A STUDY ON COBALT ADAPTATION AND MEMORY RETENTION OF FRESHWATER BACTERIA ISOLATES

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A STUDY ON COBALT ADAPTATION AND MEMORY RETENTION OF FRESHWATER BACTERIA ISOLATES

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ABSTRACT

A STUDY ON COBALT ADAPTATION AND MEMORY RETENTION OF FRESHWATER BACTERIA ISOLATES

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The mucus-dwelling bacteria previously isolated from the surface of a freshwater fish species (*Alburnus alburnus* from Lake Mogan, Ankara), were studied to discover their cobalt resistance. The minimum inhibitory concentrations (MIC) were determined for a total of thirty six bacterial isolates. The results of the resistance studies led us to design experiments on adaptation to cobalt and subsequent memory retention. Three selected isolates were exposed to an inhibitory cobalt concentration as a mixed culture and individually. The delayed formation of colonies along with competitive exclusion of one of the isolates in the mixed culture were recorded. The delay for colony formation was followed up for liquid culture conditions. After some of our isolates acclimated to cobalt and started to exhibit constant time of

growth period, it is assumed that they were adapted. We regarded adaptation as a result of memory formation. Next, we did a further study to find out how long this memory could be retained via serial multiple passages in cobalt free medium. We expressed our observations quantitatively by measuring the growth by using spectrophotometer and by performing viable counts. Interestingly, where there was a high CFU, the photometric values were very low. We interpreted the finding such that the presence of cobalt above tolerance limits were causing size reduction in the cells. So that their presence was underestimated by optic devices in visible range. Our study hinted that freshwater bacteria was adapting cobalt in a memory based mechanism and able to retain this memory for some time.

Key Words: fish mucus-dwelling bacteria, freshwater, cobalt, resistance, MIC, adaptation, memory retention, spectrophotometer, viable count, epigenetic

ÖZ

TATLISU BAKTERİ İZOLATLARININ KOBALTA ADAPTASYONU VE HAFIZADA TUTMA SÜRESİ ÜZERİNE BİR ÇALIŞMA

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Bir tatlı su balığı olan *Alburnus alburnus* (gümüş) türünden önceden izole edilmiş olan yüzey mukus izolatlarının kobalta karşı dirençleri çalışılmıştır. Minimum inhibitör konsantrasyonu (MIC) toplam otuz altı izolat için belirlenmiştir. Bu direnç çalışmalarının sonuçları bizi kobalt adaptasyonuna ve sonraki bellek tutma uyumu üzerinde deney tasarımı yapmaya götürmüştür. Seçilen üç izolat, karışık kültür ve tek izolatlı kültür olmak üzere inhibitör kobalt konsantrasyonuna maruz bırakılmıştır. Karışık kültür içinde rekabet ve dışlanma oluştuğu kaydedilmiştir. Koloni oluşumundaki gecikmeler sıvı kültür içinde takip edilmiştir. Bazı izolatlar ortamdaki kobalta alıştırılmış, sabit zamanlı büyümeler elde edilmiştir ve takiben kullanılan konsantrasyona adapte

oldukları varsayılmıştır. Adaptasyon, hafızanın bir sonucu olarak değerlendirilmiştir. İlerleyen çalışmalarda kobaltsız ortamda yapılan bir çok seri pasajdan sonra izolatların, kobaltlı ortamda, kobalta adapte olma durumunu hafızalarında ne kadar süre tuttukları takip edilmiştir. Gözlemlerimizde büyüme kantitatif olarak hem spekrofotometre ile hem de canlı koloni sayımı ile tespit edilmiştir. Koloni sayım sonuçları ile spektrofotometrede, ölçülen sonuçların birbiriyle örtüşmediği belirlenmiştir. Yüksek koloni sayımının olduğu yerde, fotometrik sonuçlar düşük bulunmuştur. Tolerans limitinin üstündeki kobalt konsantrasyonunun hücrelerin boyutunda küçültmeye neden olduğu bu halde optik cihazların algısının altına düştüğü yorumu yapılmıştır. Çalışmamız izolatların bellek tabanlı mekanizmalar ile kobalta adapte olduklarını ve bir süre için bu belleği korumanın mümkün olduğunu işaret etmiştir.

Anahtar Kelimeler: balık mukusunda yaşayan bakteriler, tatlısu, kobalt, direnç, MİK, adaptasyon, hafızada tutma, spektrofotometre, koloni sayımı, epigenetik.

To My Family

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LIST OF ABBREVIATIONS

Cfu	:	Colony forming unit
FS	:	Fish surface
NB	:	Nutrient broth
Co	:	Cobalt
H.M.	:	Heavy metal
EI	:	Epigenetic Inheritence
OD	:	Optical Density
Ab	:	Antibiotic
ND	:	Not Determined

CHAPTER 1

INTRODUCTION

1.1 Heavy Metal Resistance

Metals play an important role in the entire life processes of microorganisms. Some metals are essential and function as catalysts for biochemical reactions, are stabilizers of protein structures and bacterial cell walls, and serve in maintaining osmotic balance (Bruins *et al.*, 1999).

The introduction of heavy metals, in various forms in the environment, can cause significant modifications in the structure and function of microbial communities (Doelman *et al*.,1994). Low concentrations of metals such as cobalt, copper, nickel and zinc are essential for many cellular processes of bacteria. However, higher concentrations of these metals often are cytotoxic (Abou-Shanab, *et al.*, 2007). Moreover some bacteria are able to resist some of the heavy metal even at high toxic levels (Olukoya *et al*.,1997).

Toxicity occurs through the displacement of metals from their native binding sites or through ligand interactions. Bacteria have adapted to metals through a variety of chromosomal, transposon, and plasmid-mediated resistance systems (Bruins *et al.*, 1999).

1.2 Resistance Mechanisms

The environment contaminated with metals may build up resistance systems to almost all toxic metals (Rouch *et al.,*1995). Metal resistant microorganisms can render strategies for detoxification or removal of metals from the environment.There are six known mechanisms for heavy metal resistance:

1. Metal exclusion by permeability barrier
2. Active transport of the metal away from the cell/organism
3. Intracellular sequestration of the metal by protein binding
4. Extracellular sequestration
5. Enzymatic detoxification of the metal to a less toxic form
6. Reduction in metal sensitivity of cellular targets (Bruins *et al.*,1999)

Bacteria developed Metal resistance systems since they exist in an environment that has always contained metals. These resistance systems evolved after prokaryotic life started and appear in nearly all types of bacteria (Ji and Silver, 1995).

1.3 Factors Contributing to Heavy Metal Resistance

Metal resistance and antibiotic resistance are often associated together (Nakahara *et al.*,1977; Harnett and Gyles, 1984; McEntee *et al.,*1986; Schwarz and Hobel, 1989; Belliveau *et al*.,1991). Metal resistance and antibiotic resistance are both transferred among organisms through conjugation or transduction (Harnett and Gyles,

1984). In some conditions antibiotics and metals resistance to is mediated by the same plasmid (Nakahara *et al*.,1977).

Human activities and development of antibotic resistance are both constituted environments of high selection for metals and metal resistance recorded before the use of antibiotics (Ji and Silver, 1995).

1.4 The Records on Heavy Metal Resistance in Freshwater Environments

Akinbowale *et al.* (2007) investigated the heavy metal resistance in motile aeromonads and pseudomonads from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. They determined the relationship between antimicrobial and heavy metal resistance. Since the trout farms were in situated agricultural areas, they interpreted the heavy metal tolerance observed in their study as a result of heavy metal contamination from fertilisers used in agricultural areas. Significant number of reports lend the suggestion which they indicated about the metal contamination in natural environments which have an important role in the maintenance and proliferation of antibiotic resistance.

Bar *et al*.(2007) isolated and characterized a bacterium from river Mula, to examine the response of this bacterium for Co^{+2} and Pb^{+2} and characterize the differential profiling of protein expression by using 2D PAGE and mass spectrometry. Under the effect of these heavy metals, they exposed a differential regulation of proteins to overcome with the metal toxicity by using 2D PAGE.

Olukaya *et al*.(1997) isolated a number of bacteria (228) that can resist toxic heavy metals from Lagos Lagoon sites which had a high number of bacteria were resistant to cobalt.

Ogilvie and Grant, (2007) used pollution induced community tolerance (PICT) as a tool for analysing the effects of chronic metal pollution on estuarine sediment microbial communities. They examined the associated microbial community structure by using terminal restriction fragment length polymorphism (T-RFLP). The individual acclimation and genetic or physiological adaptation to heavy metals and loss of the sensitive species due to long term exposure to a toxicant or mixture of toxicants is the basis of PICT. PICT which is could be helpful establishing if a chemical has modified a community.

Habi and Daba (2009) examined the isolates from a stream to evaluate the squeeze of feacal or metal population on heavy metal and antibiotics resistance along with plasmid incidence. According to their results they observed high resistance to Pb and low resistance to Cd in the stream water polluted with these heavy metals. By comparing the frequency of strains carriying plasmids between freshwater and urban waste water, they determined the higher number of strains carriying plasmids urban waste water than metal and/or low feacal polluted stream water. They did not find out a revelance between plasmid and metal resistance.

Wright *et al.* (2006) reported an additional mechanism in exposure to heavy metals through bacteria to sustain the antibiotic resistance in the environment. As a result of using a culture–independent bacteria that was sampled along metal contamination were more tolerant of antibiotics and metals compared to bacteria from a reference site. This evidence supports the hypothesis that metal contamination directly selects for metal tolerant bacteria while co-selecting for antibiotic tolerant bacteria.

Lee *et al.* (2009) surveyed the heavy metal and antibiotic resistance profile of bacteria isolated from giant freshwater prawn (*Macrobrachium*

rosenbergii) hatchery in Malaysia. They found out the effectiveness of antibiotic and heavy metal resistance relation to control the bacterial diseases in *M. rosenbergii.* The water sources around hatchery displayed an increasing contamination of antibiotics and heavy metals with the rate of high resistance to most of the tested antibiotics and heavy metals.

1.5 Cobalt as a Heavy Metal

Cobalt is found mainly in the Co^{+2} form, Co^{+3} is only stable in complex compounds. Co^{+2} is of medium toxicity, but cobalt dust may cause lung diseases (Nemery *et al.,*1994). Cobalt occurs mainly in the co-factor B12, by catalyzing the C-C, C-O and C-N rearrangements (Kobayashi and Shimizu,1998). To accomplish a variety of metabolic functions, for procaryotes and eucaryotes, cobalt is required as a trace element. Cobalt is an important cofactor in vitamin B12-dependent enzymes and in some other enzymes in animals, yeast, bacteria, Archaea, and plants (Ranquet *et.al*,2007).

Heavy metal toxicity has been described over the years in a range of organisms Altough there is a long-standing recognition of metal toxicity, the mechanism(s) of toxicity is not completely understood.

The toxicities of metals, including iron, copper, cadmium, and nickel, generate radicals by interaction with oxygen.The cobalt in particular has been shown to produce a spectrum of reactive oxygen species in water (Thorgersen and Downs, 2007).

1.6 Action Mechanisms of Cobalt

Microorganisms can have one or a combination of several resistance mechanisms (Bruins *et al.*,1999).

There are differences between chromosomal and plasmid-based metal resistance systems. Essential metal resistance systems are usually chromosome-based and more complex than plasmid systems. Plasmid-encoded systems, on the otherhand, are usually toxic-ion efflux mechanisms. This suggests that ion efflux mechanisms are more likely to be plasmid-borne because they can be quickly mobilized to other organisms and they reduce the gene carrying burden since they are only needed on certain occasions (Silver and Walderhaug,1992).

In most bacterial cells Co^{+2} is quickly accumulated by the CorA system (Smith *et.al*,1993; Snavely et al.1989, 1991). There is not any inducible ATP-driven uptake system was identified that is induced when the cobalt concentration is too low, but a system related to the nickel transporter HoxN from *Ralstonia eutropha* was found in *Rhodococcus rhodochrous* (Komeda *et al.*, 1997). Therefore, HoxN homoloque seems to provide Co^{+2} for the production of non-B12-cobalt protein.

The gram-negative bacteria resistance to cobalt by nodulation cell division transporter that is originated from transenvelope efflux driven resistance. Cobalt resistance appears always to be the by-product of resistance to another heavy metal, (Liesegang *et al.,* 1993; Schmidt and Schlegel,1994) or zinc (Nies *et al.,*1987). Members of the CDF protein family have a significant role in transporting cobalt. The COT1p protein from transports Co^{+2} across a mitochondrial membrane (Conklin *et al.,* 1994) and the ZntA protein brings about Co^{+2} efflux in the gram-positive bacterium *Staphylococcus aureus* (Xiong and Jayaswal, 1998). Hence,

cobalt is taken up by CorA transporters or exceptionally by a HoxN type transporter in eukaryotes and gram-positive bacteria.

In Czc as well as in other transenvelope transporters, one component transports the substrates across the cytoplasmic membrane; this transporter may be a nodulation cell division, an ABC (Table 1.1.) or a MSF protein or protein complex. The Czc system consists of three components CzcA, CzcB and CzcC that could transport Co^{+2}, Zn^{+2}, Cd^{+2} across cytoplasmic membrane, periplasm and outer membrane (Rensing, 1997). The third component, CzcC, may be an integral outer membrane protein or may contact an integral outer membrane protein.

The czc system is an efflux system has a role in removing away Cd^{+2}, Zn^{+2} and Co^{+2} that enter bacteria by simport with Mg^{+2} (Trevors *et al*.,1986). The Czc operon was first isolated in *A. Eutrophus* and has been mapped in the chromosome and on plasmid pMOL28 and pMOL30 (Nies,1992; Rensing,1997).

1.7 Bacterial Adaptation to Heavy Metals and Other Environmental Factors

Evolution of resistant populations sustaining essential microbial processes has led the microbial communities acclimate to toxic compounds. There are a number of mechanisms inspired the acclimation process: 1) induction of specific enzymes not present in populations before exposure to the toxicant; 2) genetic selection for new metabolic abilities; 3) increase in the number of microbes able to transform the toxicant to a less toxic form.

Table 1.1. Protein families important for heavy-metal transport. D. H. 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

In polluted environments each of these mechanisms plays some role in the acclimation of microbial communities to toxic compounds.

Chemical structure and concentration of the toxicant is an important factor in the acclimation process. Altough there was not any detectable acclimation of the community, Spain *et al.,* (1980), observed a minimum threshold concentration of the pollutant.

Among the microbial communities situated in mercury-polluted and control waters were examined by Liebert *et al.,* (1991), to determine the relationship of mercury resistance to the concentration and chemical speciation of mercurial compounds. They enumerated the Hg^{+2} and CH_3Hg^+ resistant bacteria from mercury contaminated ponds using a direct viable counting method. They exposed the relationship between bacterial tolerance and the *in situ* concentrations and chemical speciation of mercurial compounds. As a result, they suggested that mercury acclimation of the microbial community was specific to the *in situ* concentrations of the given mercurial chemical species present. The aquatic microbial communities have a response to mercury that associated with an increase in tolerance levels. Mechanisms that increased the rate of Hg^{+2} reduction generate the tolerance by designating the correlation between developed resistance and quick inducible loss of Hg^{+2}. A specific molecular mechanism was responsible for acclimation of natural communities to Hg^{+2}. Various environmental characteristic features, such as the amount of organic matter or ionic interactions that determine bioavailability of mercurial compounds may affect the ability of the aquatic microbial community to acclimate mercury. Thus the enumeration of mercury-resistant populations would be enable to realise how mercury affects microbial activities and how environmental factors influence mercury toxicity.

Zeng *et al*., (2009), examined the metal tolerance of a bloom-forming cyanobacterium, *Microcystis aeruginosa*, widely found in eutrophied and metal-contaminated freshwater ecosystems. They investigated its acclimation and recovery from cadmium and zinc exposure. Enhanced tolerance in cyanobacteria after metal acclimation has been reported (Turner and Robinson, 1995). Altough *M.aeruginosa* (a cyanobacteria) became very sensitive following 1 day of recovery, it became more tolerant of metal toxicity following acclimation to high Cd or Zn concentrations. The following five days of recovery as tolerance, was comparable between the acclimated-recovered cyanobacterial cells and the control group. The strong ability of these cyanobacteria to acclimate to different environments proposed an acclimation or recovery from the impairment rapidly.

The phenotypic changes of soil microorganisms at the individual level lead to the microorganism acclimation to raised concentrations of heavy metals. Moreover they may adapt to raised metal concentrations through shifts in community structure or through genotypic alteration, as through evolution of plasmids that can encode resistance systems for metal ions. Rusk *et al*., (2004), do not differentiate adaptation and acclimation but for simplicity refer to evidence of a increase in tolerance to metals with increasing exposure time as “adaptation”.

Diaz-Ravin´a and Bååth, (1996), conducted an experiment to evaluate the effect of time of exposure to Zn on microbial tolerance to Zn and found that a tolerant population was established within 2 days following initial application of Zn to the soil. The studies have concluded that metal resistance and metal adaptation capabilities are widespread among different bacterial genera. However the requirement to extract or isolate microorganisms from the soil prior to assessment of adaptation limits the range of soil functions for which adaptation can be tested, as

the extraction efficiency varies between soils, and culturable microbes. Likewise, it is possible that removal of microorganisms from their natural ecological niche may cause a stress response or other disruption to the community structure, both of which could impact on any assessment of changes in metal tolerance.

Pseudomonas fluorescens strain was isolated from oxic marine sediments of the St. Anne Bay (a moderately metal-contaminated site) displaying a high tolerance to metal contamination (Zn, Cu, Cd). Poirier *et al.,* (2008), characterized its physiological and biochemical responses to metal exposures and emphasized the site of origin is relatively polluted. Eventough the strain lives in a hostile environment, it could develop adaptation strategies to protect against heavy metal toxicity in the laboratory. They suggested newly synthesized stress-linked proteins or with elevated synthesis of constitutive proteins caused the the resistance they observed.

Casadesus and D'Ari, (2002), uses "memory" term, to refer the systems whose present state is not entirely determined by present conditions but depends on the path by which the present state has been reached, i.e., on the system's past history. Bacteria and bacteriophage display memory in this sense, since, prokaryotes only respond to their present environment and they can not utilize control over their environment, so their survival depends on adaptability. Hereby, they employ sophisticated mechanisms to adjust physiological processes to their environment, as perceived via environmental signals and appropriate signal transduction systems.

Veening *et. al.,* (2008), explained the question; 'Why do bacteria display phenotypic variation?' , with a strategy called bet-hedging.

Under challenging conditions, the production of offspring with variable phenotypes ensures that at least one offspring will be appropriate (fit) under a given situation. This is a risk-spreading or bet-hedging strategy, because not every offspring will be optimally suited for the future environment. However, the overall fitness of the genotype will increase because some offspring will have the proper adaptation. The production of offspring with variable phenotypes guarantees that at least one offspring will be appropriate under a given situation, with challenging conditions.

1.8 Measurement Techniques for Heavy Metals and Antibiotic Resistance in Bacterial Isolates Reported in Literature

By using viable counts and turbidemetric measurements for a range of bacterial species, for the purpose of evaluating the relationship between maximum specific growth rates can be determined in microbiology (Métris *et al*.,2003). There are two methods for the estimation of specific growth rates from turbidemetric data. One is based on absorbance and the other is based on transmittance measurements. Both of them are compared by viable count methods. Calibration factors are determined to correct the non-linearity of absorbance measurements, and for viable count data. Turbidity measurements are used to compute the growth parameters of bacteria as an alternative to traditional plate counts. Their use has increased and have started to focus on the quantification of the variability of bacterial responses (McMeekin *et al.,* 1993). At higher cell densities, turbidity is proportional to the cell concentration. The range of proportionality depends on the size and shape of the bacteria, which

can in turn be affected by environmental conditions (McMeekin *et al*., 1993; Begot *et al*., 1996).

Some microbiologists correlate the parameters of optical density (OD) curves directly to the viable count specific growth rate and the lag times (Métris *et al*., 2003). They fitted growth curves to OD values and those curves were used to describe the increase in cell concentration. Results were reasonably good under most, but not all, conditions (Dalgaard and Koutsoumanis, 2001). As an alternative, other authors proposed the use of the observed detection time, i.e. the time for a cell population to reach a detectable level of turbidity, instead of the whole turbidity growth curve.

Table 1.2. Different measurement techniques for MIC**,** in the literature sources starting from the year of 1995 up to now.

	Resistance	**Method**	**References**
1	Heavy Metal	Optical Density	Ghosh *et al*., 1997
2	Heavy Metal	Optical Density	Rodrigue *et al*., 2005
3	Heavy Metal	Optical Density	Ramos and Rosato, 1996
4	Heavy Metal	Optical Density	Dixit *et al*., 2004
5	Heavy Metal	Optical Density	Leedjarv *et al*., 2008
6	Heavy Metal	Optical Density	Gutiérrez *et al*., 2010
7	Heavy Metal	Optical Density	Oyetibo *et al*., 2010
8	Heavy Metal	Optical Density	Joris *et al*., 2005
9	Heavy Metal	Optical Density	Caldero´n *et al*., 2006
10	Heavy Metal	Optical Density	Chunpen R., 2006
11	Heavy Metal	Optical Density	Surve and Bagde, 2009
12	Heavy Metal	Optical Density	Mcclain *et al*., 1996
13	Heavy Metal	Optical Density	Brown *et al*., 2006
14	Heavy Metal	Optical Density	Seget *et al*., 2004
15	Heavy Metal	Optical Density	Grover and Sharma, 2006
16	Heavy Metal	Optical Density	Thompson *et al*., 2010
17	Heavy Metal	Optical Density	Paul *et al*., 1995
18	Heavy Metal	Optical Density	Konopka and Zakharova, 1999

19	Heavy Metal	Optical Density	Chatziefthimiou *et al*., 2007
20	Heavy Metal	Optical Density	Hynninen *et al*., 2009
21	Heavy Metal	Optical Density	Tanous *et al*., 2006
22	Heavy Metal	Optical Density	Cai *et al*., 2009
23	Heavy Metal	Optical Density	Mittal and Goel, 2010
24	Heavy Metal	Optical Density	Bourdineaud *et al*., 2006
25	Heavy Metal	Plate assay	Rathgeber *et al*., 2002
26	Heavy Metal	Plate assay	Bahig *et al*., 2008
27	Heavy Metal	Plate assay	Matyar *et al*., 2008
28	Heavy Metal	Plate assay	Lım and Cooksey *et al*., 1993
29	Heavy Metal	Plate assay	Harvey and Gilmour, 2001
30	Heavy Metal	Plate assay	Kaushik *et al*., 2008
31	Heavy Metal	Plate assay	Hayat *et al*., 2002
32	Heavy Metal	Plate assay	Tremaroli *et al*., 2009
33	Heavy Metal	Plate assay	Margaryan *et al*., 2010
34	Heavy Metal	Plate assay	Rossbach *et al*., 2000
35	Heavy Metal	Plate assay	Grass *et al*., 2001
36	Heavy Metal	Plate assay	Deeb, 2009
37	Heavy Metal	Plate assay	Maldonado *et al*., 2010
38	Heavy Metal	Plate assay	He *et al*., 2009
39	Heavy Metal	Plate assay	Ansari and Malik, 2006
40	Heavy Metal	Plate assay	Najiah *et al*., 2009
41	Heavy Metal	Plate assay	Lejon and Ranjard, 2009
42	Heavy Metal	Plate assay	Raja, 2008
43	Heavy Metal	Plate assay	Bell *et al*., 2004
44	Heavy Metal	Plate assay	Rau *et al*., 2009
45	Heavy Metal	Plate assay	Richards *et al*., 2002
46	Heavy Metal	Plate assay	Pike *et al*., 2002
47	Heavy Metal	Plate assay	Malik and Jaiswal, 2000
48	Heavy Metal	Plate assay	J ansen *et al*., 2004
49	Heavy Metal	Plate assay	Shirdam *et al*., 2006
50	Heavy Metal	Broth dilution	Geckil *et al*., 2004
51	Heavy Metal	Broth dilution	Jones *et al*., 1997
52	Heavy Metal	Broth dilution	Westfall *et al*., 2006
53	Heavy Metal	Broth dilution	Pal *et al*., 2007
54	Heavy Metal	Broth dilution	Narita *et al*., 2004
55	Heavy Metal	Microtiter	Teitzel and Parsek, 2003
56	Heavy Metal	Microtiter	Hetzer *et al*., 2006

57	Heavy Metal	Plate assay, Microtiter	Teitzel *et al*., 2006
58	Heavy Metal	Broth dilution, Disc Diffusion	Rossbach *et al*., 2008
1	Antibiotic	Optical Density	Groh *et al*., 2006
2	Antibiotic	Optical Density	Belley *et al*., 2009
3	Antibiotic	Optical Density	Bandow *et al*., 2002
4	Antibiotic	Optical Density	Sieradzki andTomasz, 2005
5	Antibiotic	Plate assay	Sieradzki *et al*., 2003
6	Antibiotic	Plate assay	Castillo *et al*., 1996
7	Antibiotic	Broth dilution	Colmer *et al*., 1998
8	Antibiotic	Broth dilution	Asako *et al*., 1997
9	Antibiotic	Broth dilution	Aires *et al*., 2002
10	Antibiotic	Broth dilution	Ruzauskas *et al*., 2009
11	Antibiotic	Broth dilution	Cerca *et al*., 2005
12	Antibiotic	Broth dilution	Xu and Lee *et al*., 2001
13	Antibiotic	Broth dilution	Bonnefoy *et al*., 1997
14	Antibiotic	Broth dilution	Walberg *et al*., 1996
15	Antibiotic	Broth dilution	Kadurugamuwa and Beveridge, 1996
16	Antibiotic	Broth dilution	Odenholt *et al*., 1998
17	Antibiotic	Broth dilution	Hu *et al*., 2002
18	Antibiotic	Broth dilution	Cendejas *et al*., 2009
19	Antibiotic	Broth dilution	Sieradzki *et al*., 1997
20	Antibiotic	Broth dilution	Moreira *et al*., 1997
21	Antibiotic	Broth dilution	Grare *et al*., 2009
22	Antibiotic	Broth dilution	Langevelde *et al*., 1998
23	Antibiotic	Broth dilution	Devirgiliis *et al*., 2009
24	Antibiotic	Microtiter (OD)	Cunningham *et al*., 2010
25	Antibiotic	Microtiter (OD)	Chait *et al*., 2007
26	Antibiotic	Microtiter (OD)	Stubbings *et al*., 2004
27	Antibiotic	Disc diffusion	Xavier *et al*., 2010
28	Antibiotic	Disc diffusion	Miller *et al*., 2003
29	Antibiotic	Plate assay, Broth dilution	Watanabe and Tadeta *et al*., 1997
30	Antibiotic	Plate assay, Broth dilution	Singh *et al*., 2000
31	Antibiotic	E Test	Walters *et al*., 2002
32	Antibiotic	E Test, Disc diffusion	Gustafsson *et al*., 2003

1	Antibiotic/Heavy Metal	Microtiter / Plate assay	Barchhiesi *et al*., 2008
2	Antibiotic/Heavy Metal	Plate assay / Broth dilution	Lee *et al*., 2009
3	Antibiotic/Heavy Metal	Broth dilution / Plate assay	Filali *et al*., 2000
4	Antibiotic/Heavy Metal	Disc diffusion / Broth dilution	Pal *et al*., 2005
1	Other Agents	Optical Density	Nascimento *et al*., 2000
2	Other Agents	Optical Density	Curylo *et al*., 2008
3	Other Agents	Optical Density	Franke *et al*., 2002
4	Other Agents	Plate assay , Broth dilution	Kotani *et al*., 2005
5	Other Agents	Plate assay	Samant *et al*., 2009
6	Other Agents	Broth dilution	Rehman *et al*., 2010
7	Other Agents	Broth dilution, Disc diffusion	Burton *et al*., 1996
8	Other Agents	Broth dilution	Hilliard *et al*., 1999
9	Other Agents	Broth dilution	Delling *et al*., 1998
10	Other Agents	Broth dilution	Bayer *et al*., 2006
11	Other Agents	Plate assay	Severina *et al*., 1998
12	Other Agents	Plate assay	Heipieper *et al*., 1996
13	Other Agents	E Test, Plate assay	Moore *et al*., 1999
	Other Agents/AB. and H.M.	Plate assay / Broth dilution	Mima *et al*., 2007

Ab ; antibiotic
H.M.;heavy metal

Starting from the year of 1995 up to now, reports (Table 1.2.) which used the spectrophotometric analysis of culture growth and MIC in case of heavy metals 24 prefered this technique out of 58 reports. For antibiotics (antimicrobial agents), 4 prefered this technique alone from 32 reports. Other agents that inhibit growth of bacteria 3 prefered spectrophotometric analysis technique out of 13 reports. Few of the reports relied on two or more techniques.

1.9 Aim of This Study

The aim of this study was to investigate the extent and nature of heavy metal resistance in freshwater bacteria isolates, which were previously shown to exhibit high multiple antibiotic resistance, by experimenting with different cobalt concentrations. During the experiments two interesting phenomena were detected. First was the high adaptation capacity to cobalt and the second was the disagreement between optical density measurements and viable counts in cobalt acclimated cultures. Therefore, we extended our scope to adaptation and memory retention investigations for cobalt. Consequently, appropriate experiments were performed.

CHAPTER 2

MATERIALS AND METHODS

2.1 Equipments

Table 2.1. Equipments and suppliers

Equipments	Suppliers
pH meter	Orion, Turkey
Autoclave	Nüve Turkey
Laminar flow cabinet	Esco,USA
Deep freezer (-70ºC)	Nuaire, USA
Incubator	Binder, Germany
Incubated Shaker	Medline, Korea
Magnetic stirrer	Velp scientifica
Centrifuge	Sigma, Germany
Vortex	Nüve,Turkey
UV-Vis Spectrophotometer	Shimadzu UV Mini 1240 , Japan
Class II Biological Safety Cabinet	ESCO ,USA
Incubated Shaker	Medline SI 600R,Korea

2.2 Bacteria Isolates

Bacteria isolates used in this thesis were isolated by the former M.Sc. student Tuğba Özaktaş and presented in her thesis entitled “Multiple Antibiotic Resistance Of Surface Mucus Dwelling Bacterial Populations In Freshwater Fish, 2007”. The details as follows;

Surface mucus of a freshwater fish, *Alburnus alburnus* (bleak), caught from Lake Mogan, situated in south of Ankara, was collected in

different seasons. A total of sixty bacterial isolates obtained. The mucus-dwelling bacteria were tested for resistance against ampicillin, kanamycin, streptomycin, and chloramphenicol. The resistance levels of isolates were determined by tube dilution method. About 90% of the isolates were resistant to chloramphenicol, about 84% to kanamycin, about 88% to streptomycin and about 98% to ampicillin. They found no direct relationship between the presence of plasmids and multiple antibiotic resistance. Their study indicated that multiple antibiotic resistance at high levels was among the current phenotypes of the fish mucus-dwelling bacterial populations in Lake Mogan.

2.3 Growth Conditions of Isolates

All bacterial isolates used in this study, were grown under aerobic conditions and incubated at 28˚C at 200 rpm according to Özaktaş (2007).

2.4 Media

Nutrient broth (peptone from meat 5 g., meat extract 3 g. per liter, Merck) and nutrient agar were used as growth medium for all applications. The stock solutions of cobalt salt $CoCl_2 \cdot 6H_2O$ (Sigma-Aldrich) were prepared by dissolving in H_2O. The stock solutions were sterilized by filtering through 0.22-micron filters (Pall, USA). The solutions were stored in the dark. Cobalt solution was added to the medium (broth or agar) after autoclaving and cooling down to 45-50˚C from stock solutions in laminar flow chamber.

2.5 Spectrophotometric Growth Measurements of Freshwater Isolates in the Presence of Cobalt

The 36 freshwater bacteria isolates were studied to determine their Minimum Inhibitory Concentration (MIC) values by using spectrophotometer in visible range. Altough sixty bacterial isolates were obtained by Özaktaş (2007), in this study we used 36 of them since they could not be revived, and also some of the isolates formed extensive mucus which did not permit an accurate measurement with spectrophotometer. Fresh cultures were prepared from frozen stocks and inoculated in 5 ml nutrient broth. They were incubated at 28˚C at 200 rpm in rotary shaker incubator (Medline SI 600R, Korea). All 36 isolates had their corresponding controls without cobalt. Photometric measurements were initiated when the full turbidity (loss of total transparance) was visually observed in the controls.

Different concentrations of cobalt solutions were included in to 5 ml nutrient broth inoculated with 25 µl fresh culture. The 5 ml cultures were incubated at 200 rpm at 28 ˚C the control (culture without cobalt) until full turbidity occurred (as described above). Then by using spectrophotometer (Shimadzu UV Mini 1240, Japan) at 600 nm, we recorded absorbance values for all tested isolates grown at different final concentrations of cobalt. The blanks for spectrophotometric readings consisted of 5ml nutrient broth and 25 µl of fresh culture added immediately before reading, also appropriate amount of cobalt solution. This blank represented all the conditions before the experiment started. We diluted the cultures 1:1 proportion with distilled water where the absorbance value was 2 or above. The values were recorded as MIC.

2.6 Growth of Mix Culture in the Presence of Cobalt

For mix culture experiments, three isolates (FS 48, FS 20, FS 2) were selected according to their colony pigmentation, since it would be easy to enumerate different species on agar surfaces. They formed orange, yellow, and white colonies, respectively.

Cobalt concentration to asses the growth of mix cultures was greater than the individual MIC values of the three tested isolates. The tried concentration (75 µg/ml) was inhibitory for all three isolates. Minimum inhibitory concentrations for the three isolates were 35 µg/ml, 60 µg/ml, 50 µg/ml, for FS 48, FS 20, FS 2 respectively. A mixture of these three isolates were prepared with equal volumes of inocula from each of the corresponding fresh cultures. The 10 µl of each fresh culture were transferred in 5 ml of nutrient broth and control group was prepared without cobalt solution. Totally each tube contained 30 µl of inocula. The culture tubes were incubated at 200 rpm at 28 ˚C.

Each isolates were studied in triplicates named as; FS 2 A,B,C ; FS 20 A,B,C ; FS 48 A,B,C and MIX A,B,C .

Isolates observed visually twice a day at the same time. When the cultures lost their transparency and become fully opaque visually, plate assays were done for each isolates and the mix cultures. Bacteria grown with cobalt were spread on cobalt added nutrient agar plates where control groups were inoculated on nutrient agar paltes without cobalt.

Followed by the viable counts, the liquid culture studies were initiated. The experiment repeated again by changing the amount of inoculum transferred to nutrient broths. The 75 µg /ml cobalt solution and 90 µl of a single isolate was transferred to 5 ml of nutrient broth. This time 30 µl volumes from each of the three isolates were introduced

into the nutrient broth to obtain mix culture. So that the total volume of inocula was 90 µl.

First set of mixed culture experiments hinted us that after an extended period of time some of the bacteria were growing in the presence of cobalt. In order to see the time span needed for the isolates to grow in the presence of cobalt we set up liquid cultures. Growth time was recorded when the visual turbidity was similar to that of controls (without cobalt).

2.7 Experiments on Cobalt Acclimation

Followed by establishing the cultures that grow in cobalt after a period of time, we started the acclimation studies. We kept passaging the cultures in the presence of cobalt. The passages were done upon seeing a comparable visual growth with the control. The growth was checked twice daily to see the turbidity of cultures with cobalt, in comparison to controls.

In our experiments we acclimated the bacteria isolates to cobalt through multiple passages, in an attempt to find a constant growth duration for each isolate we tried. For example, a given isolate in a trial should grow in constant time which means that if an isolate adapted to cobalt by growing in two days, next passages of this isolate should also be matured in 2 days constantly, without major fluctuations. Here we assumed that if the two day growth time does not change after 7 passages the isolate is acclimated to that concentration of cobalt. This was our starting assumption that we continued on that.

The isolates did not attain constant growth time in the presence of cobalt and the isolates did not show batch to batch consistancy were eliminated from the memory experiments described below.

2.8 Memory Retention Experiments

We accepted the determined fixed growth time that our isolates had adapted to in the cobalt added media, but we wondered how long it would take them to loose adaptation to cobalt. The cobalt acclimated cultures were passaged every day as one subculture without cobalt. Then after each passage to cobalt free medium upon growth the inocula from the culture was tranferred to cobalt containing medium again to check if there was a shift in formerly tabulated growth time.

Some of the subcultures did not grow. Therefore, the memory experiments were conducted with the growing subcultures only.

From three subcultures that adapted to cobalt containing media by growing in a fixed time, one was arbitrarily choosen for further passsaging in to nutrient broth (cobalt free) continuously. While passaging the isolates from adapted conditions to nutrient broths, we did not lose our adapted cultures, we continued to passage them into cobalt containing media, since we needed initially adapted cultures for comparison.

In memory retention experiments one cobalt adapted culture from each subcultıure was taken and 90 µl of this culture was inoculated to cobalt free nutrient broth daily. When we saw full growth as described earlier, again we inoculated 90 µl of culture to cobalt free nutrient broth.

We also compared the OD's and plate counts in adapted cultures and cultures serially passaged into cobalt free media.

Growth measurements were taken by changing the amount of the inoculum as 25 µl in order to see the effect of inoculum size.

In another experiment, we decided to wash the culture before the experiment. Here we wanted to ensure that there was not any cobalt remnant that could affect the timespan for memory retention. We washed the 1 ml of culture with phosphate buffer solution three times and then 90 µl of cobalt free culture was inoculated to cobalt free nutrient broth. When the cultures became fully opaque again we inoculated 90 µl to cobalt free nutrient broth. While passaging the isolates from adapted conditions to nutrient broths, we did not loose our initial adapted cultures, we continued to passage them, as mentioned earlier.

2.9 Construction of Growth Curves

Next, we wanted to compare the growth curves for (FS 2, FS 20). The cultures were sampled at 1 hour intervals. Samples were diluted and plated for viable count and their OD at 600 nm were also recorded. The colonies were counted after incubation at 28 ˚C.

Growth curves were plotted for three conditions for spectrophotometric recording;

1) Control group never exposed to cobalt,

2) Culture which adapted to grow in cobalt in memory experiment,

3) Culture which was adapted to cobalt and then inoculated to cobalt free nutrient broth (retention study),

Growth curves were plotted for three conditions to obtain viable counts;

1) Control group never exposed to cobalt, was inoculated to nutrient agar.

2) Culture which adapted to grow in cobalt in memory experiment was inoculated to cobalt added nutrient agar and also inoculated to cobalt free nutrient agar

3) Culture which was adapted to cobalt and then inoculated to cobalt free nutrient broth once, was inoculated to nutrient agar

The 100 µl of culture was transferred to nutrient agar plate and spreaded with glass spreaders for viable counting.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Spectrophotometric Growth Measurements of Freshwater Isolates in the Presence of Cobalt

The 36 freshwater bacteria isolates were studied to determine their Minimum Inhibitory Concentration (MIC) values for cobalt using spectrophotometer at 600 nm. We recorded the OD's of all tested isolates for different final concentrations of cobalt.

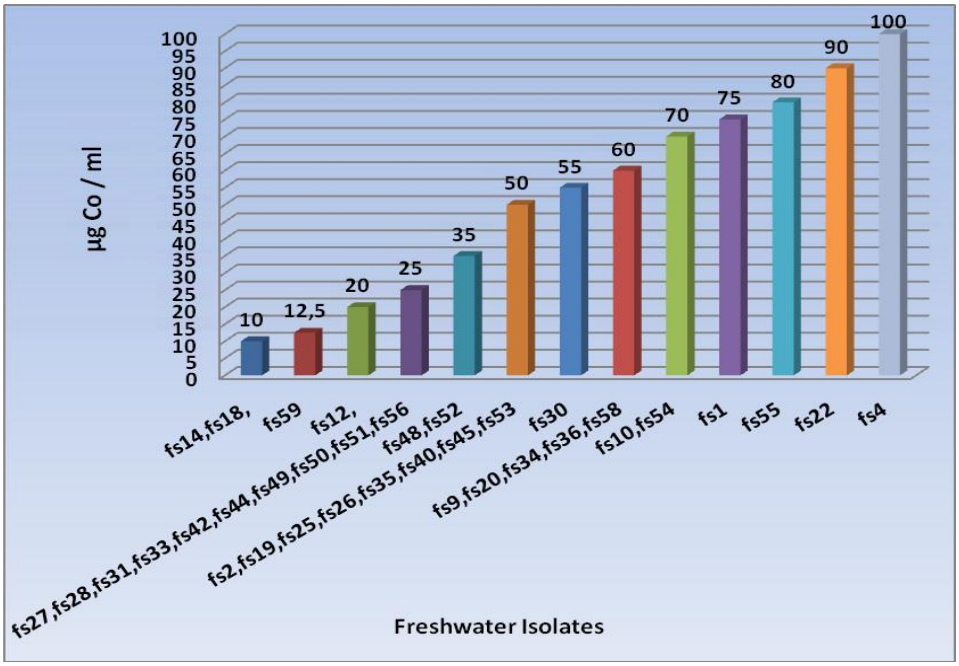


Figure 3.1. The results of Minimum inhibitory *concentration* (MIC) of cobalt on freshwater bacteria isolates expressed as µg/ml.

According to the MIC values we can say that great majority of the isolates can cope with cobalt between 25 and 60 µg/ml final concentrations (Figure 3.1.). Similarly, Tong and Sadowsky (1994), studied the metal resistance in rhizobia for levels of resistance to several heavy metals including cobalt and found that the MIC of *Rhizobium* strains for cobalt was 40 µg/ml for six isolats, 20 µg/ml for two isolates.

In another record dealing with MIC's of heavy metals, *Bacillus* exhibited high MIC values for different metals. The order of toxicity of the metals to the bacterium was Cd=Co>Cu>Ni>Zn>Mn in solid media. The effects of increasing metal concentrations to the growth rate were determined in liquid cultures in order to obtain precise patterns of resistance (Yılmaz, 2003). Additionally, In the study Co^{+2} has found to have a moderate inhibition capacity in *E. coli* (Nies, 1999).

Thus, in our further experiments regarding adaptation and memory retention, we chose the 75 µg/ml as final concentration.

3.2 Growth of Mix Culture in the Presence of Cobalt

Followed by the determination of MIC values for individual isolates we wanted to see if mix cultures could grow at higher cobalt concentrations above their individual tolerance limits. We adjusted the final concentration of cobalt as 75 µg/ml, because this concentration was inhibitory to our three choosen isolates (FS 48, FS 20, FS 2). The isolates (FS 48, FS 20, FS 2) were selected according to their colony pigmentation since it would be easy to detect the different colonies on agar surfaces.

Colonies formed in one day in control groups (cobalt free nutrient agar) of MIX A,B,C and individual isolates. However, we could not

observe colonies in cobalt added agar plates at the same time with control groups. The colonies in cobalt added plates appeared by end of 3 days of incubation.

Incubation time took longer for the colonies of MIX A,B,C and individual FS 48, FS 20, FS 2 isolates since the cobalt concentration was greater than the MIC's of each isolate determined for liquid cultures previously.

As seen in Figure 3.2. and Figure 3.3., MIX cultures inoculated on agar plates with cobalt, no orange colonies of FS 48 were encountered. This situation may be explained through the synergism-antagonism phenomenon.

Apparently, FS 48 could not cope with the given cobalt concentration in the presence of other two isolates (FS 20 and FS 2). Though it survived at the same cobalt concentration alone forming colonies much later than the others. This indicated that FS 48 was competitively excluded by FS 20 and FS 2 when cobalt present. However FS 48 continued to exist in cobalt-free medium in mix culture with a smaller population size.

The competition occurs when two populations use the same resource, whether space or a limiting nutrient. Competitive exclusion precludes two populations from occupying exactly the same niche, because one will win the competition and the other will be eliminated (Atlas and Bartha,1997).

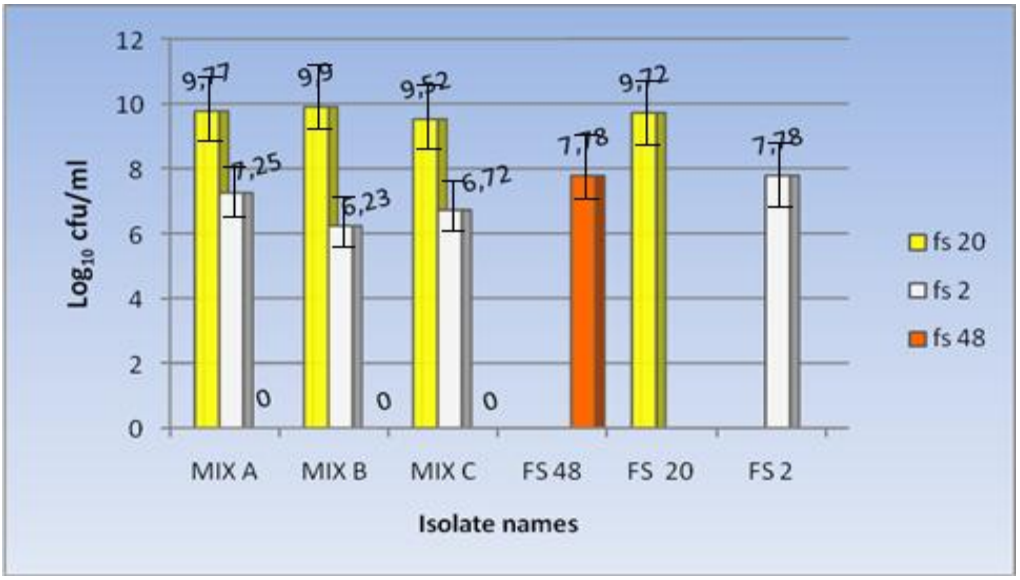


Figure3.2. Viable counts of mixed cultures A,B and C and individual FS 48, FS 20, FS 2 cultures. Counts obtained for triplicate individual culture avaraged. All cultures were grown with cobalt (75 µg/ml).

In standart MIC studies the growth is compared with the control at the same time. If there is full growth in control and no growth in experimental groups the result is assigned as negative.

In our study, we realized that the growth was occuring several days later compared to controls in cobalt containing plates. This led us to reavaluate the results of our standart MIC measurements. By concentrating on the isolates that we used in mixed culture studies we set up a new experiment to see how long would it take for bacteria to grow in above limit cobalt concentrations in liquid medium.

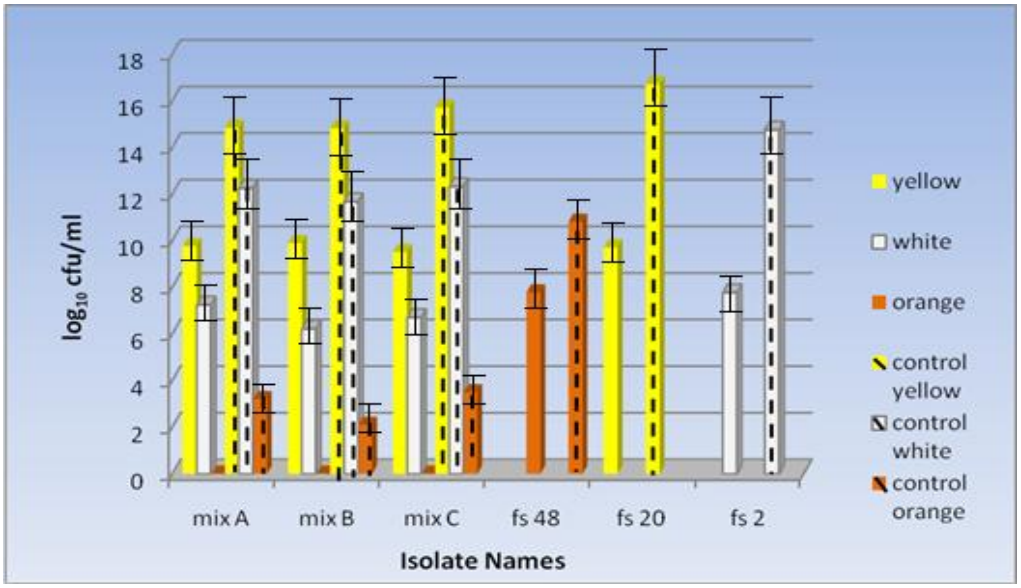


Figure3.3. Comparison between viable counts in control and in cobalt presence (75 µg/ml). MIX A,B,C (triplicate), individual FS 48, FS 20, FS 2 (avarage of triplicates).

3.3 Adaptation and Memory Retention of The Cultures

According to the results presented in Table 3.1. (first column) growth time was recorded when the full growth occured in cobalt containing broth. The recordings were done by visually comparing the growth in cobalt containing tubes with control group without cobalt. The turbidity that equal or close to the control (cobalt-free medium), occured in different timespans for isolates, even variation occurred among triplicate cultures. FS 48B showed growth 16 days later followed by inoculation in 75 µg/ml final cobalt concentration, initially in spectrophotometric measurements its MIC value was found to be 35 µg/ml.

Eventough each of the triplicate subcultures were taken from their own stock culture, the growth for the individual subcultures was not

the same. Besides FS 2A and FS 48C replicates had no visual sign of growth and we eliminated them and not included in the next set of experiments.

In our experiments we acclimated the bacteria isolates to cobalt through multiple passages in the cobalt containing media. We tried to bring the cultures to a state so that they should grow in constant time. The results were presented in the second column of Table 3.1. Afterwards, we passaged the isolates 7 more times, and after seeing continuous stable growth pattern, the cultures were assumed to be acclimated.

Some of our isolates adapted to grow in the presence of cobalt as it was shown under “Specific Adaptation Time Period”. FS 20 A,B,C adapted to grow in cobalt added liquid media, in two days and FS 2 B,C adapted to grow in cobalt in 1 day. FS 48 A,B,C showed inconsistent growth time in passages that it did not have a specific adaptation time as in other two isolates. Thus, we eliminated this isolate from our experiments. Moreover, FS 48 did not reach opaque turbidity status visually close to its control (cobalt free medium).

Then the question we raised was how long they can retain this adaptation in the absence of cobalt. To generate an answer we started to make passages of cobalt acclimated cultures in cobalt free media.

After each passage to cobalt free media, a subculture was inoculated into cobalt added media to find out if adaptation time would change. FS 20A adapted the cobalt presence by growing in two days and after 20 pasages the full growth occur in 4 days. In the case FS 20B the specific adaptation time of 2 days after 12 passages increased to 7 days. Figure 3.8. and Figure 3.10. summerizes the observations.

Moreover after each passage to cobalt free media, a subculture was inoculated into Co added media to find out if adaptation time would change. FS 2B adapted the cobalt presence by growing in one day and after 7 pasages the full growth occur in 2 days. In the case FS 2C the specific adaptation time of 1 day after 6 passages increased to 2 days. Results are shown as ‘Retention Time’ in Table 3.1.

Table 3.1. Growth of selected isolates upon inoculation into Co containing media. Cobalt concentration was above predetermined MIC values. Number of passages where constant growth time span observed. Specific adaptation time recorded after additional 7 passages. Loss of specific adaptation time followed by serial passages in Co-free media.

Isolate name*	First full growth after inoculation (+Co)	Number of passages after which constant growth time obtained	Specific Adaptation Time Period	Number of passages to adapt +Co in a specific adaptation time	Number of passages that we obtained a constant retention time	Retention Time
FS20 A	8 days later	6 passages	2 days	7	20 passages	4 days
FS20 B	9 days later	9 passages	2 days	10	12 passages	7 days
FS20 C	9 days later	7 passages	2 days	8	11 passages	4 days
FS2 B	10 day later	9passages	1 days	6	7 passages	2 days
FS2 C	10 days later	8 passages	1days	6	6 passages	2 days

*In the memory retention experiments FS 2A, MIX A,B,C and FS 48 A,B,C were not studied since some of them did not show any growth or we did not get and constant growth time span for adaptaion experiments.

Figure3.4. shows the viable counts (CFU) for FS 20B for the control, cobalt adapted and retention study cultures. The CFU's for the retention study cultures were greater than control and cobalt adapted cultures in seven day culture.

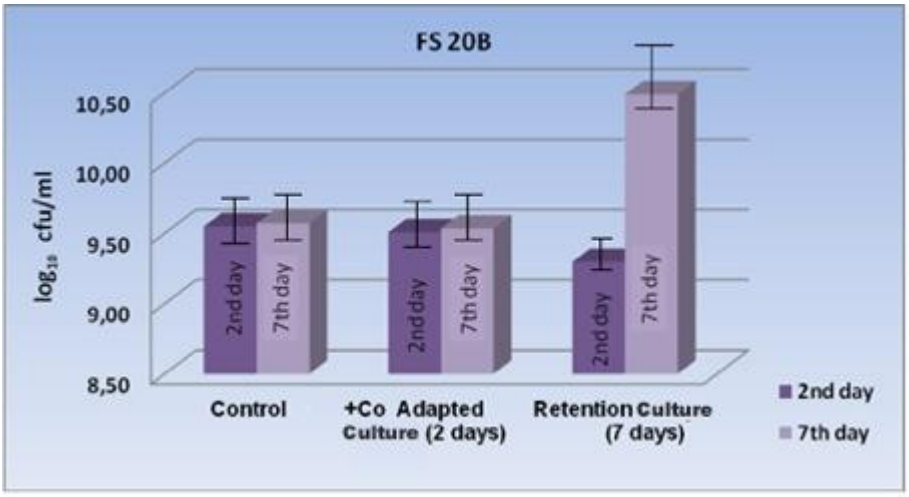


Figure3.4. FS 20B viable counts for cobalt adapted (2days) culture, Retention culture (7days) and control culture.

Figure 3.5. shows the optical density at 600 nm for the control, cobalt adapted and retention cultures. Eventough retention study cultures had the biggest viable counts, the OD values were the smallest compared to control in seventh day. This may be explained by size reduction of bacteria.

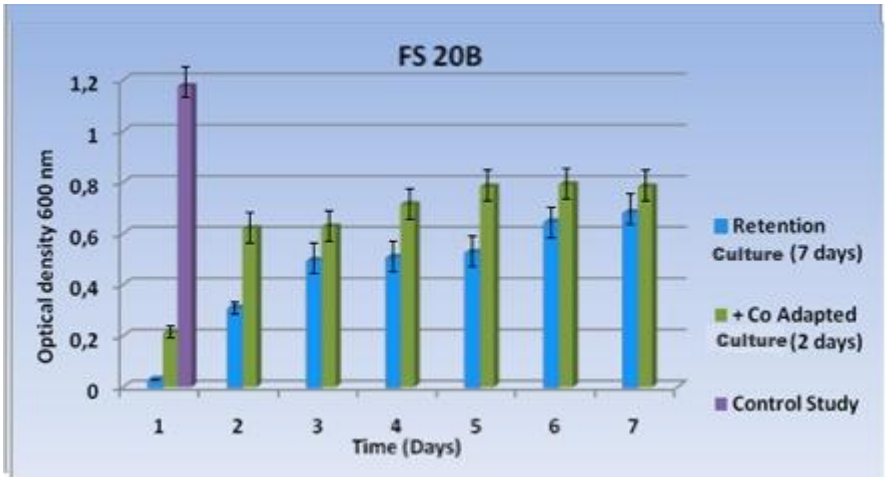


Figure 3.5. FS 20B Optical density records for cobalt adapted (2days) culture, Retention culture (7days), and control culture.

Apparently the numbers were not decreasing but the size was getting reduced. This was further discussed in section 3.4. The photograph in Figure 3.8. shows the visual difference in turbidities between 2ndand 7th day for FS 20B.

FS 20A and FS 20C adapted the cobalt presence in two days and then retained the adaptation for about 20 and 11 passages in cobalt free media. After that the cultures started to mature visually in 4 days. At this point the cultures were assumed to loose the memory perhaps gradually.

Figure 3.6. shows the viable counts (CFU) of FS 20A for the control, cobalt adapted and retention time study cultures. Retention study cultures had CFU's similar to control and cobalt adapted cultures.

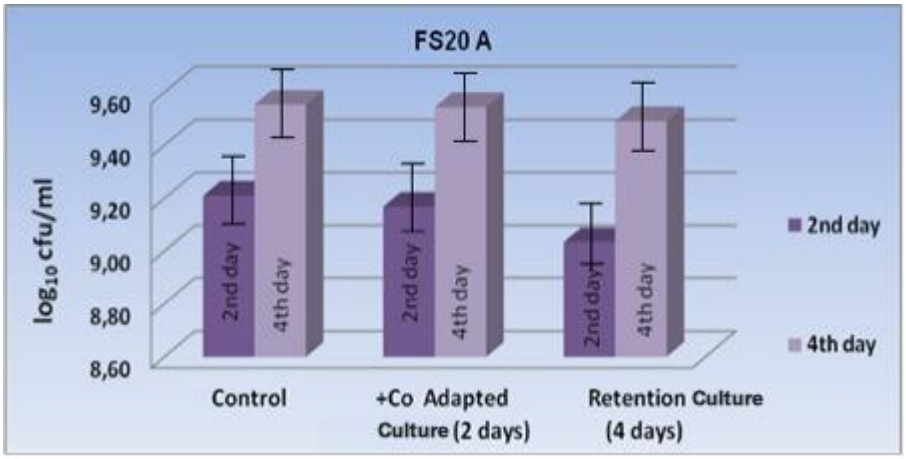


Figure 3.6. FS 20A viable counts for cobalt adapted (2days) culture, Retention culture (4days), and control culture.

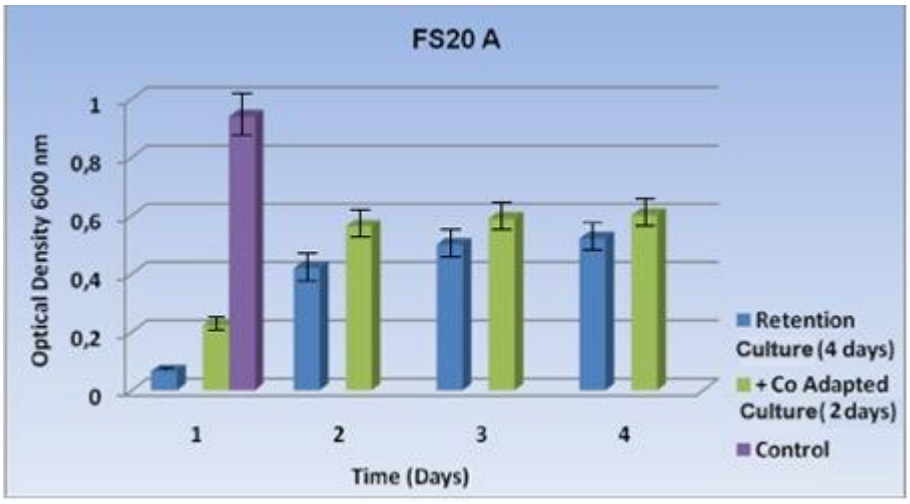


Figure 3.7. FS 20A Optical density records for cobalt adapted (2days) culture, Retention culture (4days), and control culture.

The OD values of cobalt adapted culture was almost half of the control, but it did not mean that their viable counts were less than that of control. It again led us to take the size reduction phenomenon into serious account (Figure 3.7.).

Retention study cultures' viable counts had a big difference between 2nd and 4th day, but optical density at 600 nm the difference

was almost negligible. The phographs in Figure 3.10. and Figure 3.11. show the visual difference in turbidities between 2ndand 4th day.

We came across a peculiar situation where OD measurements and viable counts were not corresponding as they should under normal circumstances. That is to say, when we measure a very low OD the plate counts were very high comparable to that of control cultures. Then we decided to investigate this as well.

3.4 Cell Size Reduction

We attributed the situation that we faced, to reduction in cell size. The bacteria was there but they were so small that they can not be evaluated through spectrophotometer correctly. Also, we saw that they were forming corresponding small colonies on agar surface. Therefore, it can be said that numbers were not dropping down dramatically bu there was considerable amount of reduction in cell sizes. We realised that measuring growth solely with spectrophotometers for example in MIC (Minimum Inhibition Concentration) studies may be somewhat misleading under certain circumstances. The best way to estimate the MIC needs more than one method.

A lake located in Italy, has been exposed to the metal (copper), acid pollution over fifty years. This lake was studied by Cattaneo *et al*.(1998), to analyse the fossil remains in terms of their size. As a result of increase in pollution of the lake, the size of some living organisms like; diatoms, the camoebians, and cladocerans got smaller. Both the reduction of the size and the trophic levels of organisms from different kingdoms indicate the selective features of the environment. Short-lived, fast-reproducing small organisms have better ability to live in

pollution stressed environments rather than the large organisms since they are less sensitive than the large ones. The inverse relationship between the size of freshwater biota and the effects of both internal and external environmental stress factors have been noticed. External stress, as heavy-metal pollution, may have a dramatic effect.

Studies conducted by Cattaneo *et al.* (1998), illustrated a distinct decline in the size of algae, protozoa, and zooplankton in the lake. After copper exposure to lake, the shift in the size of individual organisms and taxonomic compositions had been observed (Sigee, 2004).

In this study we did not measure the sizes of bacterial cells. We tried to approximate the size issue by using colonies. This is of course an implication only. It is necessary to measure the cell sizes in further studies.

3.5 Visual Observation of Growth in Broth Media

In Figure 3.8. and Figure 3.9., we also compared the inoculum size effect on the cultures visual turbidity. The measurements of growth with spectrophotometer was different for first three days of retention cultures (Figure 3.5.). Eventually, the retention time (7 days for FS 20B) did not change depending on the inoculum size (Figure 3.9.).

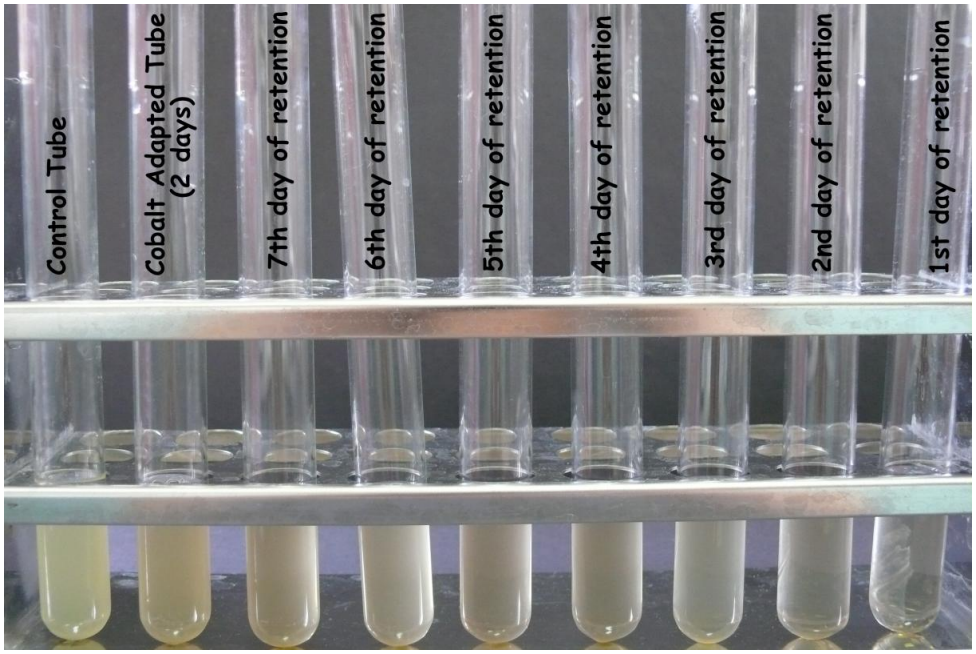


Figure3.8. FS 20B, Control, cobalt adapted (2 days) and retention cultures of 7 days. (Inoculum was 90 µl.)

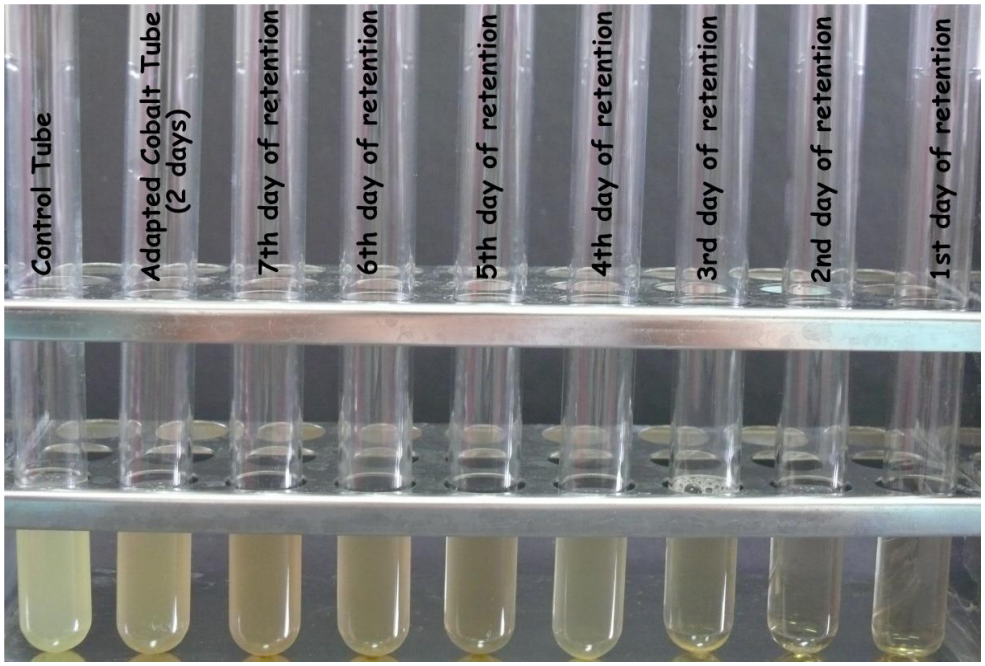


Figure 3.9. FS 20B, Control, cobalt adapted (2 days) and retention cultures of 7 days. (Inoculum was 25 µl)

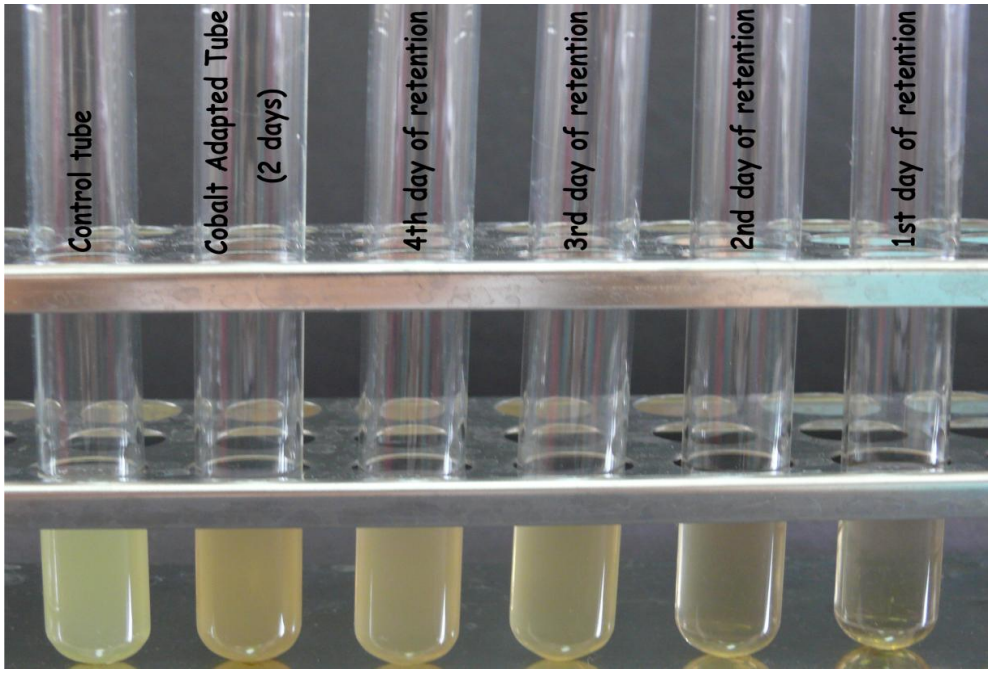


Figure3.10. FS 20A, Control, cobalt adapted (2 days) and retention cultures of 4 days. (Inoculum was 90 µl.)

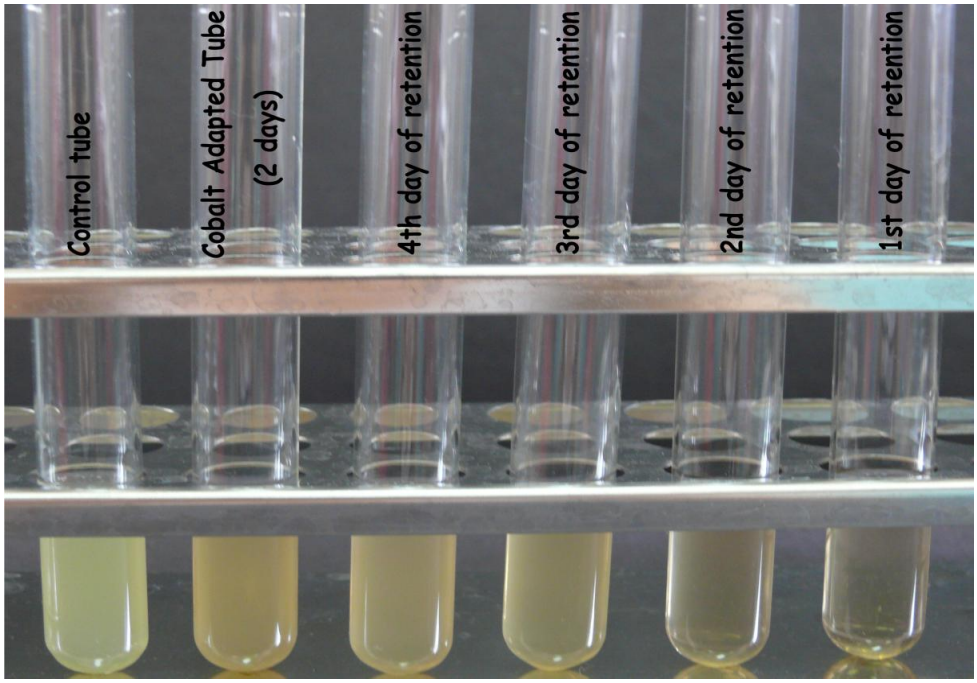


Figure 3.11. FS 20A, Control, cobalt adapted (2 days) and retention study cultures of 4 days. (Inoculum amount was 25 µl).

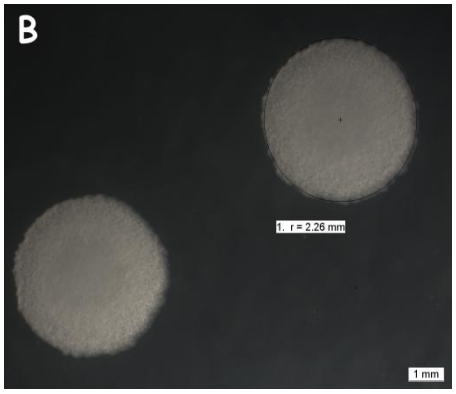
3.6 Effects of Cobalt on Colony Size

The photograps were taken through a stereomicroscope attached to a computerized system (specifications given in section 2.1). The size bars are automatically assigned as 1mm by the software. Then the radius of the colonies were measured accordingly and put in the photos. All photographs were taken under same magnification.

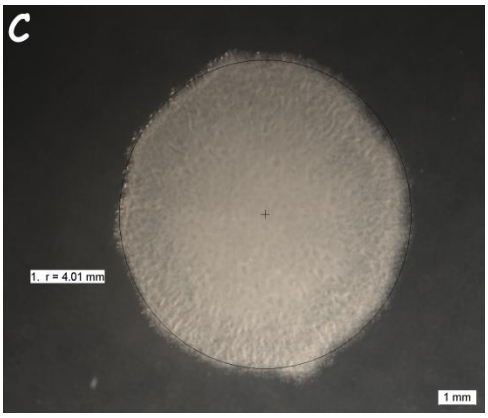
The colony size differences were obvious in Figure 3.12. In the 1st hour of the growth, the smallest colonies were measured for the cobalt adapted culture in cobalt containing nutrient agar as shown in panel A (Figure.3.12.). The largest colonies formed on nutrient agar (without Co) surface inoculated with a culture never exposed to cobalt as seen in panel C. When cobalt adapted culture was inoculated onto cobalt free nutrient agar the colony size got bigger as seen in panel B.

Furthermore when cobalt adapted culture was transferred to a cobalt free liquid media once and spreaded onto cobalt free nutrient agar the colonies (panel D) got even larger but did not reach the size of the control. Figure 3.13 shows the colony sizes measured upon 6th hour of growth in liquid culture. In terms of the colony sizes, the patterns described above was sustained. However there was an overall decrease in radius for all of them (Table 3.2. and Table 3.3.).

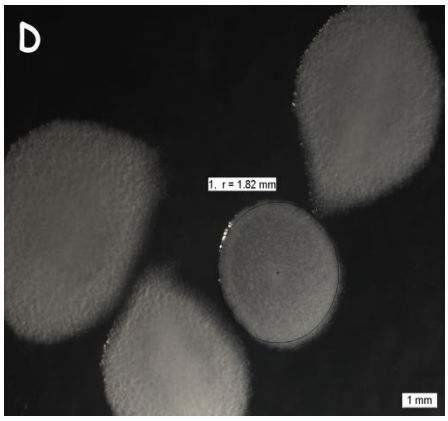
A) Cobalt adapted culture, inoculated on nutrient agar with Co. 1st hour of growth.



B) Cobalt adapted culture, inoculated on nutrient agar plate, 1st hour of growth.



C) Control never exposed to cobalt on nutrient agar, 1st hour of growth.

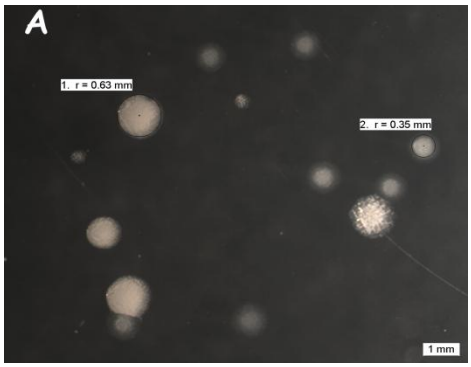


D) Culture which was adapted to cobalt, inoculated to cobalt free nutrient broth, then inoculated to nutrient agar. 1st hour of growth.

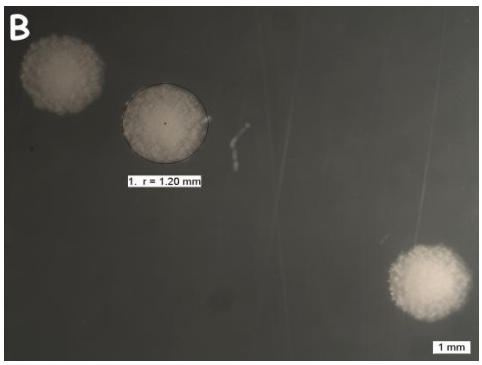
Figure 3.12. White coloured FS 2C colonies on agar plates in memory experiment. They were the colonies representing the 1st hour of growth, for A, B, C, and D.

Table 3.2. Measured radius of the colonies in A, B, C, and D of Figure 3.12

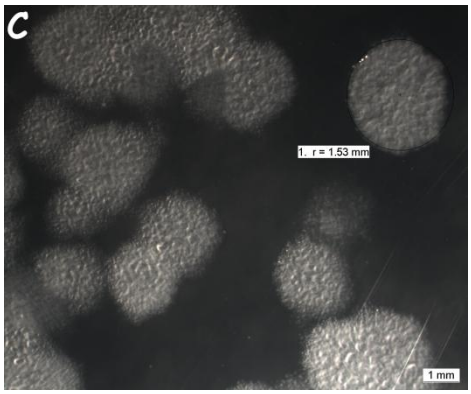
FS 2C (1st hour)	**A**	**B**	**C**	**D**
Radius	**1.49 mm**	**2.26 mm**	**4.01 mm**	**1.82mm**



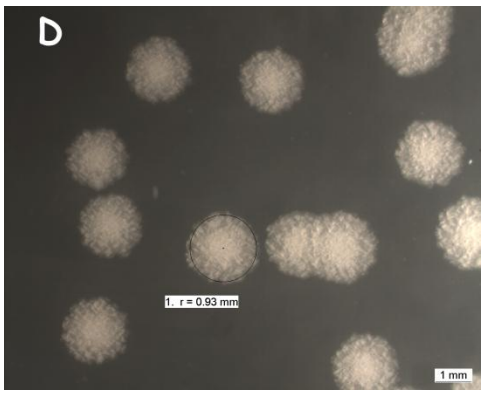
A) Cobalt adapted culture, inoculated on nutrient agar with Co. 6th hour of growth.



B) Cobalt adapted culture inoculated on cobalt free nutrient agar plate, 6th hour of growth.



C) Control never exposed to cobalt on nutrient agar, 6^{th} hour of growth.



D) Culture which was adapted to cobalt, inoculated to cobalt free nutrient broth, then inoculated to nutrient agar. 6^{th} hour of growth.

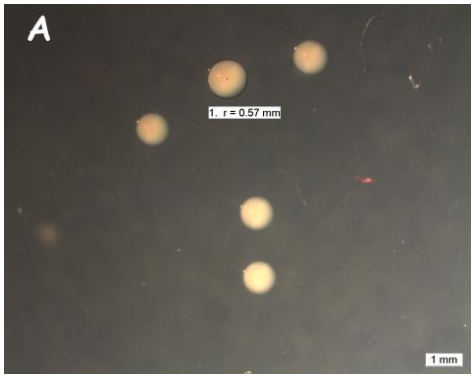
Figure 3.13. White coloured FS 2C colonies on agar plates in memory experiment. They were the colonies representing the 6^{th} hour of growth, for A, B, C, and D.

Table 3.3. Measured radius of the colonies in A, B, C, and D of Figure3.13

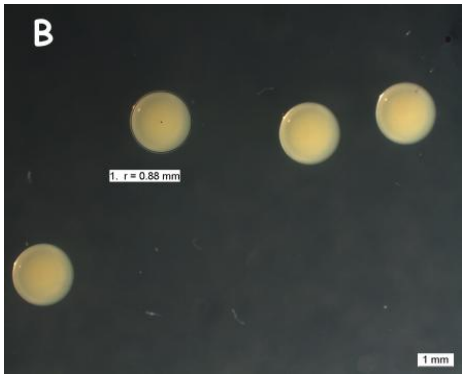
FS 2C (6^{th} hour)	**A**	**B**	**C**	**D**
Radius	**0.63 mm**	**1.20 mm**	**1.53 mm**	**0.93 mm**

Corresponding colonies of the FS 20B culture were obtained after 10 hours of growth. The photographs showing the colony sizes were given in Figure 3.14. The radius measurements were given in Table 3.4. Here also the the pattern of colony size relations repeated as mentioned for FS 2C.

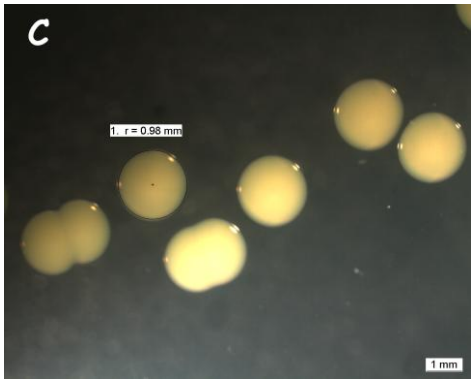
For FS 48A culture the colony sizes were measured after 2nd, 5th and 7th hours of growth. As seen in Figure 3.15., 2nd hour of growth the panel A represents the colonies coming from FS 48A grown in cobalt containing liquid media and inoculated onto nutrient agar with cobalt. The radius was 0.36 mm. The control had colony radius of 1.03 mm (panel B). Figure 3.16. shows the photographs of the colonies for the same isolate in 5th hour. In the presence of cobalt the radius was 0.35 mm and in control the radius was 1.08 mm. In the 7th hour the radius of the colony measured as 0.2 mm and that of control was 2.5 mm (Figure 3.17.).



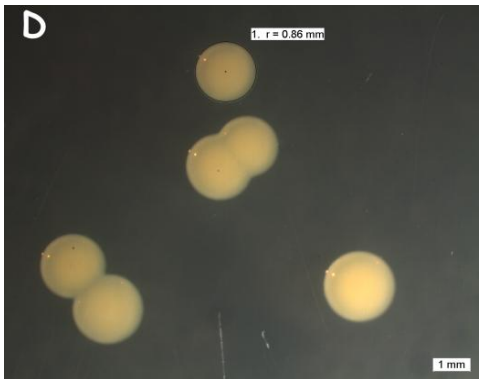
A) Cobalt adapted culture, inoculated on nutrient agar with Co 10th hour of growth.



B) Cobalt adapted culture, inoculated on cobalt free nutrient agar plate,10th hour of growth.



C) Control never exposed to cobalt on nutrient agar, 10th hour of growth.



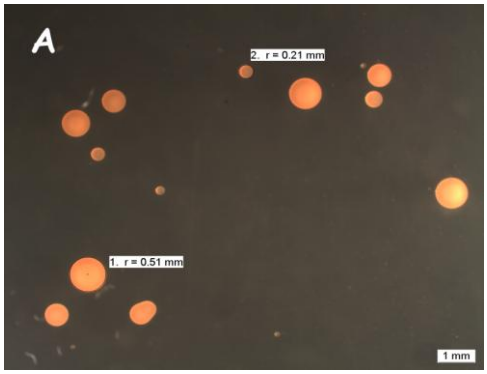
D) Culture which was adapted to cobalt, inoculated to cobalt free nutrient broth, then inoculated to nutrient agar 10th hour of growth.

Figure 3.14. Yellow coloured FS 20B colonies on agar plates in memory experiment. They were the colonies representing the 10th hour of growth, for A, B, C, and D.

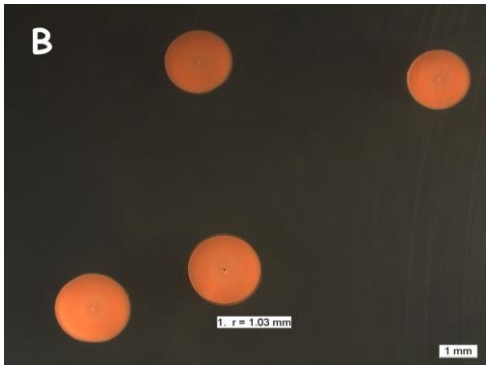
Table 3.4. Measured radius of the colonies in A, B, C, and D of Figure 3.14

FS20B(10th hour)	**A**	**B**	**C**	**D**
Radius	**0.57 mm**	**0.88 mm**	**0.98 mm**	**0.86 mm**

FS 48 had different distinct size colonies as seen in Figure 3.15 A. It was hard to acclimate this isolate to cobalt, FS 48 took long time to grow in cobalt containing liquid media in first inoculation (FS 48A 14 days, FS 48B 16 days, FS 48C no sign), furthermore it was showing inconsistent growth times in acclimation experiments. Different colony sizes may idicate that adaptation is rather idiosyncratic. As if different measures were taken by individual subclones rather than a single decision for all.

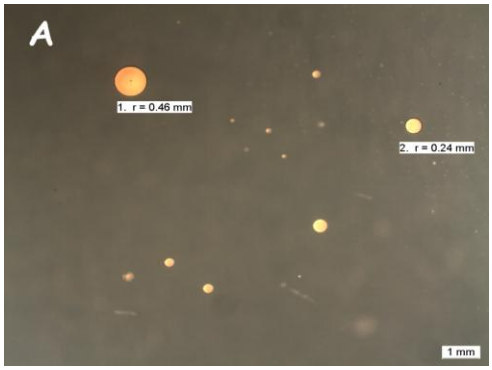


A) Cobalt adapted Culture, inoculated on cobalt added nutrient agar, 2nd hour of growth.

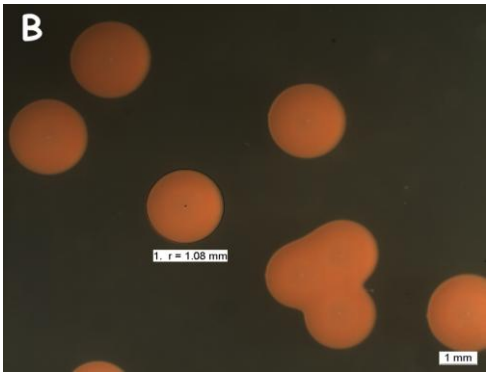


B) Control never exposed to cobalt on nutrient agar, 2nd hour of growth.

Figure 3.15. Orange coloured FS 48A colonies on agar plates in memory experiment. They were the colonies representing the 2nd hour of growth, for A, B, C, and D.

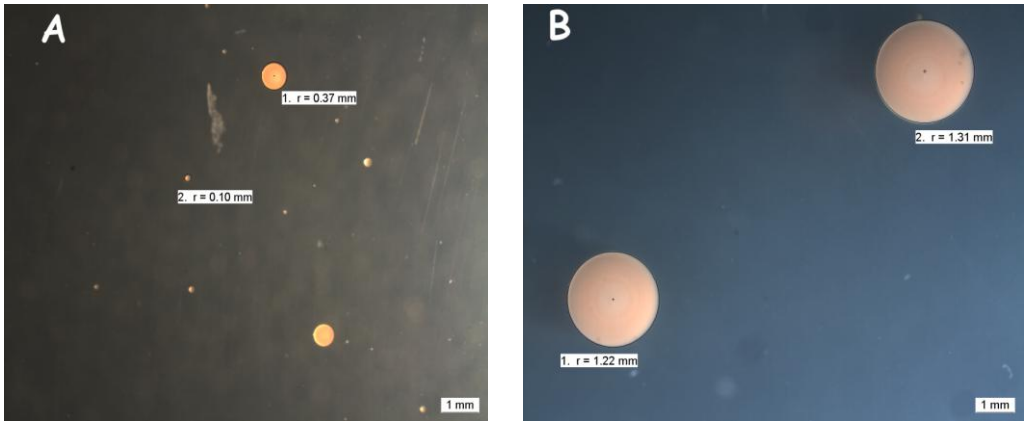


A) Cobalt adapted Culture, inoculated on cobalt added agar plate, 5th hour of growth.



B) Control never exposed to cobalt on nutrient agar, 5th hour of growth.

Figure 3.16. Orange coloured FS 48A colonies on agar plates in memory experiment. They were the colonies representing the 5th hour of growth, for A, B, C, and D.



A) Cobalt adapted Culture, inoculated on cobalt added agar plate, 7th hour of growth.

B) Control never exposed to cobalt on nutrient agar, 7th hour of growth.

Figure 3.17. Orange coloured FS 48A colonies on agar plates in memory experiment. They were the colonies representing the 7th hour of growth, for A, B, C, and D.

Furthermore, the growth curve (Figure 3.34.) of FS 48 A showed a sudden viable count drop at the 2nd hour. Spectrophotometric value at that time kept its regular prophile. This discrepancy between the viable count and the OD measurements is obviously due that the photometer can not discriminate between dead and alive cells (Madigan *et al*. 2006). The photographs in Figures 3.18. and Figure 3.19. show general appearance of the colonies in nutrient agar plates where the radius measurements were performed.

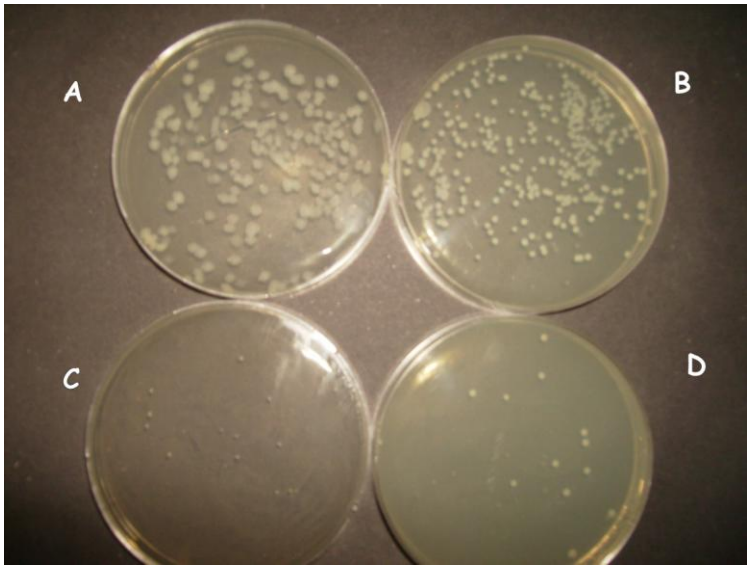


Figure 3.18. Colonies of FS 2C on nutrient agar. A) Control never exposed to cobalt, B) Culture which was adapted to cobalt, inoculated to cobalt free nutrient broth, then inoculated to nutrient agar C) Cobalt adapted culture, inoculated on nutrient agar with cobalt D) Cobalt adapted culture,inoculated on cobalt free nutrient agar

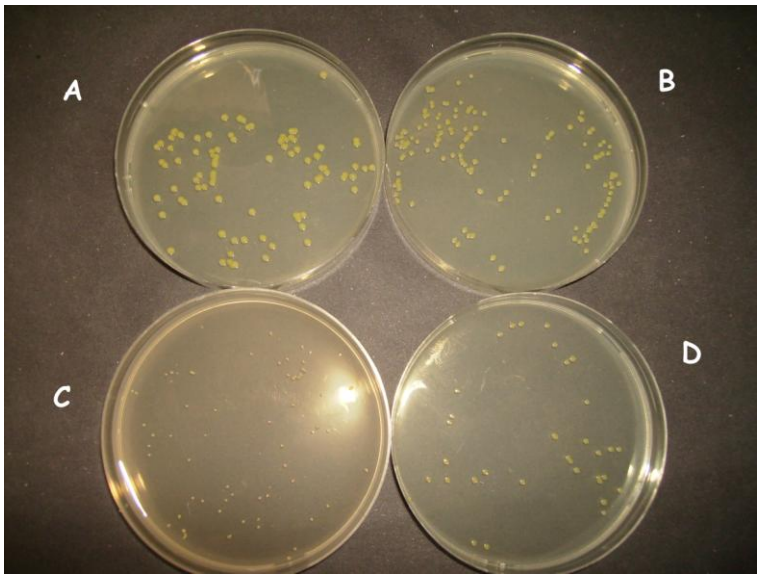


Figure 3.19. Colonies of FS 20B on nutrient agar. A) Control never exposed to cobalt, B) Culture which was adapted to cobalt, inoculated to cobalt free nutrient broth, then inoculated to nutrient agar C) Cobalt adapted culture, inoculated on nutrient agar with cobalt D) Cobalt adapted culture,inoculated on cobalt free nutrient agar.

3.7 Growth Curves Related to Cobalt Adaptation and Memory Retention Experiments

In order to calculate the effect of cobalt on growth of isolates, it was necessary to calculate the generation times. Therefore, growth curves were generated by performing viable counts and measuring optical densities in the time of samplings. The following curves provides the measurement of growth in terms of cfu/ml and OD at 600 nm.

3.7.1. Growth Curves for FS 2C Culture

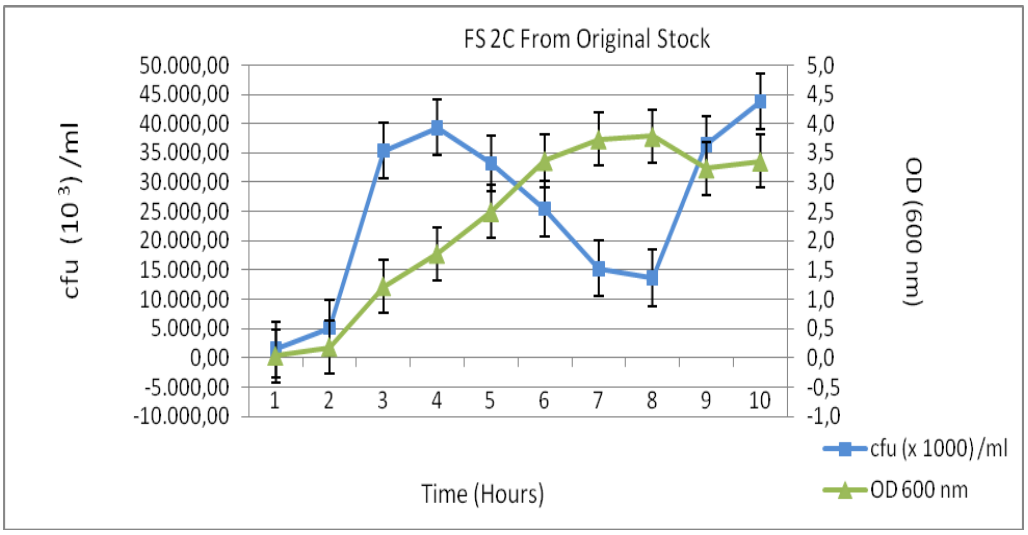


Figure 3.20. Growth curve for FS 2C control culture, never exposed to cobalt, not acclimated, from original stock.

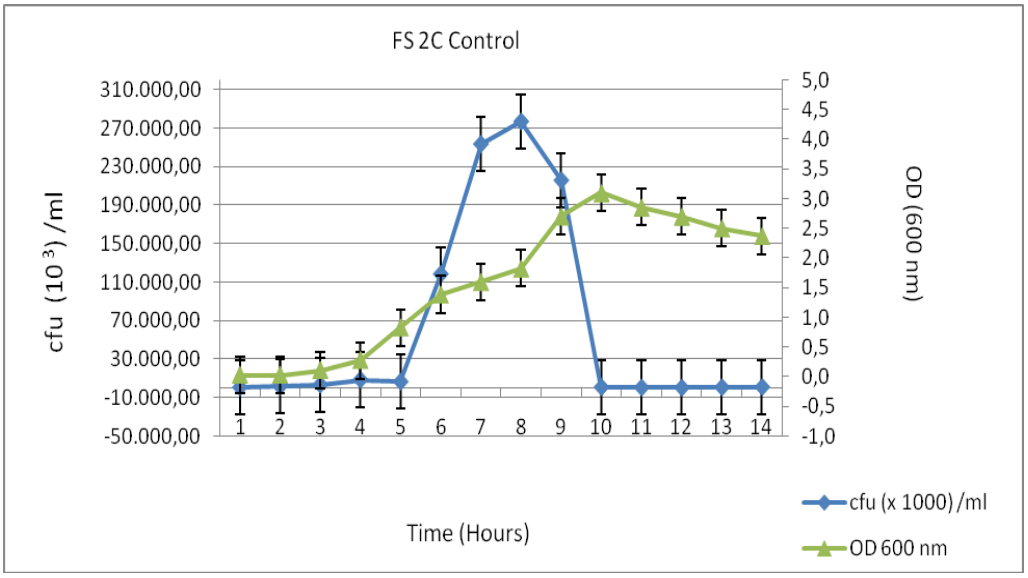


Figure 3.21. Growth curve for FS 2C culture sequentially passaged (99 times) in cobalt free medium followed by cobalt acclimation.

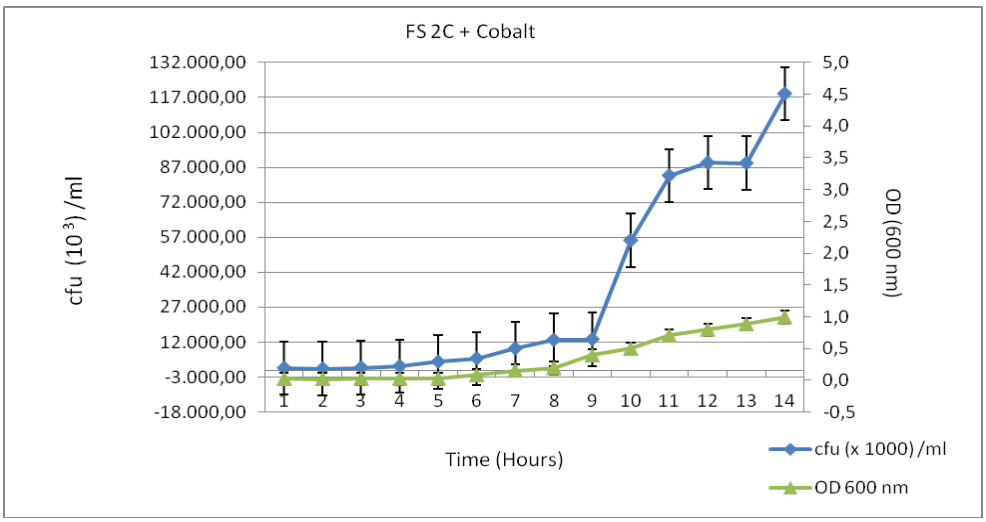


Figure 3.22. Growth curve for FS 2C retention culture in cobalt containing medium (followed by 99 times passage in cobalt)

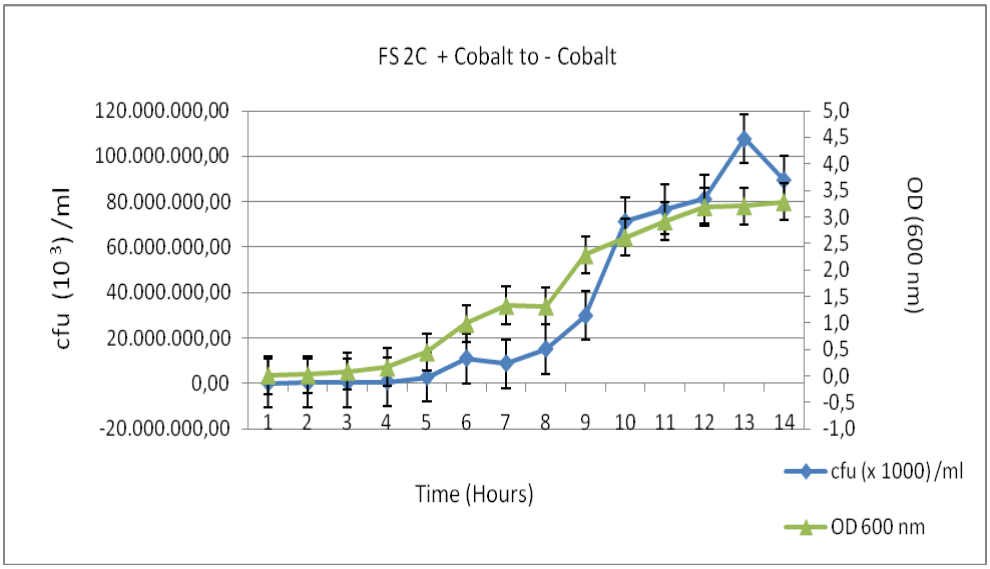


Figure 3.23. Growth curve for FS 2C Co adapted culture inoculated to nutrient broth without cobalt once (Followed by 99 times passage in cobalt containing medium)

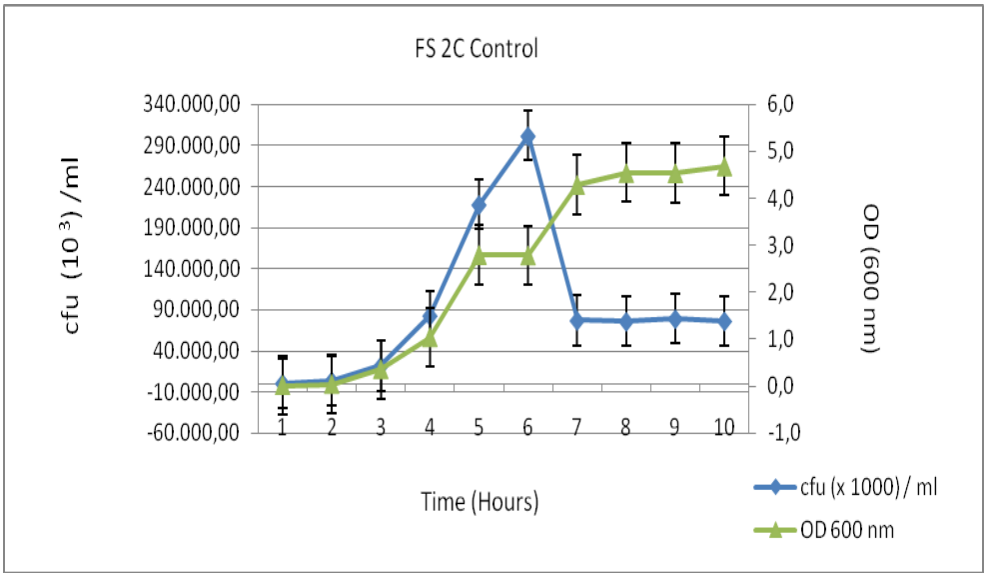


Figure 3.24. Growth curve for FS 2C culture sequentially passaged (140 times) in cobalt free medium followed by cobalt acclimation.

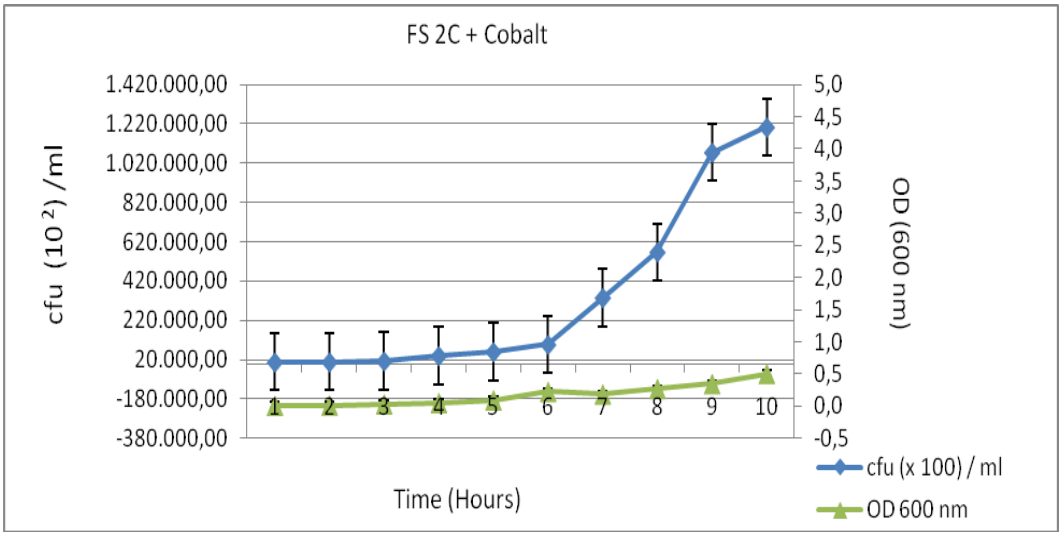


Figure 3.25. Growth curve for FS 2C retention culture in cobalt containing medium, (followed by 140 times passage in cobalt medium).

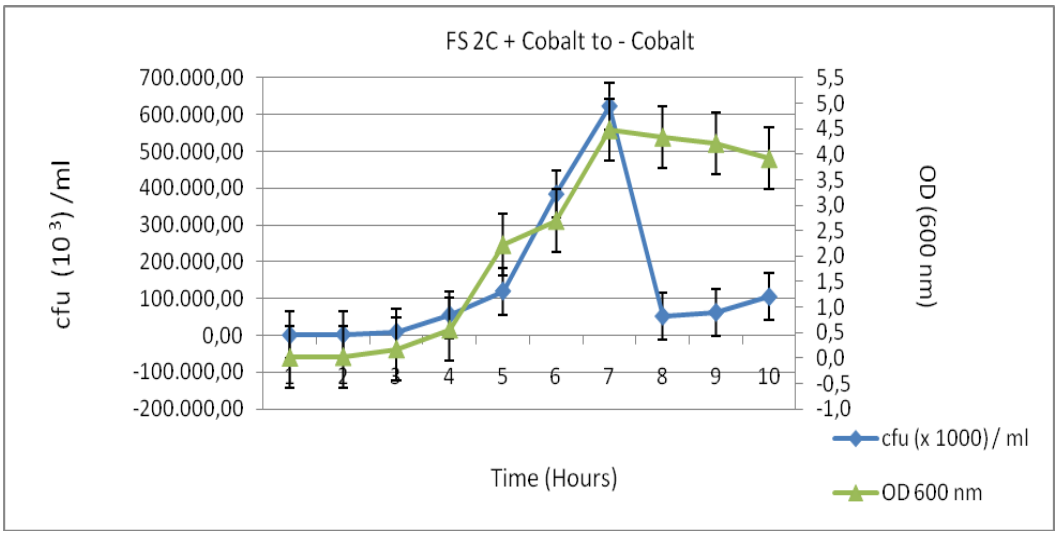


Figure 3.26. Growth curve for FS 2C Co adapted culture inoculated to nutrient broth without cobalt once (Followed by 140 times passage in cobalt containing medium).

3.7.2. Growth Curves for FS 20B Culture

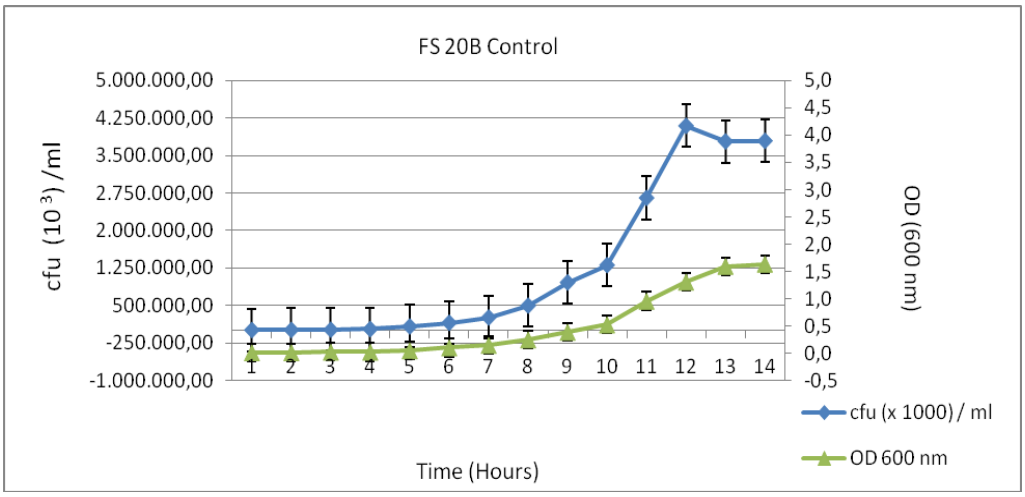


Figure 3.27. Growth curve for FS 20B culture sequentially passaged (99 times) in cobalt free medium followed by cobalt acclimation.

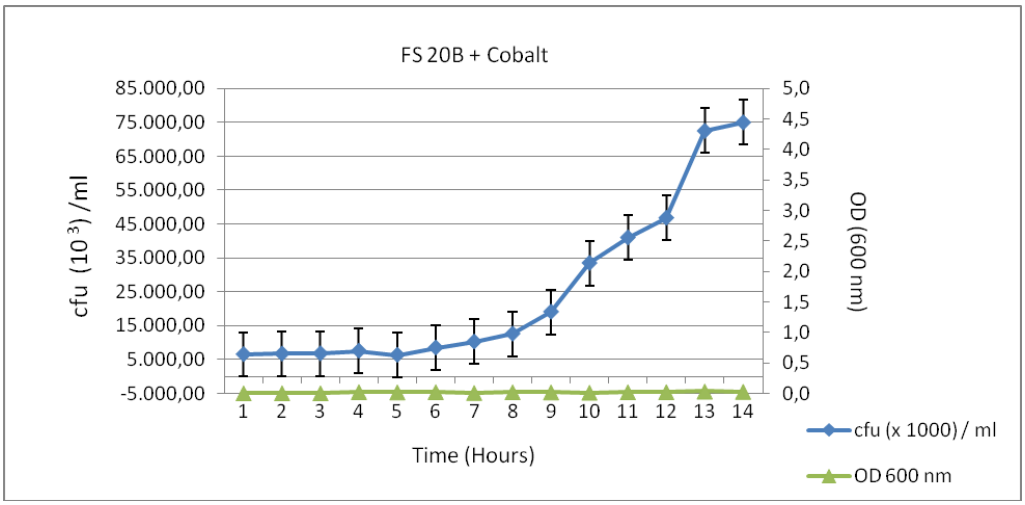


Figure 3.28. Growth curve for FS 20B retention culture in cobalt containing medium (followed by 99 times passage in cobalt medium).

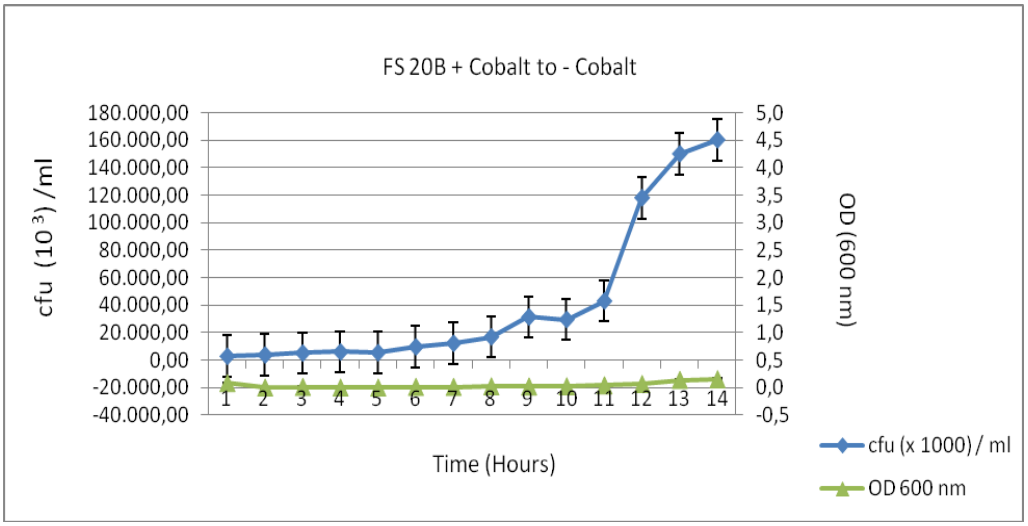


Figure 3.29. Growth curve for FS 20B Co adapted culture inoculated to nutrient broth without cobalt once (Followed by 99 times passage in cobalt containing medium).

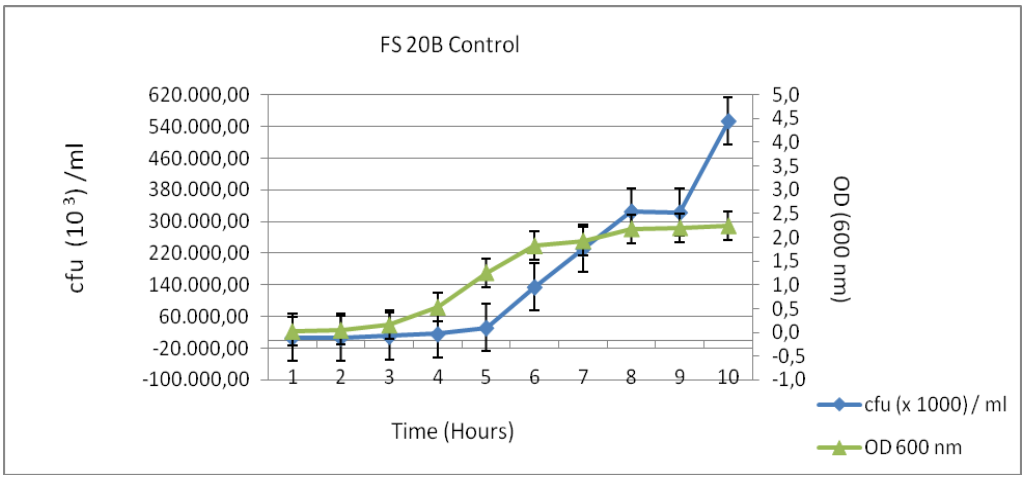


Figure 3.30. Growth curve for FS 20B culture sequentially passaged (140 times) in cobalt free medium followed by cobalt acclimation.

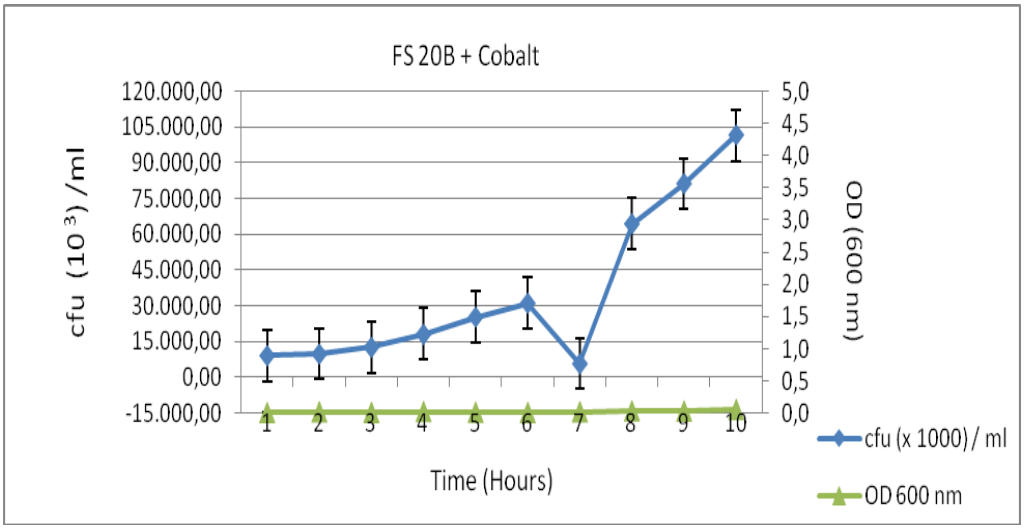


Figure 3.31. Growth curve for FS 20B retention culture in cobalt containing medium, (followed by 140 times passage in cobalt medium).

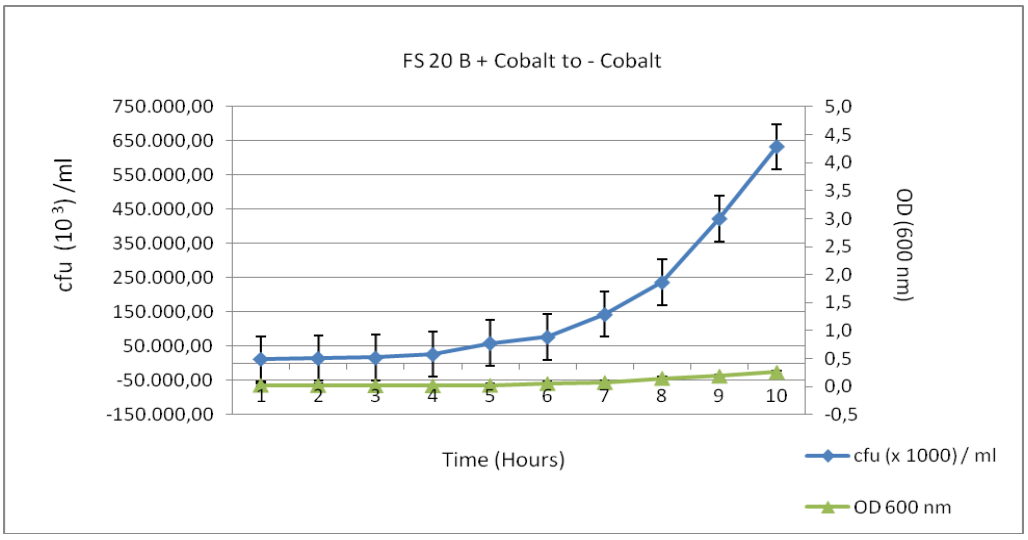


Figure 3.32. Growth curve for FS 20B Co adapted culture inoculated to nutrient broth without cobalt once (Followed by 140 times passage in cobalt containing medium).

3.7.3. Growth Curves for FS 48A Culture

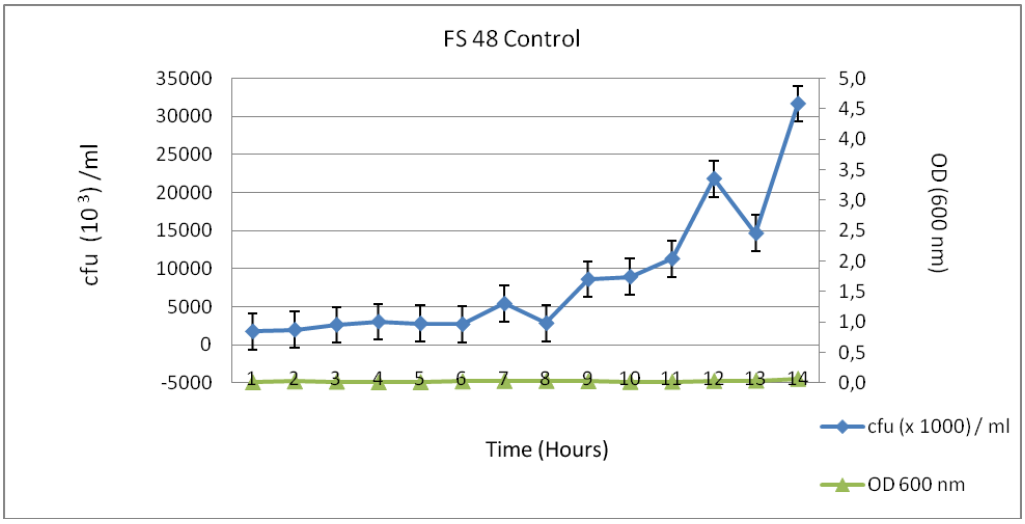


Figure 3.33. Growth curve for FS 48 control culture never exposed to cobalt (no acclimation) from original stock

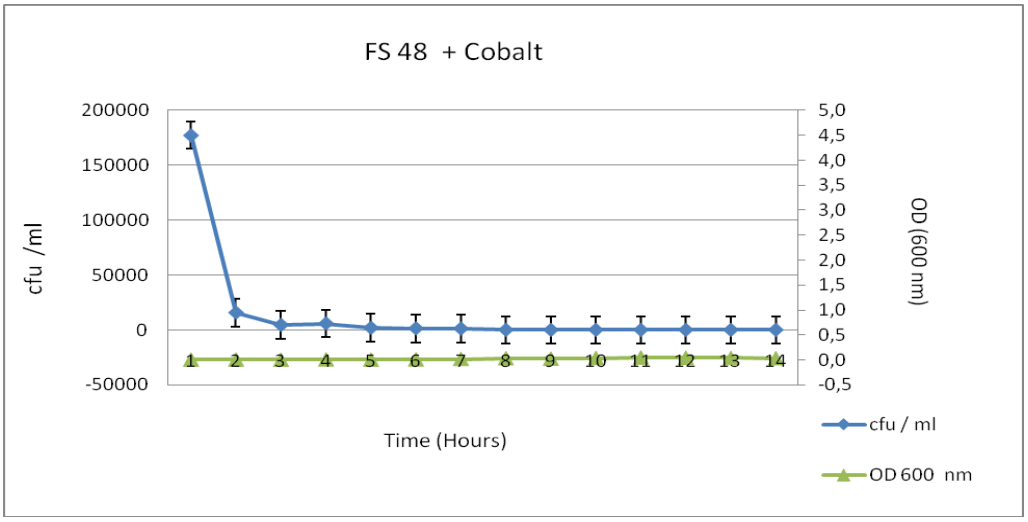


Figure 3.34. Growth curve for FS 48 from original stock inoculated to cobalt containing medium (no previous exposure or acclimation to cobalt).

3.8 Generation Times

The cell counts at exponential phase were used in order to calculate the generation times. Generation times were calculated by using the following formula (Madigan *et al.* 2006).

$$N = \frac{\text{Log } N - \log N_0}{\log 2}$$

In the formula, n standards for the number of generation (number of times the cell population doubles during the time interval) and generation time is calculated with g= t / n, where t simply represents the hours or minutes of exponential growth.

Table 3.5. Generation times of cultures in minutes

	FS 2C	FS 20B
Before acclimation	42.6	ND
Control 99 passages in cobalt free media	55.2	67.2
Cobalt adapted culture 99 passages with cobalt	67.8	134.4
Cobalt adapted culture 99 passages in cobalt and once in NB	76.2	67.8
Control 140 passages in cobalt free media	43.2	94.8
Cobalt adapted culture 140 passages with cobalt	79.8	115.2
Cobalt adapted culture 140 passages in cobalt and once in NB	36	72.6

ND: Not determined

According to Table 3.5. the longest generation time meaning the slowest growth occurred in FS 20B. This culture was continuosly grown in cobalt containing medium. It was found to attain full growth visually in 4 days. Later on memory retention experiments showed that it lost adaptation after 12 passages and started to attain full growth in seven days. Meanwhile FS 2C propagated under the same conditions had a generation time of 67.8 minutes. This isolate was found to loose adaptation after 6 passages. In adapted condition the full growth was attained in 1 day. However upon loss of adaptation the full growth was obtained in two days. Even after 140 passages in cobalt upon introduction into cobalt free medium FS 2C was found to grow rapidly even faster than the original stock culture. The data shows that the isolates generation times were changing depending on the tried conditions.

As it is well known that by evolution of resistant populations, microbial communities acclimate to toxic compounds.

Acclimated populations sustain crucial microbial processes and several mechanisms which would result in the acclimation process have been declared as we mentioned before. Each of these mechanisms plays some role in the acclimation of microbial communities in polluted environments (Liebert *et al.,*1991).

In literature reviewing, we experienced with a definition of ‘’Epigenetic inheritance (EI) ‘’, which infers the passage of cellular states from one generation to the next, without alterations of the genome’’. The basic example of epigenetic inheritance is methylation, transferring of a phenotype by modifications to the DNA. Furthermore other examples for epigenetic inheritance are prions, genomic imprinting, and histone modification (Veening *et. al.,* 2008).

Our results show that two isolates (FS 20 and FS 2) adapted to cobalt presence in a number of passages. Our starting question to be answered was how long they can retain this adaptation in the absence of cobalt ? Do they have a memory to retain its former adaptation capacity ?

Veening *et. al.,* (2008), mentioned " Bacterial Persistence'' with a well-known example; bacterial bet-hedging strategy. Persister cells are not only simply antibiotic resistant but also demonstrate a transient growth arrested state. The switch from normal growth to persistence and vice versa is stochastic and epigenetic in nature. Recent mathematical modeling proposed that bacterial persistence can be considered as a social characteristic and can be affected by kin selection (Gardner *et al.,* 2007).

Since state of a biological system is not determined only by present conditions, it also depends on its former history, so we can conclude that the system has memory. (Casadesu´s and D'Ari, 2002). Bacteria and bacteriophage have a several memory mechanisms, some of which seem to convey adaptive value. The heritable memory is the type of genetic programme that displays inversion of the specific DNA sequences which results in switching between alternative patterns of gene expression. On the other hand, inheritable memory also is based on epigenetic circuits, in which a system have two possible steady states is locked in one or the other state by a positive feedback loop (Casadesu´s and D'Ari, 2002).

Finally we decided that our two isolates could retain the adaptation to cobalt and this perhaps be explained by the epigenetic inheritance which is the passage of cellular states from one generation to the next, without alterations of the genome. Whenever the state of a biological system is not determined solely by present conditions but

depends on its past history, that indicates the state of memory and we can say that our system has memory as well.

CHAPTER 4

CONCLUSION

- The 36 freshwater bacteria isolates exhibited different growth inhibition profiles in the presence of cobalt. Majority of the isolates (30 out of 36) exhibited growth inhibition under 70 µg/ml and the upper limit was 100 µg/ml cobalt for one isolate.

- Mix culture studies composed of three isolates, one was lost through competitive exclusion by the others in the presence of cobalt as determined through viable counts.

- In liquid cultures, there was growth pattern inconsistency among triplicate batches.

- After cobalt acclimation constant growth time of 2 days for FS 20 A,B,C and 1 day for FS 2 B;C was tabulated. At this point the cultures were assumed to be adapted to the tried cobalt concentration.

- These constant growth durations changed upon sequential repeated multiple passages in cobalt-free medium. In memory retention experiments, we found that FS 20A after 20 and FS 20C after 11 passages started to grow in four days instead of 2 days in cobalt containing media. FS 20B after 12 passages

started grow in 7 days instead of 2 days. FS 2B after 7 passages and FS 2C after 6 passages started to grow in 2 days instead of 1 day.

- The presence of cobalt and prolonged exposure to cobalt altered the generation times of the cultures.

- Under the presence of inhibitory compounds such as cobalt where viable count is high the OD measures were low. We interpreted this occurence with size reduction phenomenon.

- Our study suggests that MIC studies should be performed cautiously especially with the environmental isolates with high adaptation capabilities. Since our experiments conclusively showed that OD can not be used as a sole growth measurement method.

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APPENDIX A

Optical Density Comparisons on Growth Curves

Different absorbances (600 nm, 590 nm, 540 nm) were compared for the isolate Fs 2C**.**

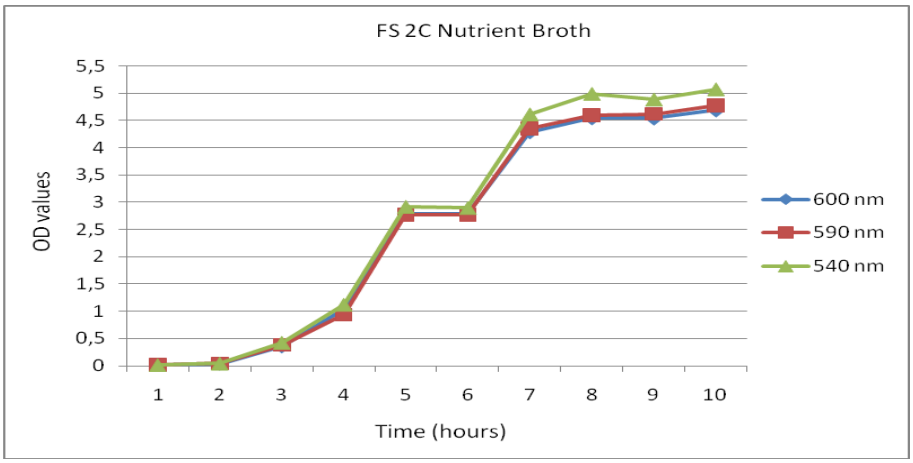

Figure. Growth curve of control FS 2C analysed in different wave lenghts (540nm, 590nm, 600nm)

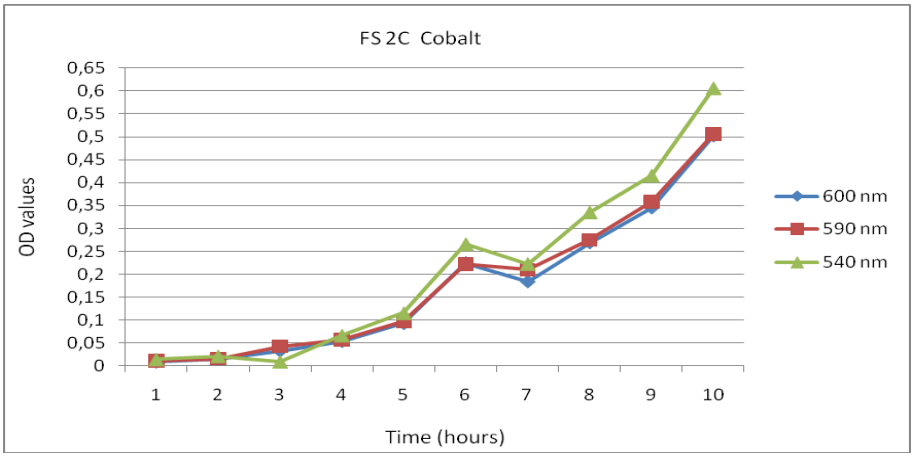

Figure. Growth curve of cobalt FS 2C analysed in different wave lenghts (540nm, 590nm,600nm)