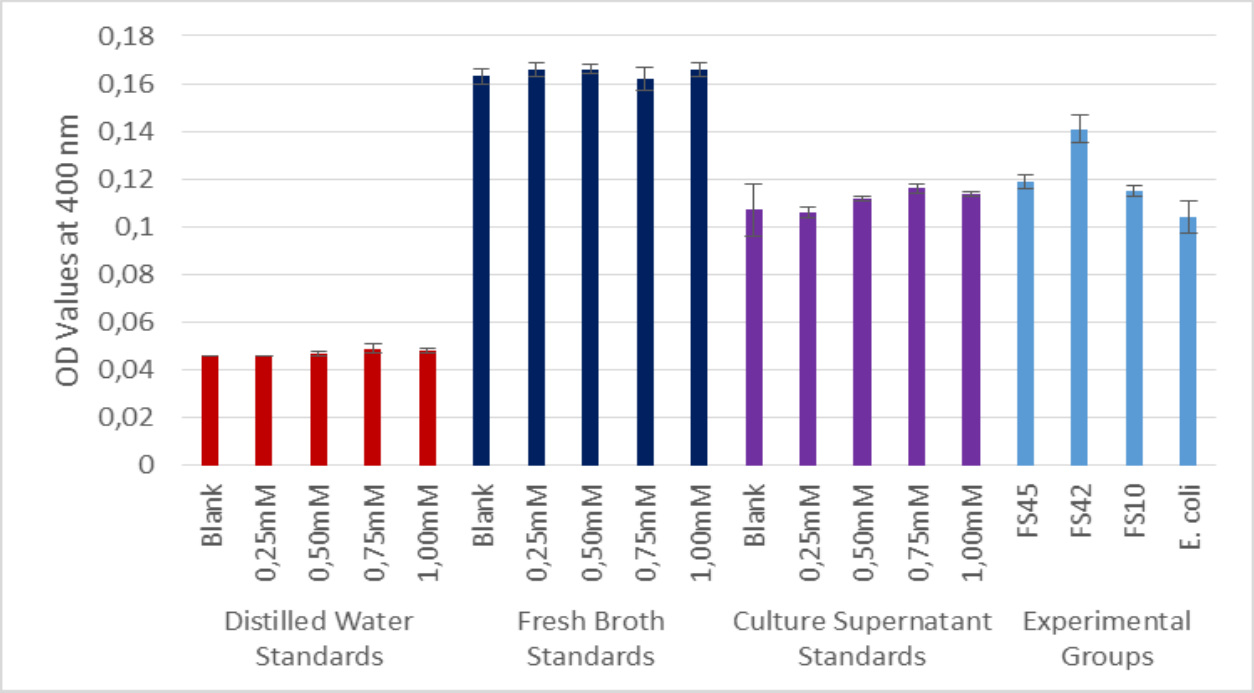

Figure 3.7 Standards prepared with different solvents and their comparison with experimental groups. Red bars indicate OD values for distilled water standards; dark blue bars indicate OD values for fresh broth standards; purple bars indicate OD values for culture supernatant standards, and light blue bars indicate OD values for experimental groups.

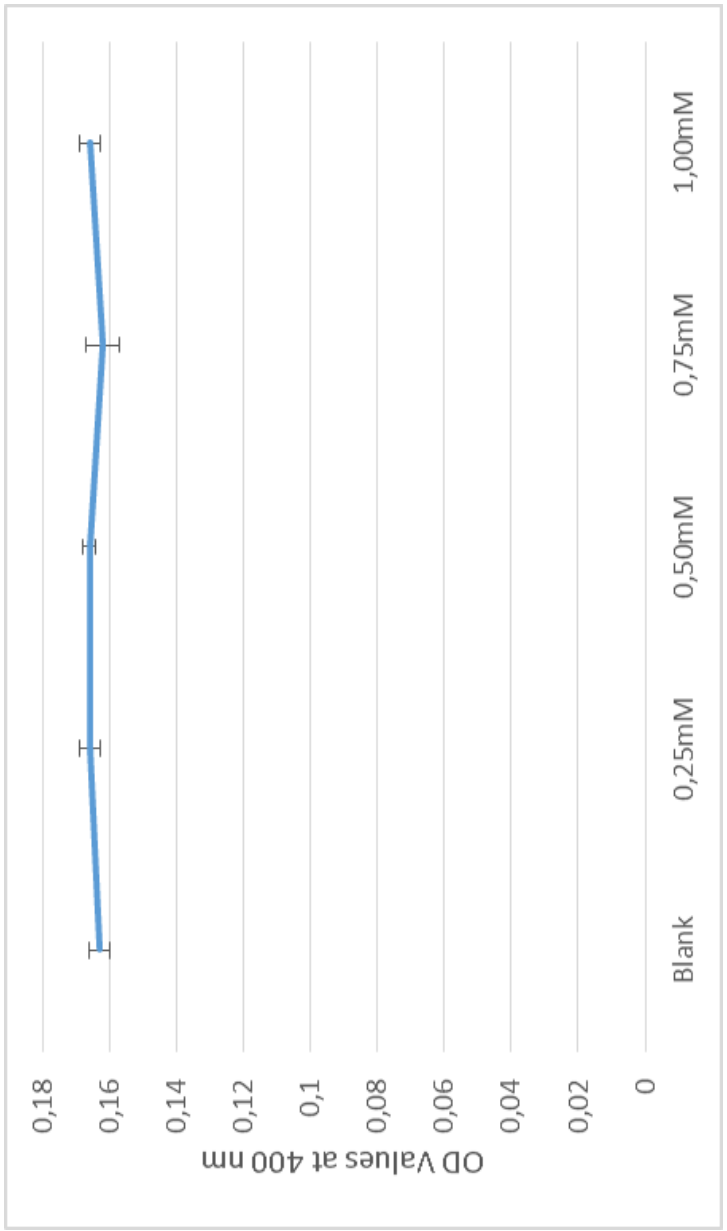

Figure 3.8 Standard curve derived from fresh broth standard absorbances measured at 400 nm.

3.2.3 ATR-FTIR Experiments

3.2.3.1 Band Frequency Analyses

In his study, Gamo (1961) analyzed infrared spectra of hydrated metals such as $NiCl_2.6H_2O$. For $NiCl_2.6H_2O$, he found two absorption peaks (Figure 3.9), whose positions were at 3370 cm^{-1} and 1610 cm^{-1}. In our ATR-FTIR measurements, we obtained two peaks for pure $NiCl_2.6H_2O$, whose positions were 3406 cm^{-1} and 1608 cm^{-1}, similar to his findings (Figure 3.10). We also observed these same peaks in our bacterial culture spent media (Table 3.1) (see also Appendix III).

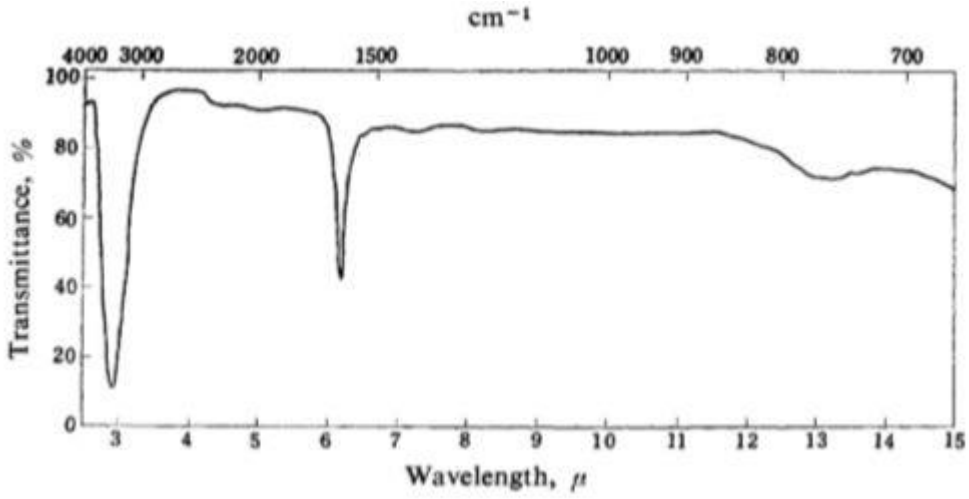

Figure 3.9 Absorption bands of $NiCl_2.6H_2O$ according to Gamo (1961). Peak positions are at 3370 cm^{-1} and 1610 cm^{-1}.

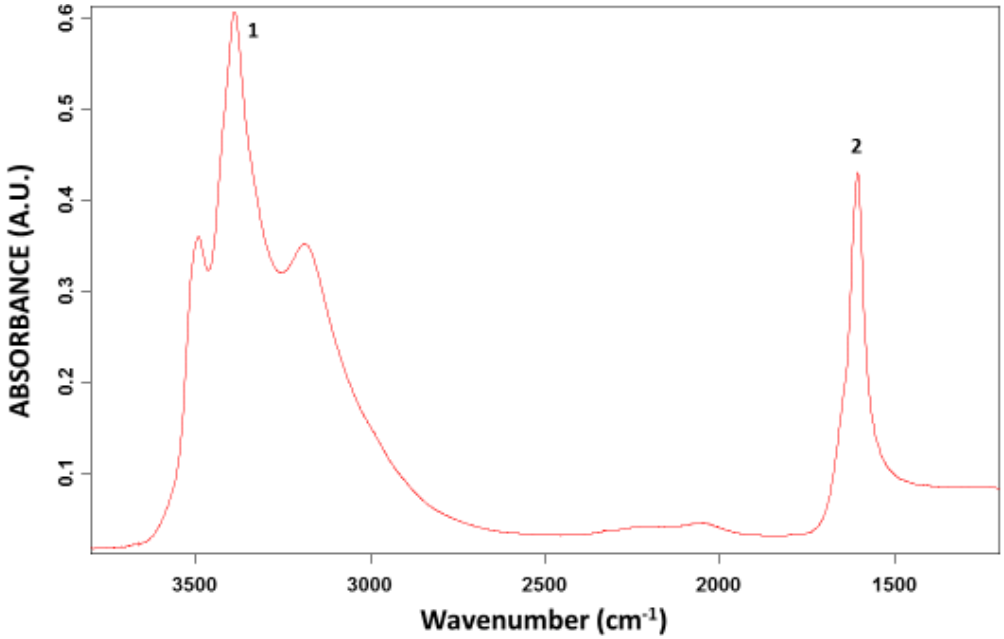

Figure 3.10 Representative ATR-FTIR spectrum of pure $NiCl_2.6H_2O$, non-water-subtracted, at 3800-1200 cm^{-1} spectral region. 1, 2 represent the positions of $NiCl_2$ specific bands.

Table 3.1 Band frequencies for pure, non-water-subtracted $NiCl_2.6H_2O$ and experimental groups. All the controls contained 1mM $NiCl_2$ added spent medium following the completion of culture time.

	Peak Band 1	Peak Band 2
Pure Ni	3406.4 ± 0.001	1618.7 ± 0.078
E.coli control	3403.9	1617.7
E.coli spent medium	3402.8 ± 0.072	1617.1 ± 2.056
FS10 control	3402.7	1617.7
FS10 spent medium	3402.4 ± 0.184	1618.4 ± 0.303
FS42 control	3401.0	1617.7
FS42 spent medium	3402.2 ± 0.371	1618.1 ± 0.617
FS45 control	3401.3	1617.8
FS45 spent medium	3401.9 ± 0.050	1618.8 ± 0.232

According to Gamo (1961), one cannot directly observe $NiCl_2$ itself in FTIR-spectrum, but observes the bands caused by its interactions with crystalline water.

There are other studies, indeed, that has recorded FTIR measurements of several Ni containing compounds. For example, Nowsath Rifaya *et. al.* (2012) studied FTIR spectrum of NiO nanoparticles at the wavenumber interval of 4000 cm^{-1} and 400 cm^{-1} at room temperature; which was similar to ours. They claimed that the presence of a band at wavenumber 418,57 cm^{-1} proved the existence of NiO. Moreover, they argued that the bands at the wavenumbers 1629,90 cm^{-1} and 3431,48 cm^{-1} indicated the existence of water.

Behnoudnia & Dehghani (2014) recorded the FTIR spectra for $NiC_2O_4.2H_2O$, $Ni(OH)_2$, and NiO. Similarly, they used 4000 cm^{-1} and 400 cm^{-1} interval. For the FTIR spectrum for $NiC_2O_4.2H_2O$, they detected a band at 3399 cm^{-1} and claimed that this band corresponds to the presence of an hydroxyl group, which is in a stretching vibration mode. They also recorded a band at 1626 cm^{-1} for the compound $Ni(OH)_2$ and attributed this band to the bending vibration of water molecules.

We obtained two bands which were at around 3406 cm^{-1} and 1618 cm^{-1}, apparently, very close to the values discussed in the articles mentioned above. One can deduce from these results that what we measured was actually traces of water. This is not likely, because we dried our samples with gaseous nitrogen and made the measurements afterwards as mentioned in Materials and Methods. Moreover, these two articles used FTIR without ATR in their studies, so traces of water in their samples were likely to have been seen.

For further clearance, we also measured infrared spectrum of water in ATR mode and subtracted it from our measurement of pure $NiCl_2.6H_2O$. However, water subtraction did not affect the peaks (Figure 3.13).

As it can be seen in Figure 3.13, FTIR bands of water are wide and cover almost entire spectrum in regions where the other molecules are often detected. Subtraction of water bands provides sharpening and detailing of the other peaks otherwise masked by water spectrum. If no difference is observed after water

subtraction, this means that there is no water in the environment and the bands are purely because of the other molecules' presence (Severcan & Haris, 2012; Gurbanov, 2010). So, in our experiment, the bands were purely because of the presence of the Ni compound itself.

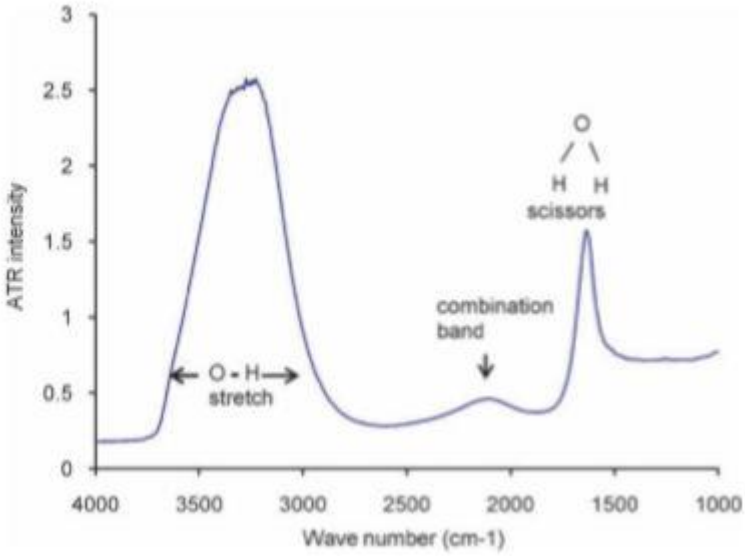

Figure 3.11 ATR-FTIR spectrum of water according to Mojet *et. al.* (2010). The band with the greatest intensity shows the O-H stretch, the band with the lowest intensity shows the combination, and the intermediate band shows the O-H scissors.

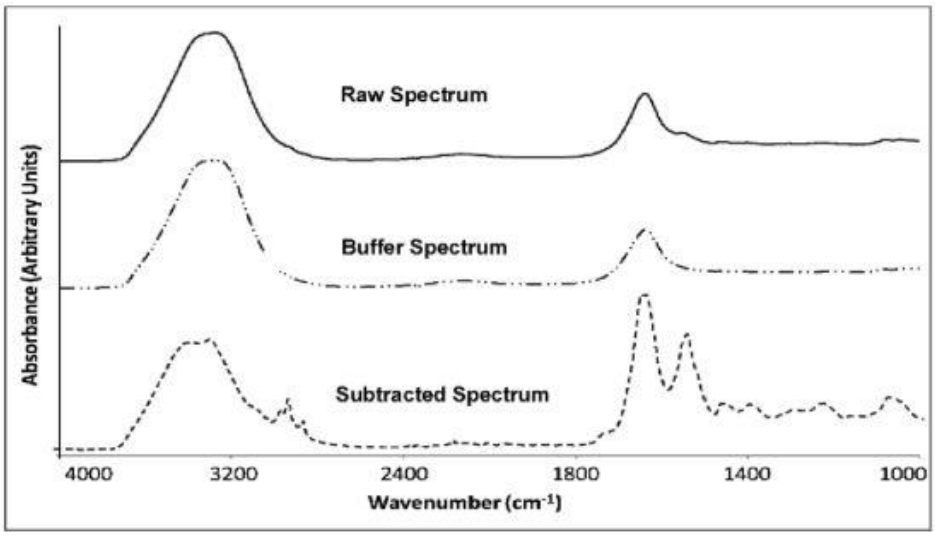

Figure 3.12 Example spectra for before and after water subtraction (Severcan & Haris, 2012).

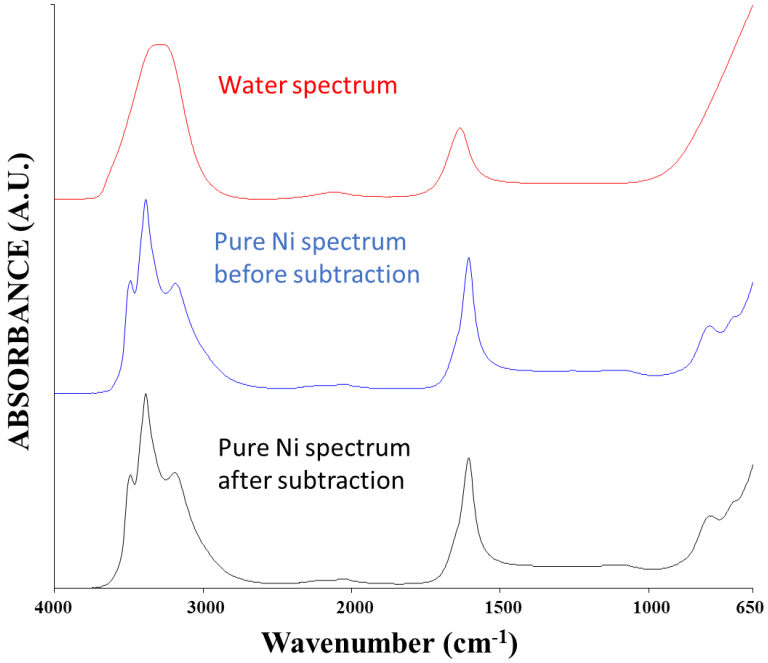

Figure 3.13 Average ATR- FTIR spectrum of pure $NiCl_2.6H_2O$ before and after subtraction in the 4000–650 cm^{-1} frequency range.

To sum up, with ATR-FTIR we can infer the element's presence from its intra- and intermolecular interactions.

3.2.3.2 Measurement of Ni Concentrations in Culture Supernatants as Indicators of Biosorption

Quintelas *et. al.* (2009) studied percent biosorption of several metals in *Escherichia coli* biofilms, including Ni. They found out that biosorption for different amounts of Ni was around 85%. In our study, surprisingly, percent Ni biosorption was nearly 98% for our *Escherichia coli* group as measured by taking a intensity of a band at around 3402 cm^{-1} (see Table 3.2 and Figure 3.14). Quintelas *et. al.* (2009) and Lameiras *et. al.* (2008) also worked on the biosorption kinetics of *E. coli* and found out that there are two steps: The first step is related with external cell surface, and the second step is intracellular accumulation or reactions. Moreover, according to Krishnasmawy & Wilson (2000) and Jasper & Silver (1977), *E. coli* cells had the ability to accumulate Ni by using their magnesium transporters. This might also be true for our *E. coli* strain.

Interestingly, *Microbacterium oxydans* (FS42) showed no biosorption at all. This might be because this type of bacteria has a very efficient efflux pump mechanism for Ni (Nies, 1999). The other *Microbacterium oxydans* species (FS45), however, showed some Ni biosorption. If this species also uses efflux pumps for the removal of Ni, then we can say that it is not as efficient as the efflux pump of FS42.

Becerra-Castro *et. al.* (2011) isolated *Microbacterium oxydans* strain SA62 from *Alyssum serpyllifolium* (a plant species) and found out that this strain had the ability to mobilize Ni. Interestingly, they also found out that there was no noticable relationship between Ni mobilization and Ni resistance. Their strain also survived at 1mM of Ni, similar to our bacteria. Our biosorption results appeared to be consistent with their study regarding to our *Microbacterium oxydans* isolate.

FS10, an unidentified *Microbacterium* species, showed similar biosorption

with *Microbacterium oxydans* FS45. From this result, we infer that these two species may have similar efflux pump mechanisms for Ni.

Table 3.2 Percent biosorption values according to band intensities.

Bands	Biosorption Percentage
E.coli 3402.8 Band	97.8%
E.coli 1617.1 Band	30.6%
FS10 3402.4 Band	6.3%
FS10 1618.4 Band	7.4%
FS42 3402.2 Band	0%
FS42 1618.2 Band	0%
FS45 3401.9 Band	2%
FS45 1618.8 Band	7.7%

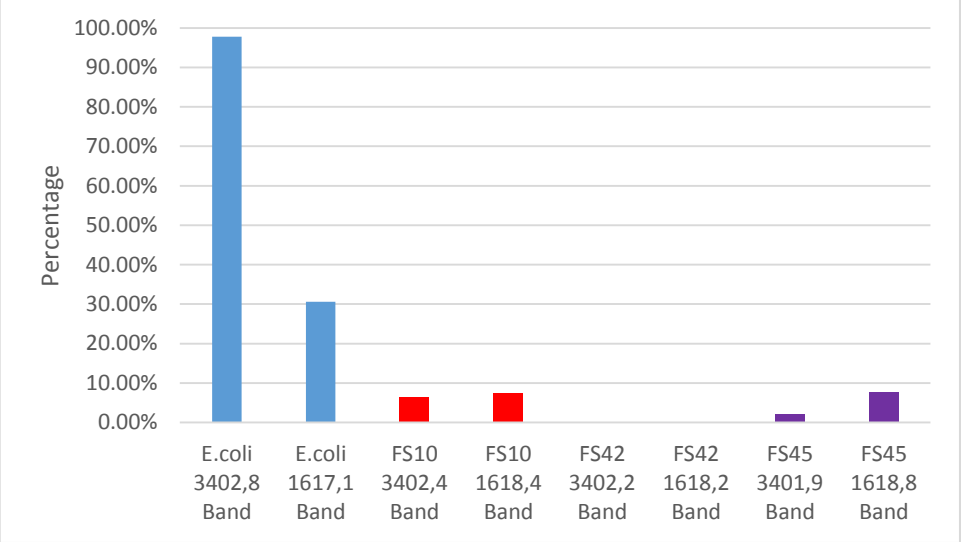

Figure 3.14 Percent biosorption values according to band intensities.

To sum up, we might say that *Escherichia coli* exhibits better Ni accumulation, compared to *Microbacterium* species we have tried in our studies. We were able to show that, rather than UV range optic measurements, infrared range served better for quantitation of Ni in bacterial culture supernatants .

CHAPTER 4

CONCLUSION

- *E. coli* and *Microbacterium* species investigated in this study have the common MIC of 3mM. They all grew in all 1mM Ni-containing media, reproducibly and stably.
- Determination of Ni concentrations in bacterial culture supernatants was not possible on the basis of absorbance data recorded with UV-Vis spectrophotometer, at least, for the concentrations we studied. However, the absorbances provided visible differences in biosorbance: FS42 > FS45 > FS10 > *E. coli*. Highest absorbance corresponded to highest Ni amount in the solution; hence lowest biosorbance.
- Our study showed that in ATR mode FTIR water spectrum subtraction from the Ni bands did not alter the band frequency recordings.
- Calculation of biosorbed Ni was done by measuring Ni in culture supernatant with ATR-FTIR.
- In our study, every bacteria showed different biosorption capacity.
- FS42, FS45, and FS10 were weak Ni accumulators. However, they might be good choices for Ni mobilization, worth checking.
- *Escherichia coli* (ATCC 8739) showed the highest biosorption compared to freshwater isolates.
- ATR-FTIR spectroscopy was proved to be useful in measuring metals in bacterial culture supernatants.

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APPENDIX A

CFU TABLES FOR MIC EXPERIMENTS

Table A.1 CFU table for 3mM Ni.

	Control			
	FS45	FS42	FS10	E. coli
Day 1	3.935.000.000,00	4.506.666.667,00	4.053.333.333,00	2.290.000.000,00
Day 7	345.000.000,00	213.333.333,00	155.000.000,00	560.000.000,00
	Experimental NA			
	FS45	FS42	FS10	E. coli
Day 1	48.333.334,00	86.666.667,00	86.666.667,00	20.000.000,00
Day 7	3.333.334,00	8.333.334,00	23.333.334,00	0,00
	Experimental Ni			
	FS45	FS42	FS10	E. coli
Day 1	0,00	0,00	0,00	0,00
Day 7	0,00	0,00	0,00	0,00

Table A.2 CFU table for 1mM Ni.

	Control			
	FS45	FS42	FS10	E. coli
Day 1	3.981.667.000,00	5.056.666.500,00	4.751.666.500,00	1.700.000.000,00
Day 7	101.666.650,00	136.666.700,00	186.666.650,00	470.000.000,00
	Experimental NA			
	FS45	FS42	FS10	E. coli
Day 1	463.333.300,00	163.333.350,00	658.333.350,00	560.000.000,00
Day 7	680.000.000,00	3.083.333.500,00	2.973.333.500,00	1.230.000.000,00
	Experimental Ni			
	FS45	FS42	FS10	E. coli
Day 1	345.000.000,00	21.666.665,00	50.000.000,00	650.000.000,00
Day 7	881.666.700,00	1.386.666.500,00	4.436.666.500,00	1.320.000.000,00

APPENDIX B

PLATE ASSAY RESULTS FOR BIOSORPTION EXPERIMENTS

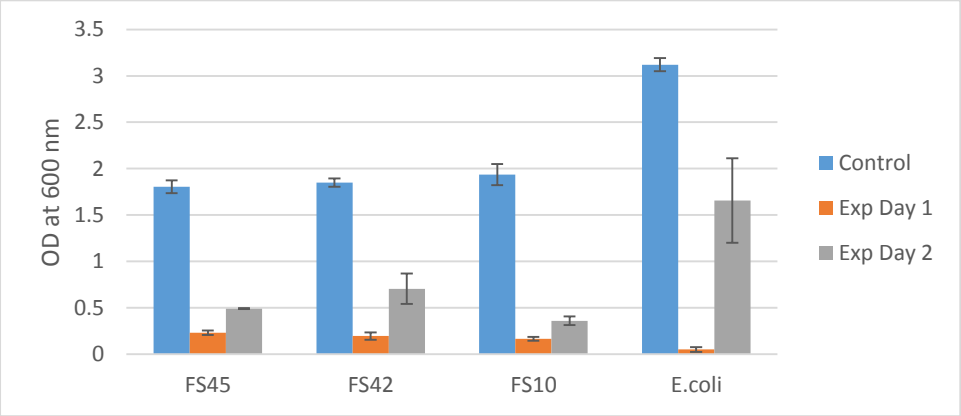

Figure B.1 Cell densities of biosorption experiments at 600 nm.

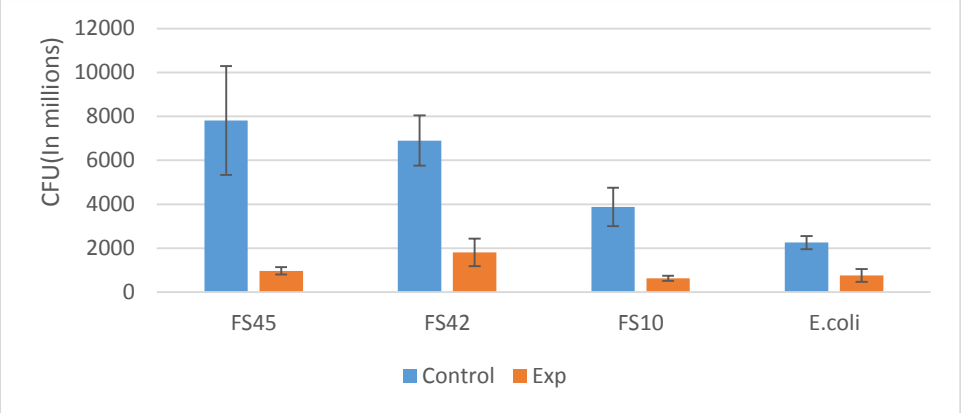

Figure B.2 Plate assay results of biosorption experiments.

APPENDIX C

FTIR GRAPHICS FOR BIOSORPTION EXPERIMENTS

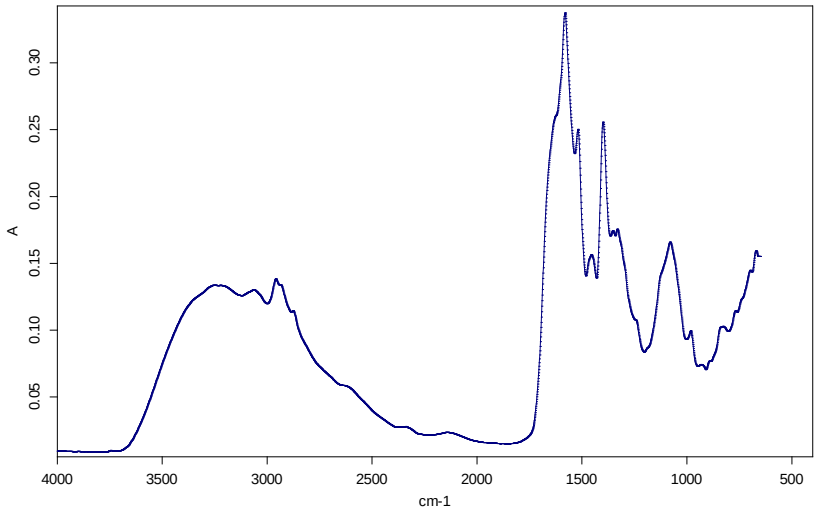

Figure C.1 Absorbance peaks for *Escherichia coli* control supernatant.

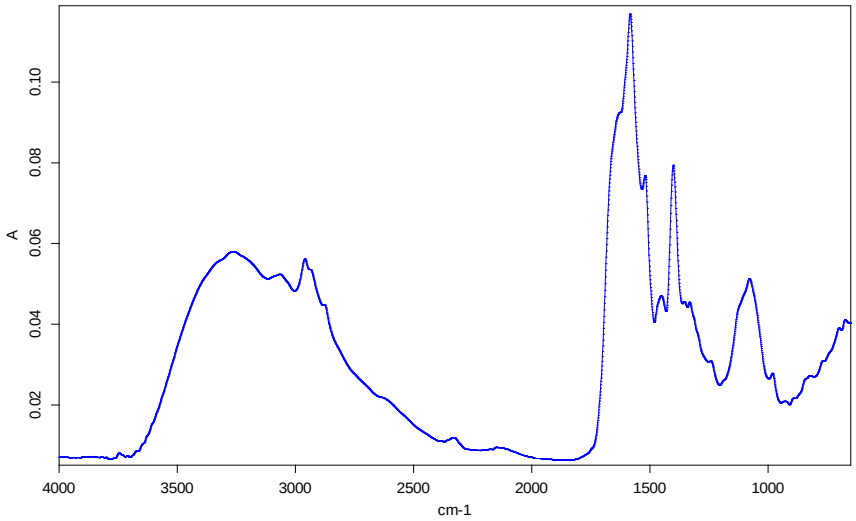

Figure C.2 Absorbance peaks for *Escherichia coli* control supernatant with 1mM Ni.

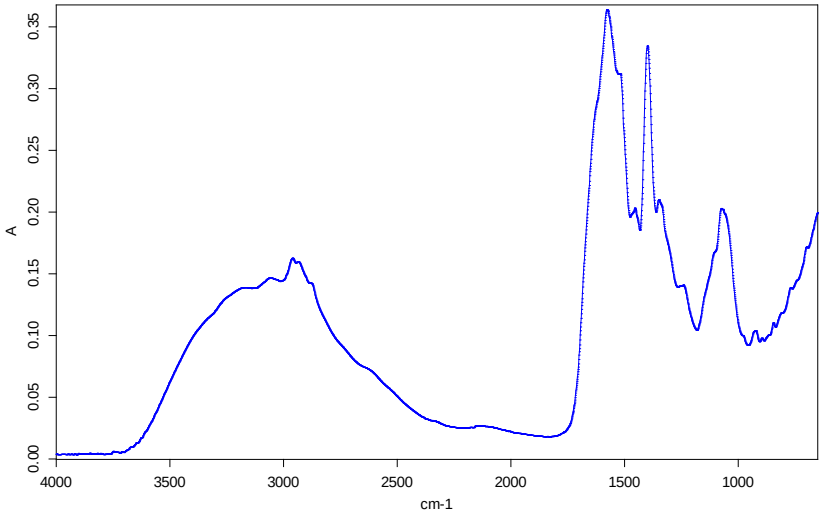

Figure C.3 Absorbance peaks for *Escherichia coli* experimental group A supernatant.

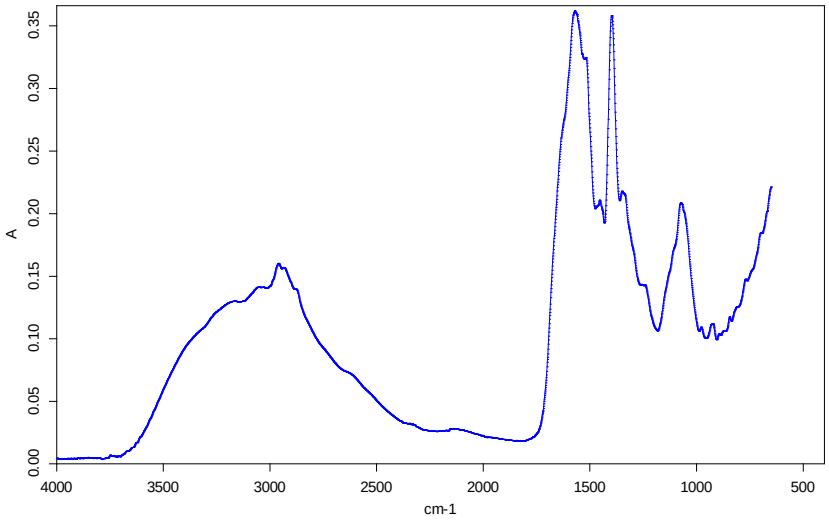

Figure C.4 Absorbance peaks for *Escherichia coli* experimental group B supernatant.

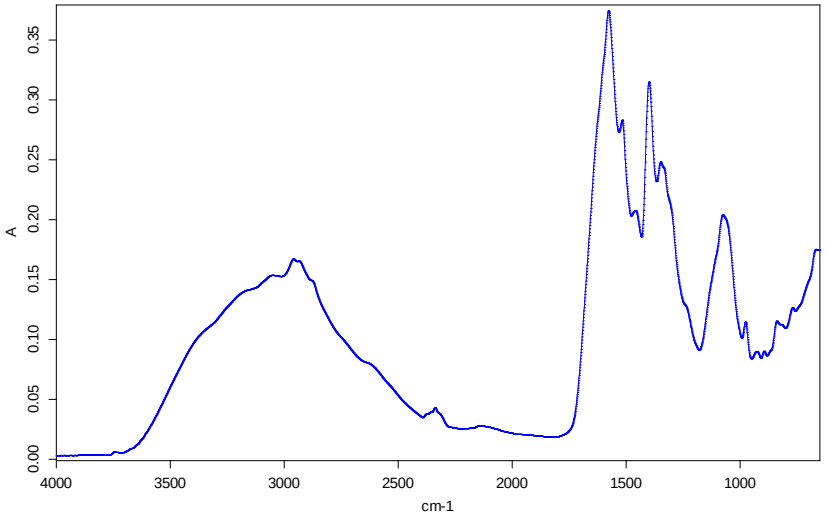

Figure C.5 Absorbance peaks for FS10 *Microbacterium spp.* control supernatant.

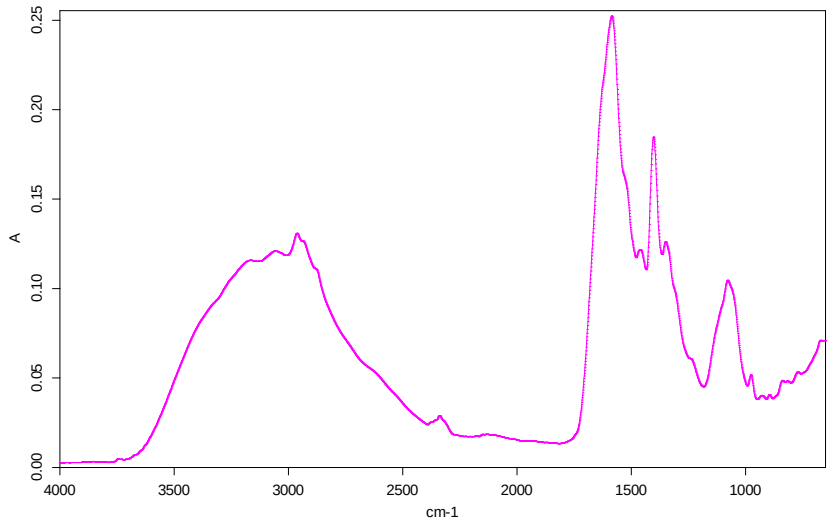

Figure C.6 Absorbance peaks for FS10 *Microbacterium spp.* control supernatant with 1mM Ni.

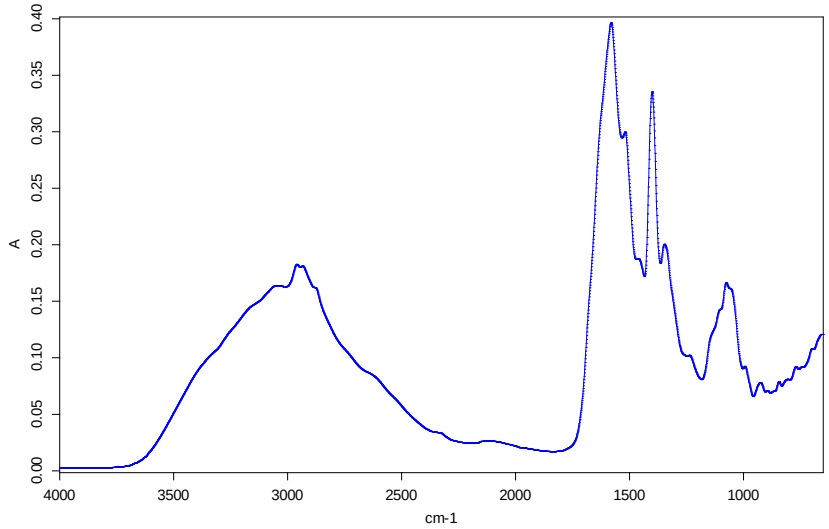

Figure C.7 Absorbance peaks for FS10 *Microbacterium spp.* experimental A supernatant.

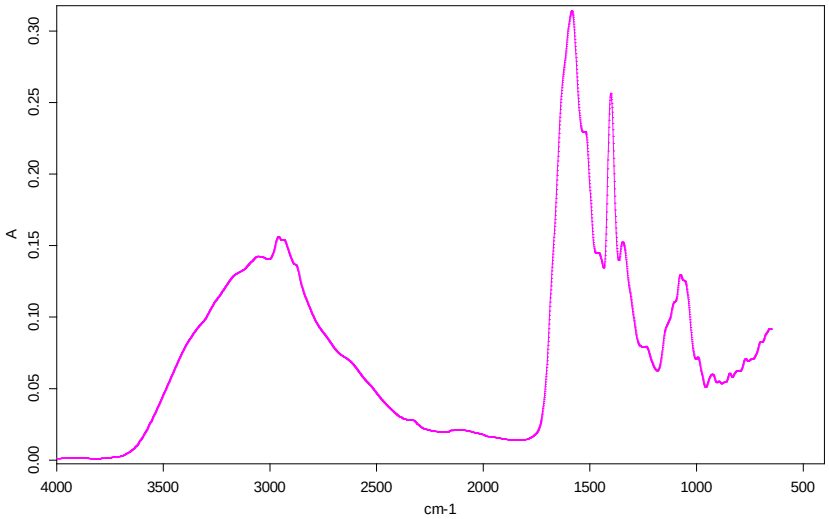

Figure C.8 Absorbance peaks for FS10 *Microbacterium spp.* experimental B supernatant.

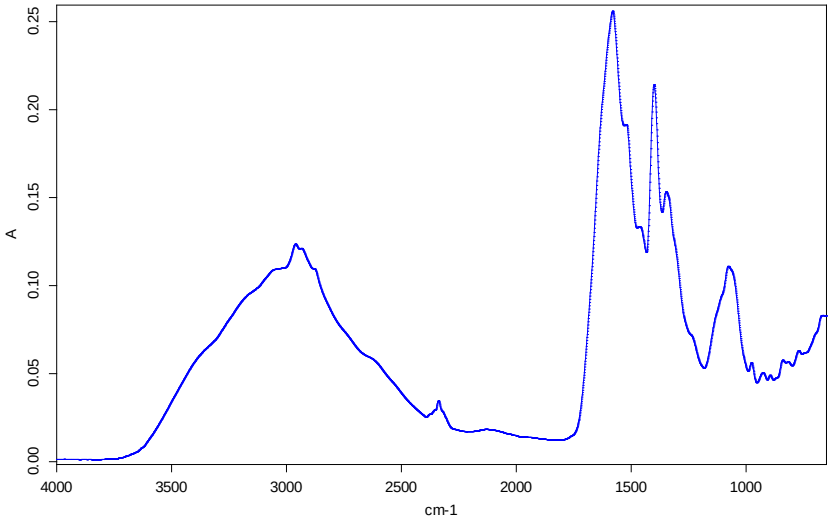

Figure C.9 Absorbance peaks for FS42 *Microbacterium oxydans* control supernatant.

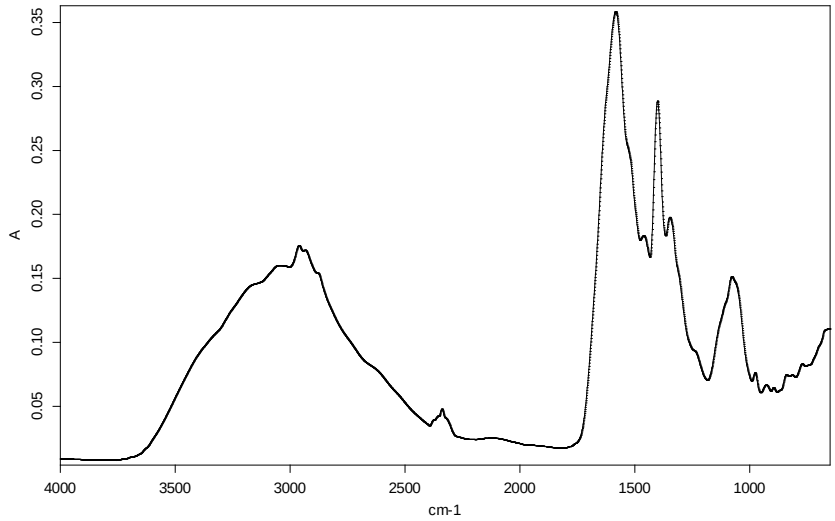

Figure C.10 Absorbance peaks for FS42 *Microbacterium oxydans* control supernatant with 1mM Ni.

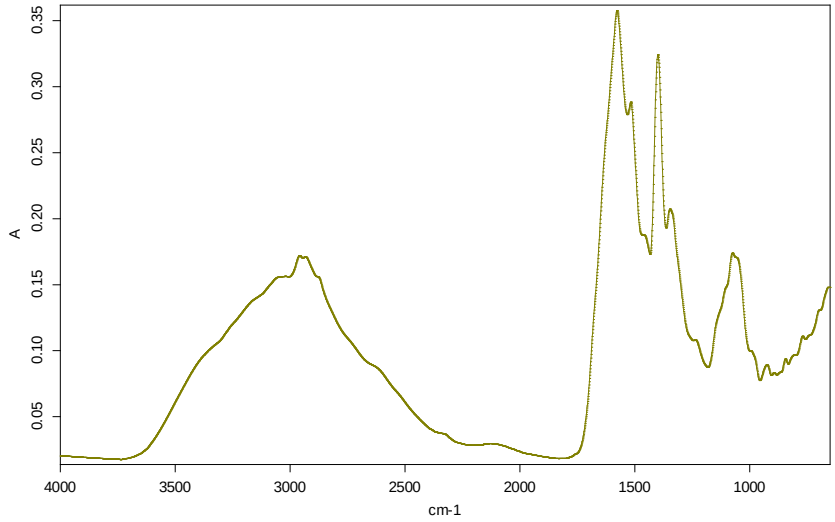

Figure C.11 Absorbance peaks for FS42 *Microbacterium oxydans* experimental A supernatant.

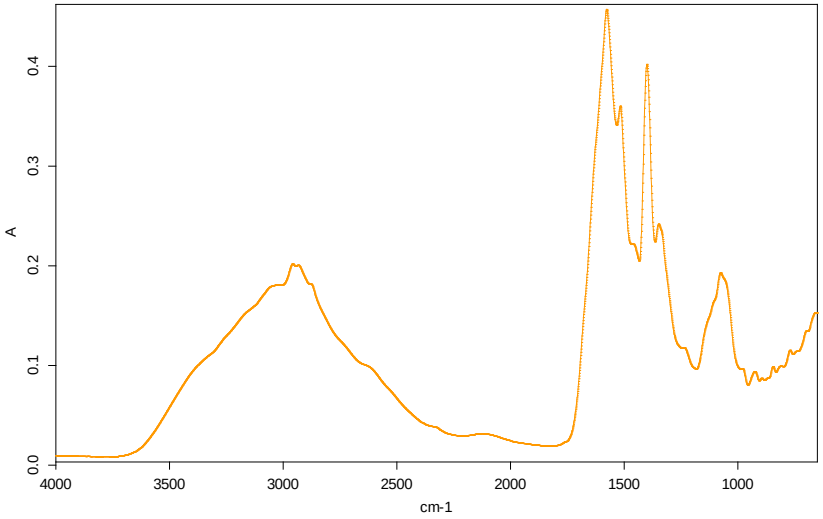

Figure C.12 Absorbance peaks for FS42 *Microbacterium oxydans* experimental B supernatant.

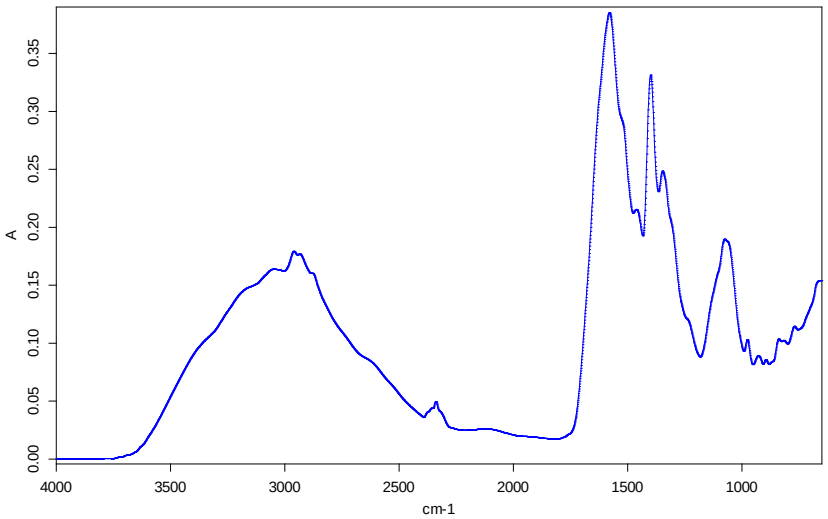

Figure C.13 Absorbance peaks for FS45 *Microbacterium oxydans* control supernatant.

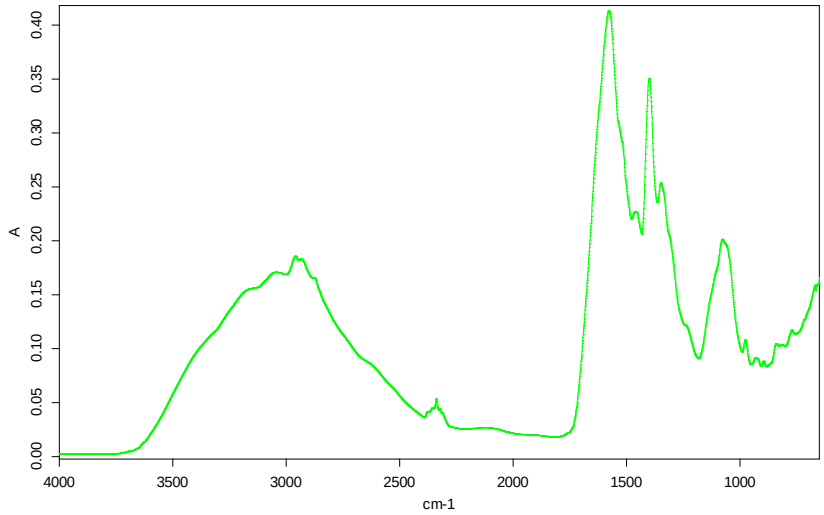

Figure C.14 Absorbance peaks for FS45 *Microbacterium oxydans* control supernatant with 1mM Ni.

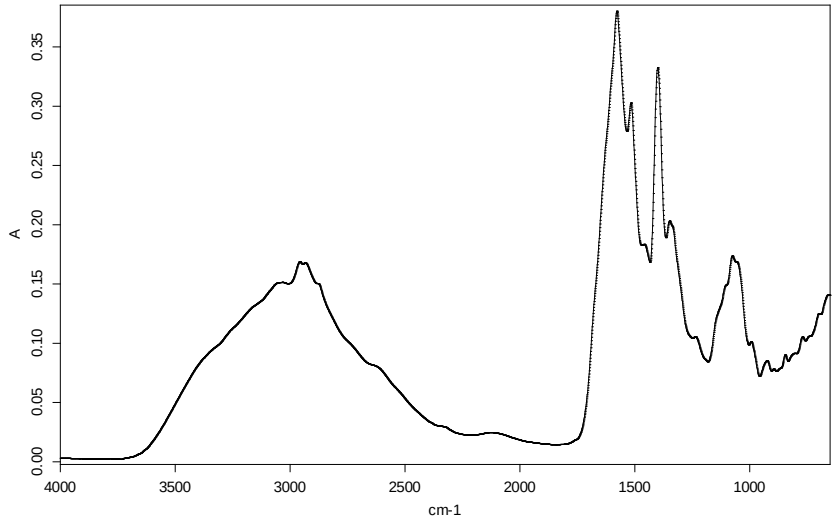

Figure C.15 Absorbance peaks for FS45 *Microbacterium oxydans* experimental A supernatant.

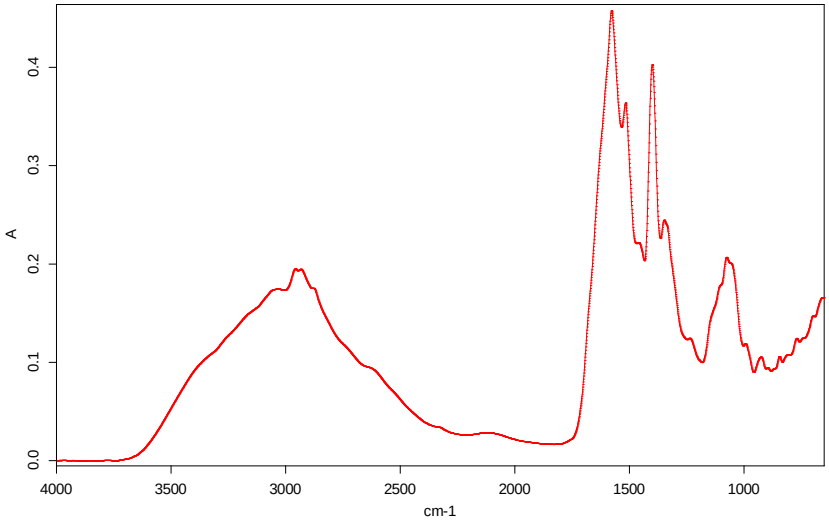

Figure C.16 Absorbance peaks for FS45 *Microbacterium oxydans* experimental B supernatant.