CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Analysis of Indigenous Bacterial Flora Found in Lead Contaminated Soil of Faisalabad and Harvesting its Ability for Bioremediation

by

Hifza Anwar

A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2021

I would like to dedicate my research thesis to my family, friends and colleagues especially to my mother whose prayers and support helped me a lot to make this project successful

CERTIFICATE OF APPROVAL

Analysis of Indigenous Bacterial Flora Found in Lead Contaminated Soil of Faisalabad and Harvesting its Ability for Bioremediation

by

Hifza Anwar

(MBS193031)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Syed Babar Jamal Bacha	NUMS, Rawalpandi
(b)	Internal Examiner	Dr. Sohail Ahmed Jan	CUST, Islamabad
(c)	Supervisor	Dr. Arshia Amin Butt	CUST, Islamabad

Dr. Arshia Amin Butt
Thesis Supervisor
December, 2021

Dr. Sahar Fazal
Head
Dept. of Biosciences & Bioinformatics
December, 2021

Dr. Muhammad Abdul Qadir
Dean
Faculty of Health & Life Sciences
December, 2021

Author's Declaration

I, **Hifza Anwar** hereby state that my MS thesis titled "**Analysis of Indigenous Bacterial Flora Found in Lead Contaminated Soil of Faisalabad and Harvesting its Ability for Bioremediation**" is my own work and has not been submitted previously by me for taking any degree from Capital University of Science and Technology, Islamabad or anywhere else in the country/abroad.

At any time if my statement is found to be incorrect even after my graduation, the University has the right to withdraw my MS Degree.

(Hifza Anwar)

Registration No: MBS193031

Plagiarism Undertaking

I solemnly declare that research work presented in this thesis titled "**Analysis of Indigenous Bacterial Flora Found in Lead Contaminated Soil of Faisalabad and Harvesting its Ability for Bioremediation**" is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been dully acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Capital University of Science and Technology towards plagiarism. Therefore, I as an author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of MS Degree, the University reserves the right to withdraw/revoke my MS degree and that HEC and the University have the right to publish my name on the HEC/University website on which names of students are placed who submitted plagiarized work.

(Hifza Anwar)

Registration No: MBS193031

Acknowledgement

I would like to acknowledge the efforts of my supervisor **Dr. Arshia Amin Butt**, without her assistance and dedicated involvement in every step throughout the process, this research would have never been accomplished. i would like to thank you very much for your support. I would also like to show gratitude to my all teachers and senior fellows who enables me and equipped me to complete this research.

(Hifza Anwar)

Abstract

Some microorganisms have the ability to either degrade or remove environmental pollutants and could serve an effective use in lead bioremediation. Lead is a chemically inert heavy metal and if present in toxic amount in environment, can have negative health effects by becoming a part of the natural food chain. The main objective of this experiment was to identify lead tolerant bacterial species present in Pb contaminated soil from Faisalabad. WHO has reported elevated levels of lead contamination in water, and air across major cities in Pakistan such as Islamabad, Lahore, Faisalabad and Karachi. Faisalabad specifically also shows an elevated level of Pb contamination in soil. It majorly affects children aged 3-5 years and the urban population residing in major cities in Pakistan. Major source of Pb exposure in Pakistan are industrial wastes and effluents discharged directly into environment and it has toxic effects on the human nervous, renal and reproductive systems. Molecular Docking via CB dock is performed taking some bacterial proteins as receptors molecules and lead salts as ligand molecule for insilico analysis of bacterial strain involved in biosorption or biotransformation process.To identify lead tolerant bacteria, soil samples were collected from Pb contaminated sites near Faisalabad, followed by inoculation and purification of bacterial colonies in lead nitrate based culture media. UV spectrophotometry was performed and the colony showing maximum growth rate was subjected to 16s rRNA and Phylogenetic analyses. The identified bacterial species possesses considerably high lead tolerance and further analyzed for potential lead biosorption capabilities.

Keywords: Lead tolerance, Molecular docking, 16s RNA Analysis, Phylogenetic Analysis, UV Spectrophotometry, soil microbiota, lead nitrate.

Contents

List of Figures

List of Tables

Abbreviations

A.T.P	Adenosine triphosphate
B.C	Before christ
CB	Cavity blind
CADD	Computer aided drug discovery
CDC	Center for disease control
Cd	Cadmium
Ca	Calcium
DALYs	Disability adjusted life years
DNA	Deoxy ribonuclecic acid
Fe	Iron
I-TASSER	Iterative threading assembly refinement
IQ	Intelligence quotient
NCBI	National center for biotechnology information
Pb	Lead
PPEs	Personal Protective Equipments
PDB	Protein Data Bank
RNA	Ribonuclecic acid
spp	specie
TSA	Trptic soy agar
UV	ultra violet
WHO	World health organization
Zn	Zinc
3D	Three dimensional

Chapter 1

Introduction

1.1 Background

The use of living microorganisms for the degradation of environmental pollutants or to inhibit pollution is termed as bioremediation [1]. The environment could be either aqueous, terrestrial, or may be both. Bioremediation is all about the cleaning of contaminated environment through the exploitation of various metabolic capabilities of microorganisms. This remediation could be done either at the site of waste present or waste could be transported to some other place for treatment [2]. There are several ways like absorption, adsorption etc through which waste binds to some biological agent. The enzymatic attack of microorganisms is must for bioremediation to be effective [3].

Only a few microorganisms have the ability to degrade a segment of pollutants and only some of the contaminants are biodegradable. Therefore, the study of biodegrading potential of microorganisms is worth considering. Application of bioremediation also involves the manipulation of environmental parameters to permit microbial growth and degradation at faster rate [1].

Lead is denoted by the symbol "Pb" and holds an 82 atomic number. In its natural state, it is a soft, malleable and stable heavy metal which gives an initial silver-blue color that turns gray in open air. Lead is chemically inert, though its

oxides are amphoteric i.e. they covalently react with acids and bases. Lead also forms chains, rings & polyhedral structures by bonding with itself. If present in excessive amount in the environment, lead bio-accumulates into the natural food chain, resulting in detrimental health effects [4].

The analysis revealed, rather than geogenic, the main sources of lead contamination turned out to be the anthropogenic activities [5]. Smelting, mining, electroplating, and paint industry are the main causes of elevated lead concentration in the ecosystem. This Pb is then transported from soil and ground water to the biological systems and that's how lead enters the food chain [6].

Ingestion of lead contaminated food and water and inhalation of Pb contaminated aerosols or dust particles are the two main routes of lead exposure. In the human body, the highest % of lead is absorbed in kidneys, liver, brain and heart tissues [7].

Lead contamination could cause severe impacts on bone marrow, nervous system, digestive and cardiovascular system, reproductive, immune systems and kidney of humans [8]. Lead is mostly absorbed in the bloodstream after ingestion. The ability of lead to interfere with the proper functioning of enzyme is the main cause of its toxicity. Lead binds to the sulfhydryl groups present on most enzymes, preventing metal cofactors like Ca, Fe, and Zn from binding with the enzyme [9]. Lead can cross the blood brain barrier by mimicking calcium. Lead interferes with the function and structure of neurons by degrading the myelin sheaths of neurons, reducing their numbers, decreases neuronal growth, and also causes hindrance in the neurotransmission routes. Upon entry into the body, lead binds with ferrochelatase, an enzyme involved in the incorporation of iron into protoporphyrin IX, and halts its activity.

This, in turn, leads to insufficient production of heme, eventually leading to microcytic anemia. Other symptoms associated with Pb exposure are nephropathy, stomach aches and weakness in digits, wrists and ankles. Children suffering from lead exposure display signs of neurological, psychological and behavioral abnormalities. Symptoms associated with low blood Pb levels in children include hearing

problems and decreased IQ. Whereas, high blood Pb level are often fatal,leading to acute encephalopathy and dementia. According to WHO and The Centre for Disease Control (CDC), lead concentration in blood over 510mg/dL is considered elevated [10]. Bacteria are often favored for bioremediation of heavy metals as they can efficiently resist heavy metal toxicity by different biochemical processes like absorption, adsorption, methylation and redox reactions [1].

The lead bio sorption depends upon the pH of bacterial strain. 16s rRNA revealed that lead tolerant strains belonged to four genera; *Bacillus, Paenibacillus, Brevibacterium,* and *Staphylococcus* [11]. It may be possible to identify, isolate and assess local lead consuming bacterial species from environmental samples found near lead contaminated industrial zones of Pakistan and finding the pathways involving in bioaccumulation and biosorption of lead into bacterial cell, and what necessary metabolities should be added to enhance the abilities of bacterial strain to biotransform the Pb containing salts into less toxic form. In this research we focus on finding the proteins and functional groups of identified bacterial strain involved in Pb uptake so that we could find out the metabolities and important substrates of the pathway which could then provided in excess so that bacterial strain can grow faster and perform its Pb degradation function very efficiently.

Chapter 2

Literature Review

2.1 Lead Exposure Pre-Industrial Revolution

The lead was described clearly for the first time in the third century BC. There was no surprise in the fact that people were not much interested in it. In the earlier days, the people that suffered from this disease were the artisans i.e. the craftsman, mechanics, etc. They mostly belonged to the lower class and their symptoms were not projected in general [26].

The sewage and water systems were a great step in the hygienic conditions in ancient Rome. It gave the representation of the most important element to prevent the development of epidemic episodes in one of the greatest cities of that period. As the material that the pipes were made of was lead, it gave the metal salts to the water that passed through them.

It resulted in many diseases in the people, who drank that water i.e. the people who ruled the Empire. The diseases included shorter expectancy of life, lower rates of birth, and a short life expectancy because of the great plasmatic amount of the lead [12]. In the fifteenth century, because of the development in the culture and economy, the instructors of the workshop plus the trainers started moving towards the big cities. Due to the usage of the colors i.e. the carbonate (white lead) that

was based on the lead, the painters were more exposed to the lead in comparison to the other workers [30].

2.2 Lead Pollution and Exposure during the Industrial Revolution

By the 19th century the adverse effects of metal intake were quite well known and the knowledge of the recurring lead poisoning was also made sure at the same time. At the time of the industrial revolution the precautionary measures if present were inadequate which increased the amount of workers affected by the continued metal poisoning and the resultant interest of doctors towards them [13]. From ancient times lead has been used for various purposes and with the association of this use many harmful effects of lead on human body has been seen. In early second century physicians come to know that there exist a link between lead exposure and mental disorders. There are several professions in which adults exposure to lead is very high and these occupations are metal welding, making lead storage batteries, lead smelting and mining, painting and construction work [14].

2.3 First Ever Preventive Strategies Against Lead Exposure

The first preventive strategies in factories were introduced during the mid-20th century, such as exhaust ventilation, the elimination of the use of lead, soaking dusty processes, and PPE's (personal protective equipment's). Chelating Agents were also introduced and used as a therapeutic tool against the lead poisoning. At the same time, some other therapeutic and preventive practices were also introduced along with the scientific and a technological advance, feeding the workers with 1 liter of milk per day was one of them [15]. Milk, due to its whiteness, was considered to be a purifying substance. Some of the scientists also agreed on the

belief of assuming the role of calcium in hindering and delaying the lead absorption [16]. The development of new methods for the measurement of lead in biological media continued in the late 1960s, whereas, the international debates focused on finding the "safe" level of lead exposure [17]. The identification of sub-clinical effects of lead intoxication, during the last decades, have led to an acknowledgement of the damages of the environmental pollution caused by the burning of TEL to the general population.

Children were found to be more sensitive to the soil pollution caused by lead because of their hand to mouth activity [12]. Therefore, TEL was slowly substituted by benzene and decrease in the blood lead level was documented soon after in the general population, during the late eighties and early nineties in the industrialized countries [16]. Therefore, in many countries of the world lead is an important public health problem. Recently the World Health Organization (WHO) global burden of disease estimated and found 9.8 million disability-adjusted life years (DALYs) as a result of lead induced mild mental retardation, however, 3.1 million DALYs each year are recorded as a result of cardiovascular disease. Since 1988, Environmental lead has been recognized as a public health problem in Pakistan. High blood lead levels formed the basis of phasing out lead from petrol in 2001, since several small- scale studies have highlighted the issue of lead in blood [18].

2.4 Assessment of Lead Exposure Across Pakistan

The World Health Organization (WHO) has reported lead contamination level in ground and waste water being greater than acceptable limit, in various regions of Pakistan.The highes concentration of lead recorded by WHO in Pakistan is 4.7mg/L. Similarly, elevated atmospheric lead concentration can be seen in various cities of Pakistan. For example, despite the decline in lead concentration over the past few years by the use of Pb-free gasoline, Islamabad shows elevated Pb concentration of up to 4.7 g/m3 [1]. From the combustion of industrial oil and

coal, approximately 450 million kg per annum of lead is released. Lead emission from natural sources is around 30 million kg per annum.Vegetable species in Pakistan have elevated levels of lead, up to 44mg/kg.The main causes of high lead contamination levels are soil exposed to industrial runoff, waste water & mining activities [1].

2.5 Major Communities and Work Environments in Pakistan Affected by Lead Exposure

A research in Agha Khan University, Karachi has shown 10 μg/dl of lead concentration in the blood of 80% of children aged 3 to 5 years. Elevated lead concentrations in blood was associated with the population living near the city centre, children hand to mouth activities, and application of surma in the eyes. In Pakistan, survey for lead-poisoning in lead factory showed blood lead levels of 61.20 μg/dl in the factory workers.

In Islamabad, traffic policemen have elevated blood lead levels as shown by the studies. However, in another research on policemen from Karachi, has shown that they have even greater level of lead in their blood as they have a greater contact with public and private transport vehicles as compared to policemen in Islamabad [7].

Table 2.1: Lead Contamination Levels Observed in Water, Air and Vegetables across Various Regions of Pakistan [37].

Source	**Region**	**Province**	**Safe Pb levels by WHO & EU**	**Pb-Concentration**
Ground Water	Zhob River	Baluchistan	0.01mg/L	0.05mg/L
Ground Water	Sialkot	Punjab	0.01mg/L	0.49mg/L
Ground Water	Karachi	Sindh	0.01mg/L	0.02-.073mg/L

Ground Water	Hattar	KPK	0.01mg/L	2.34mg/L
Ground Water	Pearl Valley	Azad Jammu & Kashmir	0.01mg/L	<0.03-4.7mg/L
M.sylvestris (Crab Apples)	Northern Areas	Gilgit-Baltistan	0.01-0.3mg/kg	20mg/kg
Spinach	Faisalabad	Punjab	0.01-0.3mg/kg	2.251mg/kg
Lettuce	Quetta	Baluchistan	0.01mg/L	5.94mg/kg
Potato	Shorkot	Sindh	0.01-0.3mg/kg	0.49mg/kg
Rural/Urban Atmosphere	Islamabad	Punjab	<0.5 g/m3	4.7g/m3

2.6 Major Sources of Lead Exposure

Natural and anthropogenic processes cause and source of distribution of lead in the environment, though anthropogenic spread and release of lead is more prevalent. Some of the major contributors of lead in the environment include the industrial releases from nonferrous smelters, battery plants, chemical plants, and disturbance of older structures that are comprised of lead- based paints. Lead is extremely determined and persistent in water and in soil as well. Lead contamination of soil, sediments, surface water and ground water is due to the direct application of fertilizers that are mainly lead-contaminated sludge, and residues of lead arsenate used in agriculture.

The major air emission of lead in the countries where lead gasoline is still used, is mainly from the mobile and stationary sources of combustion. Another source of lead exposure may arise from the foods or beverages stored, cooked or served in lead containing containers, the traditional remedies, cosmetics, and food that is grown on contaminated soils, cosmetics and other lead-containing products [18].

Lead exposure in children's blood lead levels is caused by the lead in dust and water that contribute up to 35% and 20% respectively [19].

2.7 Toxic Effects of Lead Exposure on Human Body

Ingestion of food or water contaminated with lead leads to the poisoning due to lead. However, Lead poisoning is also caused by the accidental ingestions of contaminated soil, dust or lead based paint.

Lead has some adverse effects on certain organ systems, and it is thought to be quickly absorbed in the blood stream [4].

2.8 Effect of Lead Exposure on Nervous System

The exposure of lead can reduce the cognitive functioning in the youngsters. The children exposed by the lead scores less score as compare to others. In exploring pediatric cross sectional studies on theoretical deficiencies following the exposure of lead, 3-point.

Decrements in IQ have been noted when blood lead focus expanded from 5 to 20 g/dL and 5.3-point decrements in IQ when blood lead fixation expanded from 5 to 50 g/dL.

The chronic exposure is confirmed to be more harmful for adults to cognition than acute exposure. Some examination has shown quite less learning and memory scores from the adults which are exposed by the lead.

These outcomes propose that lead exposure is especially inconvenient in case of the adults, with people old enough 55 and even above that showed lower learning and memory scores, among other reasoning decays [19].

2.9 Effect of Lead Exposure on Reproductive System

The ability of lead is that it can influence the reproductive systems of both male and female. The sperm count declines and many different changes occur in the volume of it, which normally happens when the blood level surpasses 40 μg/dL.

Exercises like motility and general morphology of sperm are additionally affected at this level. The issues with the reproductivity of females because of the exposure of lead are way more complicated.

Lead can prompt unsuccessful labors, rashes, low birth weight, and issues with improvement during adolescence because of the toxic levels.

The level of blood in mother and newborn children are generally comparable as the lead present in mother's blood goes into the fetus through the placenta and furthermore through the mother's milk [19].

2.10 Effect of Lead Exposure on Renal System

Low level of lead exposure in environment was found to be associated with enhanced deterioration and worsening of chronic renal insufficiency. Accelerated progression of chronic renal disease was predicted when both blood lead level and blood lead burden were found to be increased, even at low lead exposure level.

Persistent lead exposure is found to be the cause of high blood pressure, coronary heart disease, heart rate variability, and death from stroke, however, this evidence is limited as per shown by some of the studies done. Kidney damage caused by lead might show as protein in the urine (albuminuria), elevated blood pressure, or a higher risk of gout. Lead levels should be examined in people who appear to have kidney disease, high blood pressure, or gout. Years of lead exposure revealed in Kidney operation by moderate focal atrophy, loss of proximal tubules and interstitial fibrosis are the main causes of "Chronic lead nephropathy" [4].

2.11 Traditional Methods of Removing Heavy Metals from Environment

Older chemical methods of removing heavy metals pose a serious threat to environment. Precipitation, ion exchange, electrolytic technologies and chemical extraction etc are conventional methods which are employed for getting rid off from heavy metals but all these methods have some adverse effects on environment and mankind.

Some other methods like vapor extraction, stabilization, solidification and membrane technologies are also used for removing heavy metals but these technologies are not cost effective, efficiency of these processes are not very satisfying and handling of process is also very laborious [26].

2.12 Bioremediation a Safer Way towards Cleaning Environment

Much advancement has been made in the techniques of bioremediation. The main aim of these techniques is to clean up the environmental pollutant in an ecofriendly and low cost budget .The adoption of a particular bioremediation technique depends upon the type of pollutant which is under consideration for remediation. As different pollutant demands different remediation technique so a single bioremediation technique is not enough for all type of pollutant.

Each technique has its own pros and cons and is adapted according to the situation and conditions available [3]. The indigenous microbial flora of an environment plays a key role in removing the pollutant from a site. The activity of microbes highly depends upon the biotic and abiotic factors of environment. If optimal conditions for the growth of microbes is present then the remediation activity is very fast [20]. Bioremediation must involve some biological mechanisms for removal of pollutant from the surrounding environments [21].

2.13 Major Approaches in Lead Bioremediation

Lead bioremediation can be classified into biosorption and bioaccumulation. Biosorption is a rather propitious application of bioremediation, and plays an integral part in removing heavy metals. Biosorption is an emerging technology and a passive uptake process.

For the most part, this technology is reversible and independent of a bacteria's metabolic capability. Biosorption involves the adsorption on lead on the surfaces of biological substances. Biosorption is advantageous because cheapness, simplicity, effectiveness, and eco-friendly nature. Bioaccumulation on the other hand, is a complicated technique in which heavy metals are stored inside a

bacterial cells [22].

2.14 Lead Bioremediation by Bacteria

Extensive research has been done in utilizing living and dead bacteria as biosorbsents. Using microbial biomass to absorb heavy metals from polluted substances is a potentially promising alternative.

The natural benefit that comes from using microbes for bioremediation of lead is that microbial biomass to (e.g. Citrobacter, Pseudomonas, Streptomyces, Bacillus etc.) can be extracted as a by-product similar to fermenting industries, and the microbes absorb a considerable portion of heavy metal ions, which in turn leads to the transfer of metals to a contaminated biomass. Bacteria have a specific set of genes that drive mechanisms involved in reduction of contaminants from the environment.

Examples of some important bacterial strains used in the extraction of heavy metals from sewage water and soil include *Bacillus* and *Pseudomonas*, due to their high metal binding capabilities. Major Functional groups of bacterial cells that drive absorption of heavy metals from aqueous media include hydroxyl, carboxyl,

sulfonate, amide and phosphonate groups [23].

TABLE 2.2: Examples of Bacterial Species associated with Lead Biosorption.

Bio Absorbent	**Lead Biosorption Capacity (mg/g)**	**Citation**
Bacillus firmus	467	[24]
Enterobacter cloacae	2.3	[25]
Micrococcus luteus	1965	[26]
Alcaligenes sp	56.8	[27]

2.15 Factors Affecting Lead Bioremediation in Microorganisms

Bioremediation affinity of microbes can be reduced by multiple biotic and abiotic factors which influence their growth and metabolism. Microbes generally have a natural capability to adapt to severe environmental factors. However, these microorganisms are not without some limitations. Hence, it is essential to take such limiting factors into account in order to develop an optimal strategy for bioremediation of heavy metals [23].

2.16 Deleterious Effects of Lead on Microbes

Heavy metals can cause damaging effects on microbes which is absorbing it and the extent of damage depends upon the amount of metal uptake. Heavy metals like lead can harms bacteria by several ways even destroying its DNA and proteins [28]. The normal metabolic activities of a microorganism are disturbed when a considerable amount of heavy metal is deposited in microbial cell. The accumulation of lead in microbe leads to the destruction of whole organism. Due to lead accumulation DNA of microbe is disturbed, as DNA all the functions of cell

like biochemical reactions, shape, structure, and growth rate. So when DNA is damaged all these processes of cell are also badly affected [29].

2.17 Mechanisms Adopted by Microbe to Crash Lead

Different types of mechanisms are adopted by microbes to live in environment where heavy metals are in high concentrations. Metal oxidation, methylation, enzymatic decrease and chelation are some mechanisms adopted by microbe to tolerate heavy metals [26]. Bacterial cell wall contain polysaccharide, proteins, and lipids. These polysaccharide layers have ion exchange properties and due to this property it can bind lead to its surface. Other metals like Cd and Zn can also bind to cell surface but in less proportion. Lead due to its large ionic size and high atomic weight is considered to more efficiently adsorb at the surface of cell containing polysaccharide layer [30]. In gram positive bacteria cell wall contain teichoic acid and phosphate groups the metals present in environment could bind to these negativly charged groups of cell wall and hence heavy metals are cleaned up from environment [31].

2.18 Molecular Docking

It is technique used to estimate the strength of a bond between a ligand and a target protein through a special scoring function and to determine the correct structure of the ligand within the target binding site. The 3D structure of the target proteins and the ligands is taken as the input for docking. It represents a frequently used approach in structure-based drug design since it requires 3D structure of a target protein. It can be used to determine the correct structure of the ligand within the target binding site, and to estimate the strength of the binding between the ligand and the target proteins through a specific scoring function [22]. Each docking program uses one or more specific search algorithms, one

of which is used to predict possible compliance with the receptor-ligand complex [23]. Currently, molecular docking is becoming a key tool for drug discovery and molecular modeling applications. The reliability of molecular docking depends on the accuracy of the scoring function, which can guide the ligand pose and determine when thousands of possible lines can be generated. [24]. In addition, there are some tools like Dock, Gold, Flex X-One ICM that are mainly used for high docking inclusion [25].

Molecular docking can reveal the viability of any biochemical reaction that is performed before the experimental part of an investigation. Particularly, the interaction between small molecules (ligands) and protein targets (which may be an enzyme) may predict inhibition or activation of an enzyme. This type of information can provide raw material for interactions between a protein and ligand. [26].

2.19 Problem Statement

Chemical ways of removing heavy metals have cost, handling and toxicity problems. On the other hand enhanced biosorption and bioaccuulation mechanisms of removing waste is considered safe because these methods are cost effective and environmental friendly. Not much is known about the major bacterial species found in local flora of Pakistan that possess lead bio sorption and lead bioaccumulation properties.

2.20 Aims and Objectives

1. Estimation of indigenous bacteria present in lead contaminated regions of Faisalabad, Pakistan.

2. Screening of lead tolerating bacterial strains followed by identification.

3. Verification of biotransformation and uptake in the lab.

4. Insilico identification of transport protein involved in uptake of bioavailable lead salt.

5. Identification of enzymatic proteins involved in bioconversion of highly toxic lead salts into less toxic form.

Chapter 3

Methodology

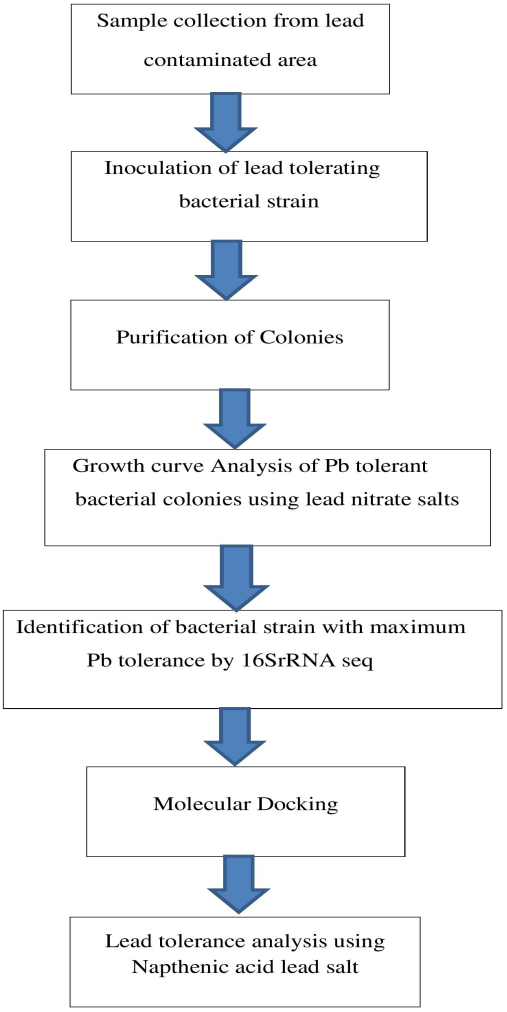


FIGURE 3.1: Flowchart of Methodology.

3.1 Sample Collection from Lead Contaminated Zones

Three main regions in Faisalabad were selected for the purpose of this experiment due to their relatively high recorded rates of lead contamination in Pakistan. These regions include Chakeera (lead concentration of 68.03 ppm) D-Type Colony (lead concentration of 50.42) and Muzaffar Colony (lead concentration of 45.02 ppm).

Since these areas have textile industries which are source of heavy metal contamination. Soil samples A, B and C were collected from Chakeera, D- Type Colony and Muzaffar Colony respectively [32].

3.2 Inoculation of Lead-tolerant Bacteria

Inoculation of bacteria from aforementioned samples was performed on lead-based culture media, comprising of 13g/L LB Broth, 15g/L Tryptic Soy Agar (TSA) and three different concentrations of lead nitrate (2g/L, 2.5g/L and 3g/L), the latter was done to analyze bacterial growth for different concentrations of lead.

The pH of prepared culture media was maintained between 6 – 6.5 (this is because pH concentration of original samples was below 6.5) and serial dilutions of up to 10-7 were prepared for each sample, and dilutions between 10-3 and 10-7 were selected for the purpose of inoculation. Inoculation was performed using glass spreader followed by incubation at 37^oC for 72 hours to ensure proper bacterial growth [33].

3.3 Purification of Lead Tolerant Bacterial Colonies

Bacterial colonies obtained from the previous procedure were then purified via streaking method. This step was performed to obtain distinct colonies of lead-tolerant bacteria, as well as to reduce the possibility of contamination. Each

colony was inoculated onto a separate culture medium containing the same concentration of lead nitrate, LB Broth and TSA as their respective culture medium. This was followed by incubation at 37^oC for 72 hours. A total of 14 distinct colonies were obtained from this procedure. These colonies were identified based on varying morphological characters including size, shape, surface tension, elevation, pigmentation and margin [34].

3.4 Growth Curve Analysis of Lead Tolerant Bacterial Colonies

3.4.1 Preliminary Analysis of Lead-tolerant Bacterial Colonies

Following purification, each colony was transferred to Broth medium containing 13g/L of LB Broth and 3g/L of lead nitrate. The Bacterial cell growth was determined by measuring absorbance percentage using UV spectrophotometer after intervals of 48 and 72 hours inoculum was incubated at 37^oC for each time interval. The purpose of this procedure was to identify bacterial colonies having the highest lead tolerance levels. Percentage absorbance values for each colony is proportional to their microbial growth i.e. higher percentage absorbance indicates higher bacterial growth rate [35].

3.4.2 Analysis of Lead Tolerance Levels in Bacterial Colonies Using Lead Nitrate Salt

Based on preliminary analysis, five bacterial colonies were further subjected to growth curve analysis via UV Spectrophotometer using lead nitrate salt. These included 3 colonies from Sample A (A3, A5 and A6) and 2 colonies from Sample C (C3 and C6). Each colony was then inoculated in LB Broth cultures, with lead nitrate concentrations ranging from 6g/L to 9g/L in order to analyze bacterial growth at varying time intervals for relatively higher lead concentrations as well

as to quantitatively analyze their lead tolerance range against lead nitrate salt. The strain showing relatively highest levels of lead tolerance range was further selected for strain identification via Gene Sequencing [35].

3.5 Selection of Protein

Structure of proteins was obtained from Protein Data Bank by entering PDB i.d of proteins which is mostly 4 letter code in .pdb format. PDB archive is the only source of information about the 3D structure of large biological molecules, including nucleic acids and proteins.

Those proteins whose structures was not available at PDB was predicted by using I-TASSER through submitting the sequence of the targeted protein in the FASTA format. I-Tasser server is designed to automatically predict the structure of full-length 3D proteins.

I-Tasser server output for all queries included up to five full-length models, the confidence scores, standard deviation of estimations and estimated TM scores and RMS [44].

3.6 Identification of Functional Domains of Target Proteins

InterPro provides effective protein analysis by separating them from families and predicting domains and active sites. To classify proteins, InterPro uses predictive forms, called as signatures.

Using a variety of information (known as member databases) and provided an Interprofessional Consortium [45]. For predicting active sites of protein SWISS Similarity tool was used.

3.7 Ligand Preparation

The 3-dimensional (3D) structure of ligands was obtained from PubChem. The PubChem is the world‘s largest repository of freely accessible chemical information database. We can search number of ligands by their names, molecular formula, structure and by other information [46]. If targeted structure is not available PubChem, then it will be drawn via ChemDraw by inserting Canonical smileys derived from PubChem. MM2 Energy minimization was identified by Chem3D ultra then Ligand structure was downloaded in .sdf form

3.8 Molecular Docking

Molecular Docking is technique used to estimate the strength of a bond between a ligand and a target protein through a special scoring function and to determine the correct structure of the ligand within the target binding site. The 3D structure of the target proteins and the ligands is taken as the input for docking. Molecular docking of protein and ligand was done through Cavity-detection guided Blind Docking (CB-Dock). CB-Dock is a method of protein ligand docking that is used to identify binding sites, calculates the size and center automatically and personalize the docking box size and perform the molecular docking through AutoDock Vina [42]. We Uploaded 3D structures of protein (.pdb) and ligand (.sdf) and submit to start docking. CB-Dock had provided an interactive 3D visualization of results in 5 different poses. Best pose was selected on basis of minimum vina score given in (kJ/m^{-1}).

3.9 Visualization of Ligand/Protein via PyMol

Docked complex of ligand and protein was visualized by PyMol. It is a free open source of molecular visualization that can generates best 3D images of proteins, small molecules, nucleic acids, and electron densities etc. This is capable of editing

molecules, ray tracing and making movies [47]. Docking poses generated via CB-Dock was visualized and saved as a molecule in .pdb form in one file for further analysis.

3.10 Analysis of Docked Complex via LigPlot

Analysis of docked complex (.pdb) was done by LigPlot, that generates automatically schematic diagram of protein ligand interactions for given PDB file. These interactions are modified through hydrophobic contacts and hydrogen bonds.

In Computational biology, LigPlot generates schematic 2D representations of protein-ligand complex, which facilitates the rapid examination of many enzyme complexes and demonstrates an informative and simple representation of the intermolecular interactions, these includes hydrophobic interactions, hydrogen bonds and atom accessibilities [48].

3.11 Analysis of Lead Tolerance Levels in Bacterial Colonies Using Naphthenic Acid Lead Salt

Five bacterial colonies were further subjected to growth curve analysis via UV Spectrophotometer using Naphthenic acid lead salt. These included 3 colonies from Sample A (A3, A5 and A6) and 2 colonies from Sample C (C3 and C6).

Each colony was then inoculated in LB Broth cultures, with naphthenic acid lead salt concentrations ranging from 6g/L to 9g/L in order to analyze bacterial growth at varying time intervals for relatively higher lead concentrations as well as to quantitatively analyze their lead tolerance range against naphthenic acid lead salt.

3.12 Sequencing of the Final Strain

Once the atomic absorption spectrometry is done and the results derived illustrate the bacterial species with the best lead absorption capability, DNA Sequencing of the specific bacterial strain will be done in the next step for the identification of the strain. The technique used for this purpose is 16S rRNA, through which the bacterial phylogeny and taxonomy is illustrated. 16S rRNA gene sequencing is basically used for the classification, identification, and quantitation of the bacteria and other microbes that are present in environmental samples and gut samples [36].

3.13 Phylogenetic Analysis of the Final Strain

After obtaining 16S rRNA results, phylogenetic analysis of identified sequence was performed using MEGA X tool, to obtain its evolutionary relationship with the closest known ancestors and published sequences in the NCBI GenBank amd to properly visualize the obtained strain's evolutionary relationship with previously known strains. Sequence alignment was performed to analyze the obtained genetic sequence relative to the retrieved NCBI sequences. Phylogenetic Analsysis was performed using Neighbor Joining method [34].

Chapter 4

Results and Discussions

4.1 Isolation and Inoculation Results

Bacterial colonies were successfully isolated following inoculation on culture medium containing LB Broth, TSA and Lead Nitrate. Colonies were successfully cultured for all three lead nitrate concentrations (2g/L, 2.5g/L and 3g/L), maximum colonies were obtained for 2g/L and minimum number of colonies were obtained for 3g/L of Lead nitrate. Optimum growth of bacterial colonies was obtained at 37^oC temperature and 6.5 pH after 72 hours of incubation. Temperature variations such as increasing temperature to 42^oC or deceasing temperature to 25^oC significantly decreased growth of bacterial colonies. Sample A (obtained from Chakeera) showed maximum growth of colonies, whereas, minimum growth of colonies resulted from sample C (obtained from Muzzafar colony).

The lead contamination relatively recorded is the highest in the three areas of Faisalabad which are: Chakeera (lead concentration of 68.03 ppm) D-Type Colony (lead concentration of 50.42) and Muzaffar Colony (lead concentration of 45.02 ppm), since these areas have textile industries which are source of heavy metal contamination. The highest concentration of lead was found in Chakeera at Paharang drain, because the wastewater of all the city were finally drained into the Paharang drain [38]. The microorganisms have existed for longer periods of time in

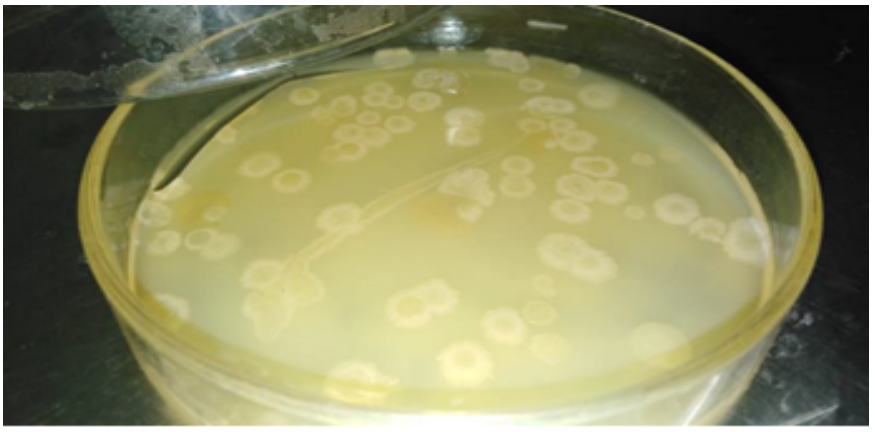


FIGURE 4.1: Inoculation results of bacterial colonies Isolated from retrieved samples.

lead contaminated areas so their tolerance to lead and absorbance is much higher and increased as they have lived as communities in these areas. The properties and ability of lead tolerance and absorbance in these microbial communities is relatively more, so if bacterial species have been living in these areas then there are chances that they might have developed an ability of tolerance and absorbance of lead and would also be able to do lead bio-sorption.

4.2 Purified Inoculum Results

A total of 18 purified bacterial colonies were obtained, out of which 14 had distinct morphological characters. Sample A and Sample C gave 6 colonies each, with sample B only showing 2 distinct colonies.

FIGURE 4.2: Purified bacterial colonies obtained from Sample.

TABLE 4.1: List of colonies and their morphological charcters.

Sample	**Colony**	**Morpholoical characters**
A	A1	Whitish
	A2	Light Brown
	A3	Colorless, Sharp margins
	A4	Fire like pattern
		Foggy surface texture
	A5	Leaf like appearance,
		Rough surface, clear margins
	A6	Circular, Hard texture, Dark brown
B	B1	Small size, Yellowish
	B2	Spots, Elevated Colonies
C	C1	Broad Margins
	C2	Narrow Margins
	C3	Dark brown coloration
	C4	Sticky Surface
	C5	Elevated Colonies
	C6	Sticky surface, Elevated brown colonies

Colonies were divided into three samples A, B and C. Sample A and Sample C gave 6 different colonies each which were labelled as A1 to A6 and C1 to C6 respectively, while sample B gave 2 colonies, indicating apparently lower soil microbial diversity in regards to lead tolerance in D-type colony. After distinguishing between morphological features and variations of each sample we can conclude that the sample which shows the most diversity has the most diverse bacterial colonies present in them, which in our case is both Sample A and Sample C which shows that they have higher microbial diversity present in their communities or colonies. This also indicates that they have more bacterial communities present which have more tolerance and absorbance capabilities in them against lead contamination &

have the ability to do lead biosorption.

4.3 Growth Curve Analysis Results

The following are growth curve analysis results of purified bacterial colonies obtained via UV Spectrophotometric analysis. Preliminary Analysis Results using UV Spectrophotometer.

4.3.1 Preliminary Analysis Results using UV Spectrophotometer

Results from preliminary analysis indicate growth of majority of bacterial colonies for the provided range of lead nitrate concentration. Results were calculated after 48 and 72 hours of incubation at 37^oC. Percentage absorbance of each colony is directly proportional to their microbial growth. Out of the 14 colonies, 5 showed relatively higher values of absorbance for highest concentration of lead nitrate (3g/L) after 72 hours, indicating a comparatively higher lead tolerance than other colonies. Out of these 5 colonies, 3 were from Sample A (A3, A5 and A6) and 2 were from Sample C (C3 and C6). These colonies will be further analyzed for lead tolerance and lead biosorption range.

Table 4.2: Preliminary Growth Curve Analysis of Purified Bacterial Colonies and lead nitrate after 48 Hours.

Sample	**Serial No**	**% Absorbance**		
		2g/L Pb $(NO_3)_2$	2.5g/L Pb $(NO_3)_2$	3g/L Pb $(NO_3)_2$
A	A1	0.514	0.389	0.404
	A2	0.365	0.333	0.286
	A3	0.286	0.787	0.532
	A4	0.426	0.342	0.436
	A5	0.242	0.302	0.398

	A6	0.584	0.314	0.980
B	B1	0.362	0.534	0.360
	B2	0.405	0.407	0.348
C	C1	0.255	0.309	0.379
	C2	0.105	0.273	0.439
	C3	0.477	1.008	0.377
	C4	0.401	0.232	0.526
	C5	0.134	0.294	0.356
	C6	0.177	0.462	0.341

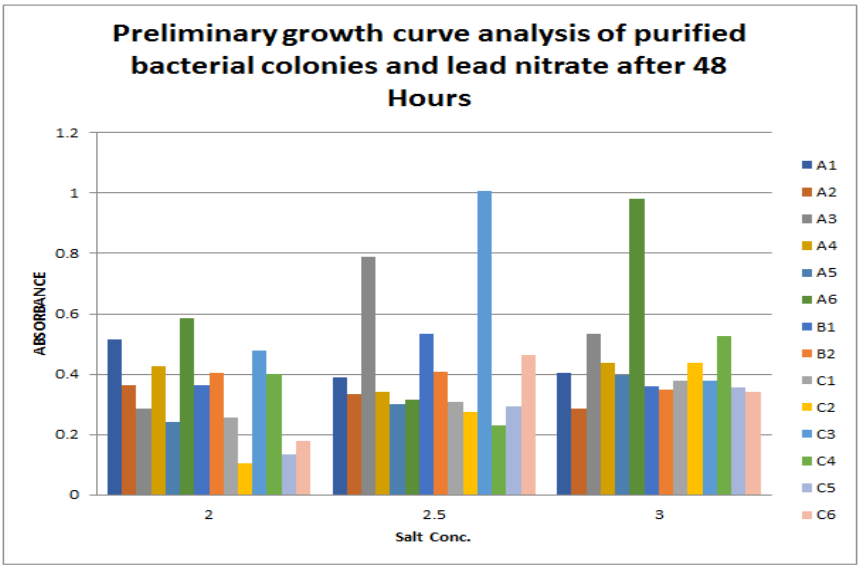


FIGURE 4.3: Preliminary growth curve analysis of purified bacterial colonies and lead nitrate after 48 Hours.

TABLE 4.3: Preliminary Growth Curve Analysis of Purified Bacterial Colonies and lead nitrate after 72 Hours.

Sample	**Serial No**	**% Absorbance**		
		2g/L Pb $(NO_3)_2$	2.5g/L Pb $(NO_3)_2$	3g/L Pb $(NO_3)_2$
A	A1	0.451	0.610	0.460
	A2	0.420	0.151	0.323
	A3	0.661	0.845	0.579

	A4	0.324	0.359	0.388
	A5	0.636	0.236	0.713
	A6	0.483	0.563	1.009
B	B1	0.468	0.505	0.404
	B2	0.638	0.362	0.197
C	C1	0.442	0.328	0.400
	C2	0.156	0.472	0.452
	C3	0.830	1.091	0.423
	C4	0.399	0.411	0.413
	C5	0.324	0.294	0.344
	C6	0.590	0.466	0.593

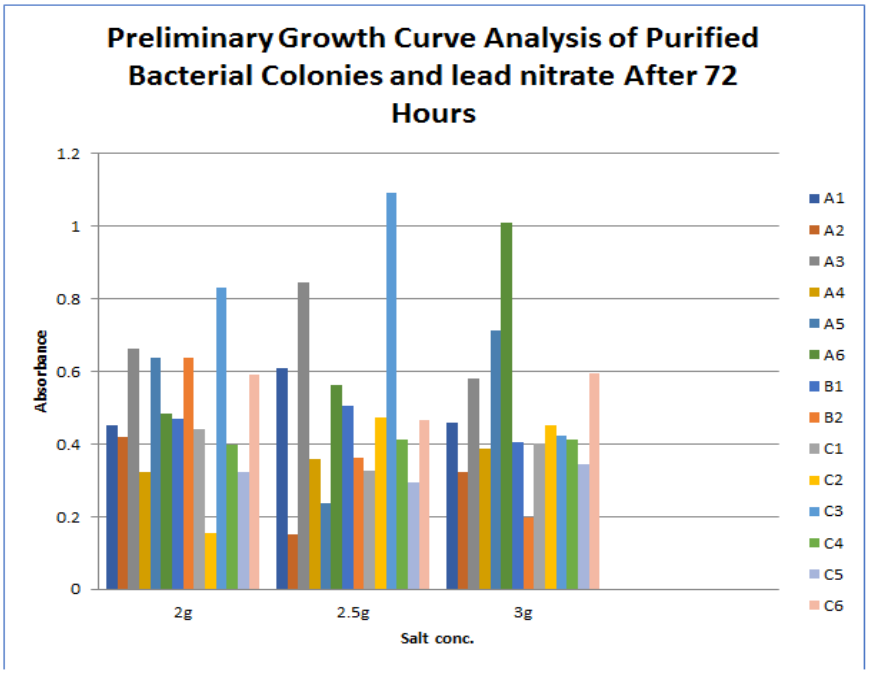


FIGURE 4.4: Preliminary Growth Curve Analysis of Purified Bacterial Colonies and lead nitrate After 72 Hours.

For Lead tolerance we started our work from a preliminary analysis in which we identified lead tolerance in all of our bacterial colonies by the usage of spectrophotometer. Results collected from the preliminary analysis showed that all 14 observed bacterial colonies were able to absorb lead to some extent. However, we finalized the best 5 colonies, which were giving the highest lead tolerance results.

Nearly all of the colonies exhibit similar growth rate when grown in broth culture containing lead nitrate. This not only confirms that Chakeera, D-Type Colony and Muzaffar Colony in Faisalabad have a highly toxic level of lead exposure[38], but also that long-term exposure has caused majority of soil microbiota in those regions to develop considerable amount of lead tolerance. Industrial waste samples were collected from 3 different areas in Faisalabad, which were continuously being contaminated by industrial waste.

In the Preliminary growth curve analysis of purified bacterial colonies after 48 hours, Sample A has 6 colonies out of which highest lead tolerance is shown in colony A6 at 3g/L (0.980), A6 at 2g/L (0.584) and A3 at 2.5g/L (0.787). Other colonies are also showing positive tolerance results but were comparatively less significant than the aforementioned colonies. Same is the case with sample B, lead tolerance is present but to a limited extent that is not so notable, highest was shown in B1 at 2.5g/L (0.534) On the other hand, best tolerance results were shown by the sample C in colony C3 at 2.5g/L (1.008).

Preliminary growth curve analysis of purified bacterial colonies after 72 Hours showed A6 at 3g/L (1.009), A3 at 2.5g/L (0.845), A5 at 3g/L (0.713), rest of the sample A colonies were also showing lead tolerant capabilities however, they were not very significant. The 2 colonies of Sample B were showing limited amount of tolerance, the highest was shown by B2 at 2g/L (0.638). Sample C had 6 colonies out of which C3 at 2.5g/L was showing (1.091) which was the highest level of tolerance among all the 14 colonies of three samples. C3 at 2g/L (0.830) other than this rest of the colonies were showing lead tolerance which was not very notable.

4.3.2 Analysis of Lead Tolerance Levels in Bacterial Colonies Using Lead Nitrate Salts

Growth curve analysis results obtained at different time intervals for different bacterial colonies shown in the following tables.

Table 4.4: Absorbance of isolates against lead nitrate salt at 48,72,96and 120 hour.

Sample ID	**Incubation Time Intervals**	**Absorbance**			
		6g/L $Pb(NO_3)_2$	7g/L $Pb(NO_3)_2$	8g/L $Pb(NO_3)_2$	9g/L $Pb(NO_3)_2$
A3	48	0.842 ±0.03	0.350 ±0.35	0.604 ±0.03	0.339 ±0.03
	72	0.383 ±0.21	0.172 ±0.35	0.849 ±0.021	0.105 ±0.01
	96	0.302±0.04	0.134±0.22	0.742±0.03	0.101±0.04
	120	0.212±0.05	0.103±0.20	0.654±0.06	0.023±0.05
A5	48	0.591± 0.02	0.454±0.32	0.279±0.04	0.743±0.21
	72	0.528 ±0.06	0.438 ±0.34	0.275 ±0.06	0.195 ±0.24
	96	0. 566±0.09	0.512±0.45	0.321±0.01	0.102±0.53
	120	0.500±0.08	0.562±0.462	0.354±0.03	0.041±0.12
A6	48	0.503 ±0.02	0.274± 0.10	0.671± 0.04	0.613± 0.31
	72	0.594 ±0.01	0.817 ±0.18	0.731 ±0.01	0.559 ±0.08
	96	0.590±0.42	0.821±0.30	0.798±0.07	0.551±0.02

Continue Table 4.4: Absorbance of isolates against lead nitrate salt at 48,72,96and 120 hour.

Sample ID	**Incubation Time Intervals**	**Absorbance**			
		6g/L $Pb(NO_3)_2$	7g/L $Pb(NO_3)_2$	8g/L $Pb(NO_3)_2$	9g/L $Pb(NO_3)_2$
A6	120	0.500±0.33	0.843±0.41	0.891±0.02	0.598±0.05
C3	48	0.538 ±0.04	0.702 ±0.03	0.545 ± 0.04	0.588 ±0.05
	72	0.863 ±0.01	0.881 ±0.07	0.593 ±0.01	0.253 ±0.01
	96	0.921±0.65	0.960±0.08	0.035±0.05	0.231±0.22
	120	0.965±0.23	0.987±0.02	0.502±0.21	0.221±0.02
C6	48	0.611 ±0.34	0.145 ±0.01	0.514 ±0.01	0.372 ±0.06
	72	0.891 ±0.65	0.115±0.08	0.565 ±0.04	0.183 ±0.03
	96	0.811±0.43	0.432±0.05	0.621±0.03	0.631±0.06
	120	0.721±0.26	0.466±0.45	0.652±0.02	0.431±0.06

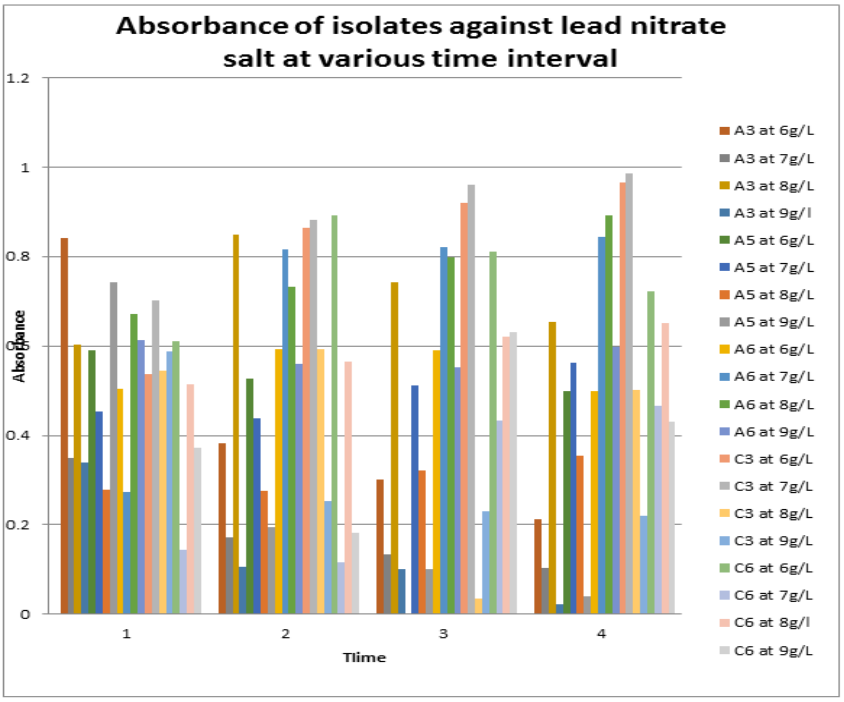


FIGURE 4.5: Absorbance of isolates against lead nitrate salt at various time interval.

Finally, 5 bacterial colonies (A3, A5, A6, C3 and C6) were selected which were again given some high levels of lead nitrate salt to check their tolerance level, compared to other observed colonies. The amount of lead was increased in this step and results were collected after 48, 72, 96 and 120 hours. We noticed that lead was absorbed but asorbance is not very much significant. Then we performed molecular docking so that we could found the lead salt which is significantly absorbed by the strain.

4.4 Proteins of *Bacillus spp*

Below is table of proteins which includes all those proteins of Bacillus spp. of bacteria which have hydrolase, nuclease, oxidation, reduction, transport, binding and ion uptake function because these proteins could be helpful for carrying out the process of biosorption or biotransformation. Some hypothetical proteins was also under consideration for predicting their role in biosorption or biotransformation process.

TABLE 4.5: Properties of some proteins retrieved from *Bacillus spp.*

S.No	P.D.B id	Actual name of protein	Function	Length/ No. of Amino Acid	Domain predicted by InterPro Scan
1	4RK9	Multiple sugar binding protein (MSmE)	Transport Protein	501	Non cytoplasmic domain 22-501 Signal peptide domain 1-22
2	5BRP	Glycoside hydrolase family	Hydrolase	568	Glycosyl hydrolase family 13 catalytic domain 22-424 Alpha amylase 37-382 Maltogenic amylase 485-558
3	1BLI	Alpha Amylase	Hydrolase	483	Glycosyl hydrolase family 13 catalytic domain 5-391 Alpha amylase 31-371

Continue Table 4.5: Properties of some proteins retrieved from *Bacillus spp.*

S.No	P.D.B id	Actual name of protein	Function	Length/ No. of Amino Acid	Domain predicted by InterPro Scan
4	1NRF	Membrane protein	Regularity Protein	601	Peptidase M56 11-308 Transpeptidase 348-590
5	2J74	Endo beta glactanse	Hydrolase	424	Glycosyl hydrolase family-53-24-421
6	4OTT	Gamma glutamyl trans peptidase	Hydrolase	585	G-glu-transpept super family43-375 G-glu-transpept super family 1-171
7	3RJL	1pyroline 5carboxylate dehydrogenase	Oxidoreductase	516	Aldehydedehydrogenase domain 50-512

Continue Table 4.5: Properties of some proteins retrieved from *Bacillus spp.*

S.No	P.D.B id	Actual name of protein	Function	Length/ No. of Amino Acid	Domain predicted by InterPro Scan
8	3OQI	Putative uncharacterized	Ligase and Transferase acitvity	249	Cyclodipeptide synthase 13-233
9		ATP binding cassete domain containing protein	Transport Protein	318	ABC transporter type domain 6-250 ATPase Domain 35-277 ABC transporter 27-177 Oligopeptidase domain 227-311 and 229-291
10		BACLD Nitrate transporter	Nitrate transmembrane transporter activity	401	Major facilitator superfamily domain 13-389

Continue Table 4.5: Properties of some proteins retrieved from *Bacillus spp.*

S.No	P.D.B id	Actual name of protein	Function	Length/ No. of Amino Acid	Domain predicted by InterPro Scan
11		Flavin reductase family protein	Binding protein	205	Flavin Rdtase like domain 20-177
12	1SCB	Subtilisin Carlesberg	Protease	379	Subtilisn carlesberg like catalytic domain 130-357 Peptidase S8 propeptide/ proteinase inhibitor I9 Domain 44-103 Subtilase family 128-368
13	5OMT	NUCB Endonucleases	hydrolase	142	Deoxyribonuclease NucA/NucB 45-137 family
14	6DZD	Hypothetical protein	Not predicted	279	Multi-copper polyphenol oxidoreductase 24-278(family)

Metal oxidation, methylation, enzymatic decrease and chelation are some mechanisms adopted by microbe to tolerate heavy metals [44]. Lead due to its large ionic size and high atomic weight is considered to more efficiently adsorb at the surface of cell containing polysaccharide layer [30].

In gram positive bacteria cell wall contain teichoic acid and phosphate groups the metals present in environment could bind to these negativly charged groups of cell wall and hence heavy metals are cleaned up from environment [31].

As Bacillus spp. have Gram positive cell wall so we choose the proteins of this bacterium for performing molecular docking.

The 3D structure of the target proteins was taken as the input for docking. It represents a frequently used approach in structure-based drug design since it requires 3D structure of a target protein.

It can be used to determine the correct structure of the ligand within the target binding site, and to estimate the strength of the binding between the ligand and the target proteins through a specific scoring function [40].

3D structures of *Bacillus spp.* proteins, retrieved from proteins data bank and these structures was cleaned by using discovery studio. Proteins whose 3D structures was not available at PDB, was obtained by I-TASSER.

4.5 Ligand Molecules

Lead nitrate and Nahthneic acids, lead salts are selected as ligand molecule. 3D structure of lead nitrate is not available at pubchem. Hence canonical smileys for lead nitrate is copied from pubchem and by using these smileys 3D structure of ligand molecule is drawn at ChemD.

3D structure of Naphthenic acid lead salt is available at pubchem .After having the 3D structures of both molecules energy minimization step is performed using chem 3D ultra.

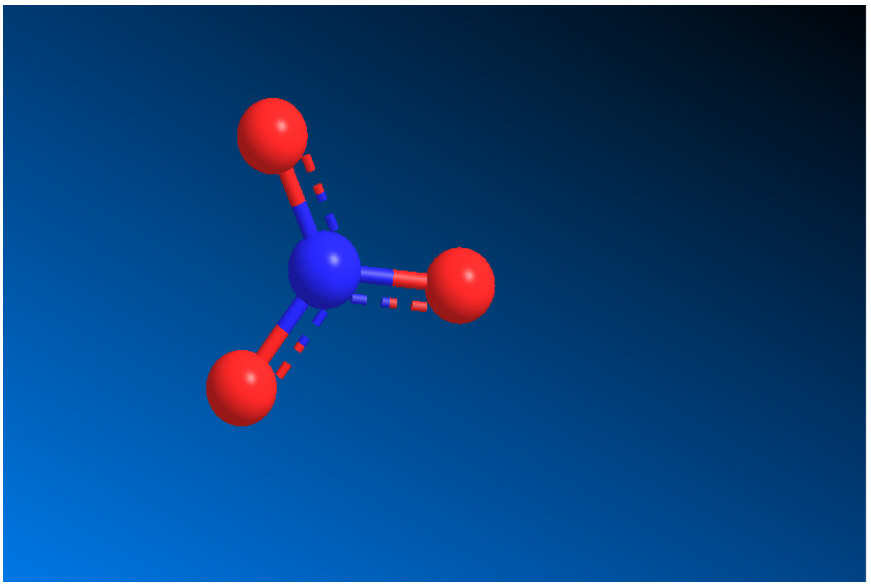


FIGURE 4.6: 3D structure of lead nitrate.

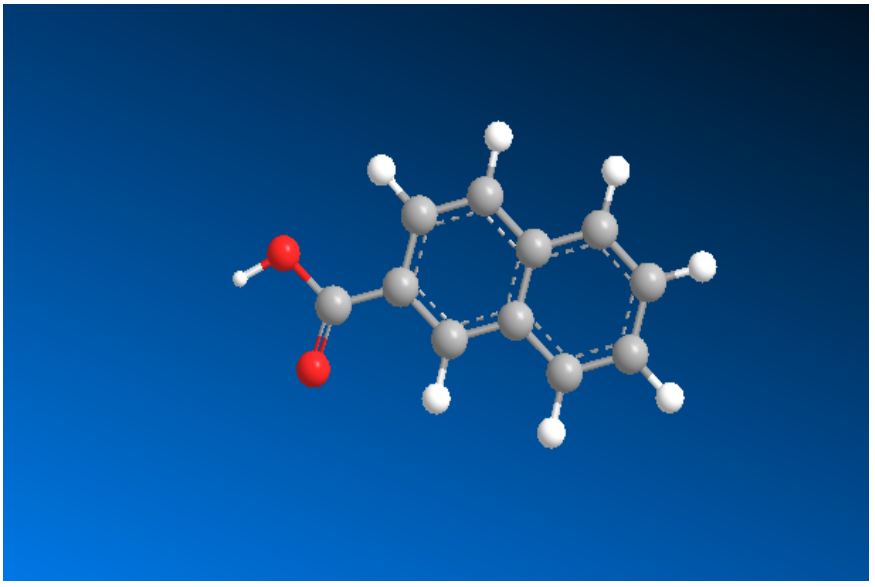


FIGURE 4.7: 3D structure of Naphthenic acids, lead salts.

TABLE 4.6: Properties of Lead nitrate and Naphthenic acids, lead salts .

Properties	**Lead nitrate**	**Naphthenic acids, lead salts**
Molecular weight	331.208	549
Number of rotatable bonds	0	0
Number of H-bond acceptors	6	4

Number of H- bond donors	0	0
Molecular Formula	N_2O_6Pb or Pb $(NO_3)_2$	$C_{22}H_{14}O_4Pb$

The ligand molecules was also prepared for the purpose of docking. This technique used to estimate the strength of a bond between a ligand and a target protein through a special scoring function and to determine the correct structure of the ligand within the target binding site. The 3D structure of the target proteins and the ligands is taken as the input for docking[43].we performed the energy minimization step of ligand which is necessary when 3D structure of ligand is downloaded from pubchem. This step is not very much necessary when we draw the molecule at chem draw but consider as a precautionary step. The energy minimization step of naphthenic acid lead salt is completed successfully but when we minimize the energy of lead nitrate it ends with energy minimization error.

4.6 Molecular Docking

Molecular docking of protein and ligand was performed through Cavity-detection guided Blind Docking (CB-Dock). Protein ligand docking is a powerful tool for computer-aided drug discovery (CADD). An interactive 3D visualization of results in 5 different poses were obtained via CB-Dock. Best pose was selected on basis of minimum vina score given in (kJ/ m^{-1}). Ligands with best binding scores were shown in Table 4.7.

Molecular docking can reveal the viability of any biochemical reaction that is performed before the experimental part of any investigation. Particularly, the interaction between small molecules (ligand) and protein targets may predict inhibition or activation of an enzyme.this type of information can provide raw material for interaction between a protein and ligand [44]. From the below table it clear that the docking score is very low when naphthenic acid lead salt is taken as ligand molecule as compared to when ligand was lead nitrate.

TABLE 4.7: Different Proteins & Ligands with best binding scores.

Actual name of proteins	**Binding score (kJ/m) lead-nitrate**	**Binding score(kJ/m) Napthenic-acid, lead salts**
Multiple sugar binding protein (MSmE)	-7.5	-3.5
Glycoside hydrolase family	Docking error	-3.4
Alpha Amylase	-6.7	-3.4
Membrane protein	-6.3	-3.4
Endo beta 1,4 glactanse	-7.7	-3.5
Gamma glutamyl transpeptidase	-7.1	-3.1
Putative uncharacterized protein	-7.6	-3.4
ATP binding cassete domain containing protein	-7.1	-2.9
BACLD Nitrate transporter	-8.2	-3.3
Flavin reductase family protein	-7.8	-
Subtilisin Carlesberg	-5.3	-2.9
NUCB Endonucleases	-6.1	-2.8
Hypothetical protein	-6.7	-3.5

4.7 Interaction of Ligands and Targeted Proteins

In Computational biology, LigPlot generates schematic 2D representation of protein-ligand complex, which facilitates the inspection of various enzyme complexes and demonstrates an informative and simple representation of the intermolecular interactions and their strength. These includes hydrogen bonds, hydrophobic interactions and atom accessibilities.

Analysis of docked complex (.pdb) was done by LigPlot, that generates automatically schematic diagrams of protein-ligand interaction for given PDB file. Following are the figures which show ligand interactions with the selected proteins.

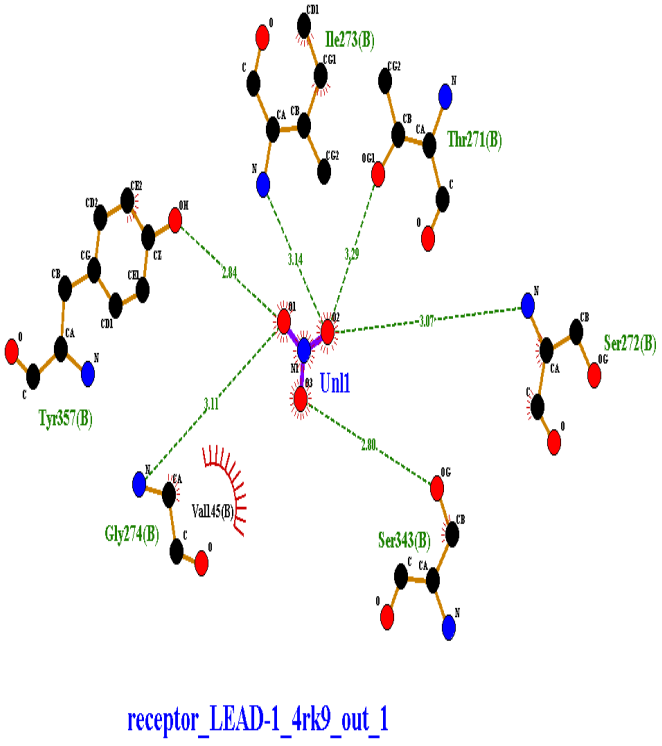


FIGURE 4.8: 2D representation of docked complex 4RK9 and lead nitrate.

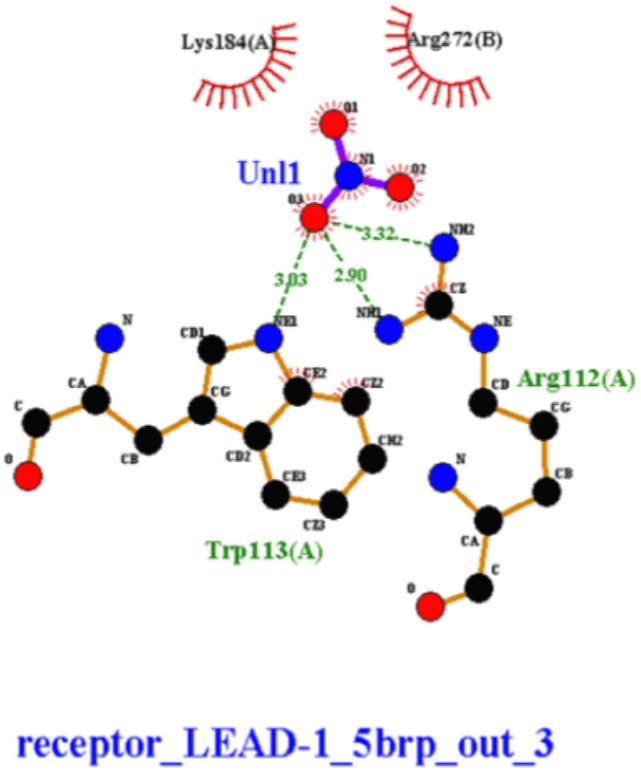


FIGURE 4.9: 2D representation of docked complex 5BRP and lead nitrate.

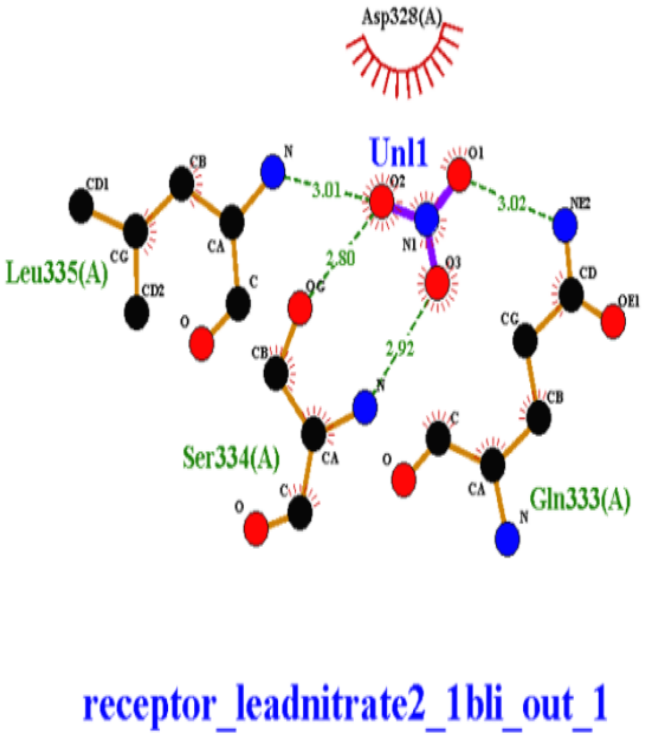


FIGURE 4.10: 2D representation of docked complex 1BLI and lead nitrate.

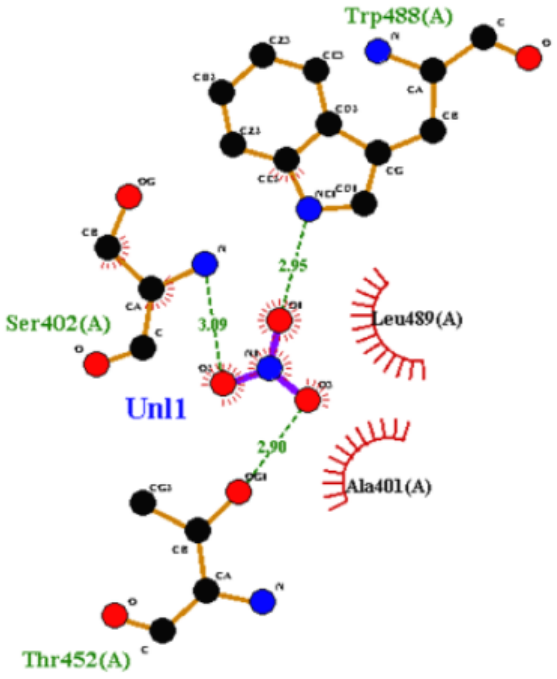


FIGURE 4.11: 2D representation of docked complex 1NRF and lead nitrate.

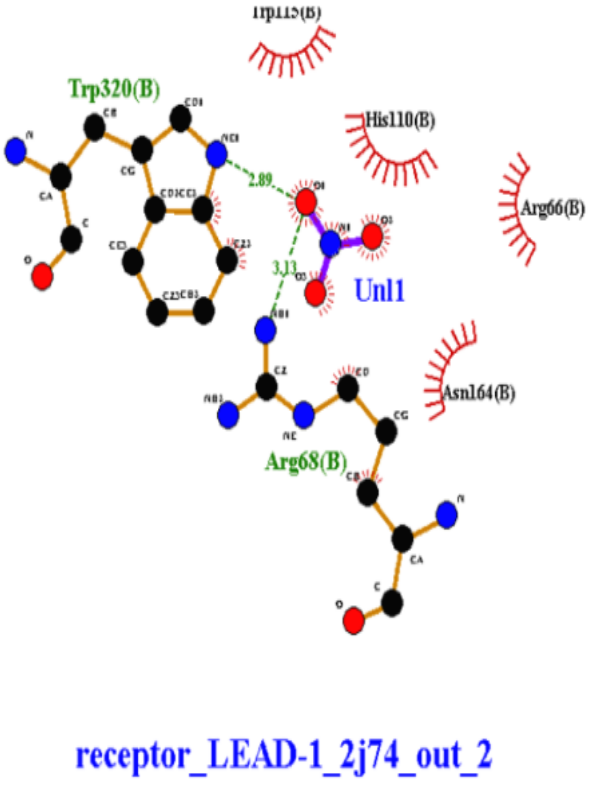


FIGURE 4.12: 2D representation of docked complex 2J74 and lead nitrate.

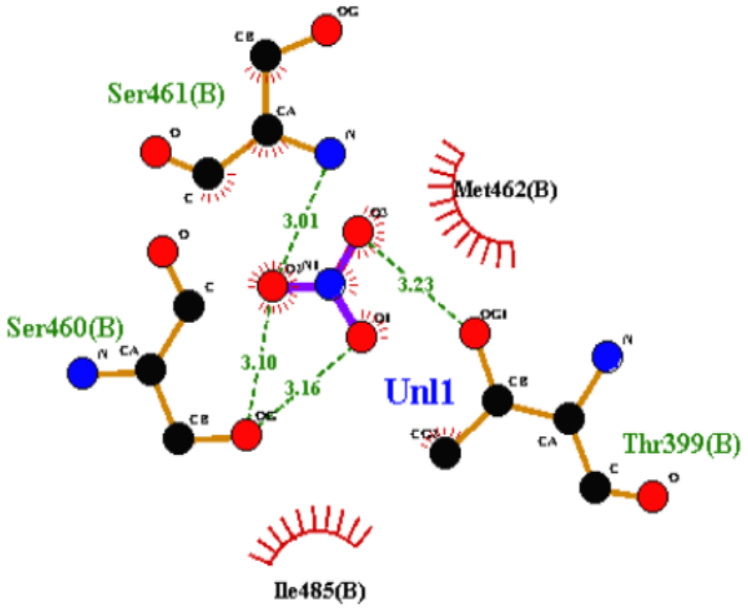


FIGURE 4.13: 2D representation of docked complex 4OTT and lead nitrate.

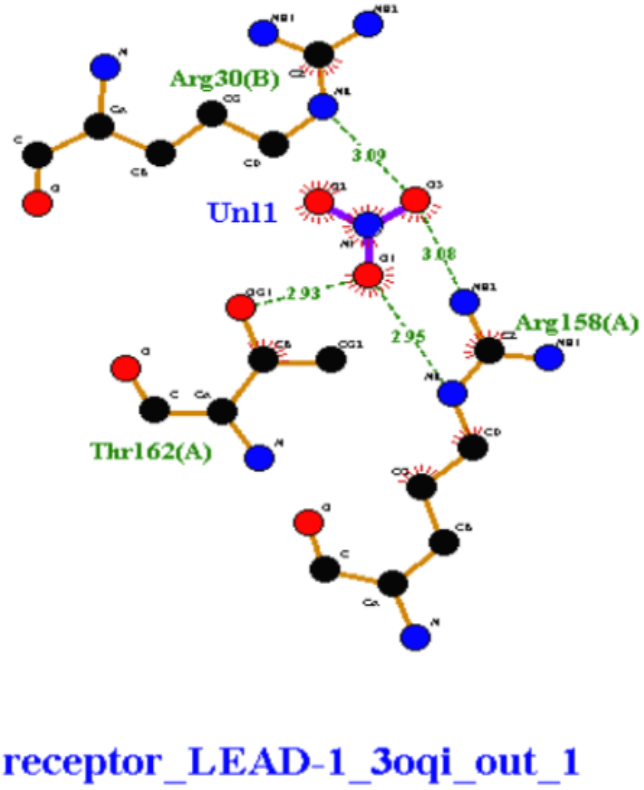


FIGURE 4.14: 2D representation of docked complex 3OQI and lead nitrate.

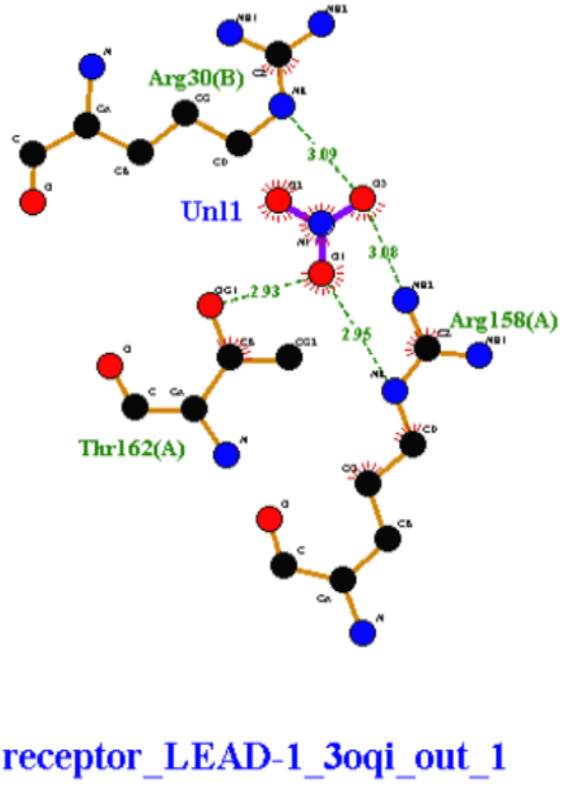


FIGURE 4.15: 2D representation of docked complex 5OMT and lead nitrate.

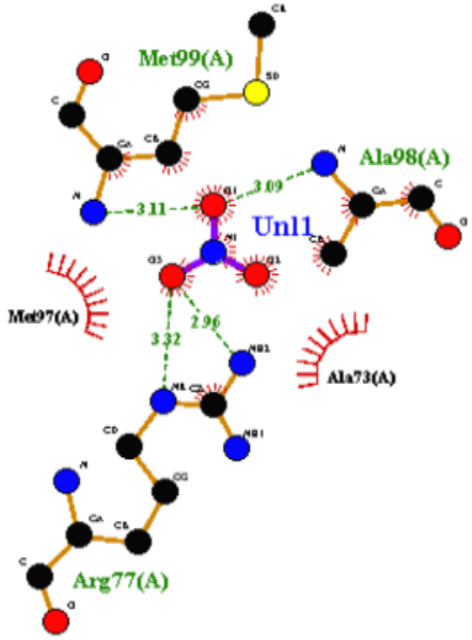


FIGURE 4.16: 2D representation of docked complex 5OMT and lead nitrate.

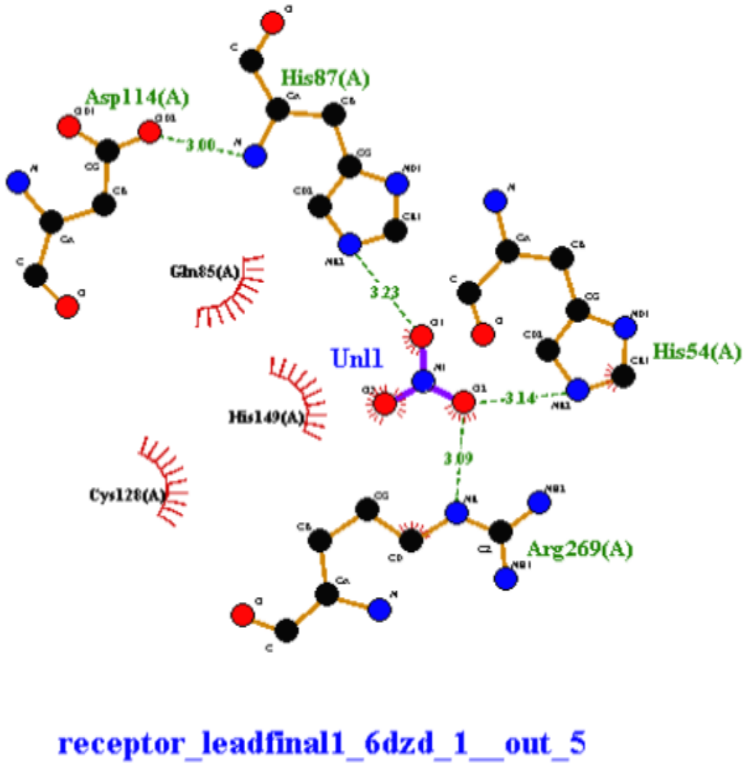


FIGURE 4.17: 2D representation of docked complex 6DZD and lead nitrate.

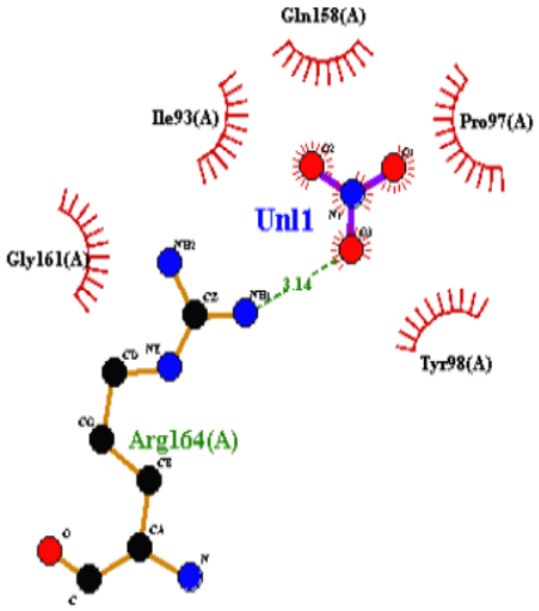


FIGURE 4.18: 2D representation of docked complex ATP cassette binding protein and lead nitrate.

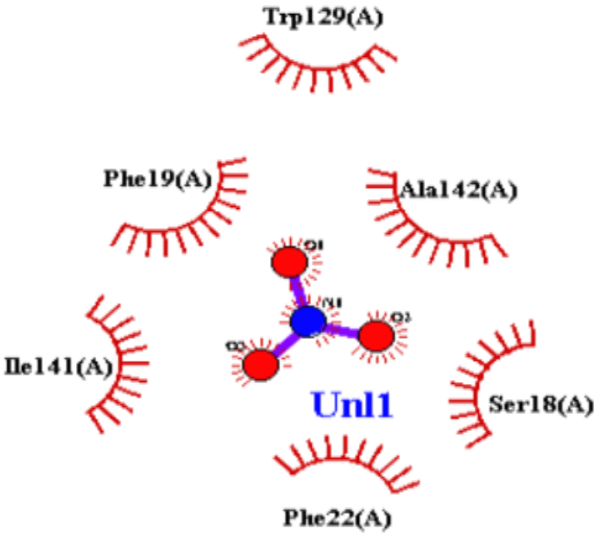


FIGURE 4.19: 2D representation of docked complex nitrate transporter protein and lead nitrate.

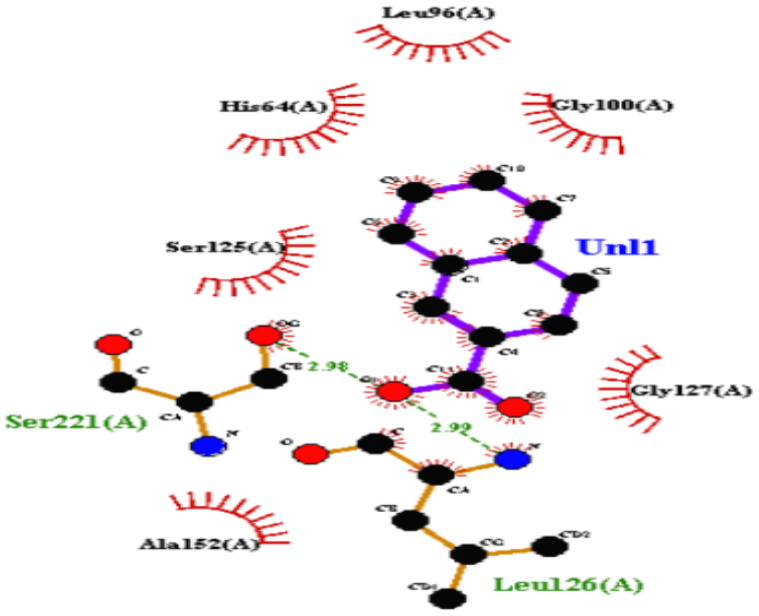


FIGURE 4.20: 2D representation of 1SCB protein with Naphthenic acids, lead salt.

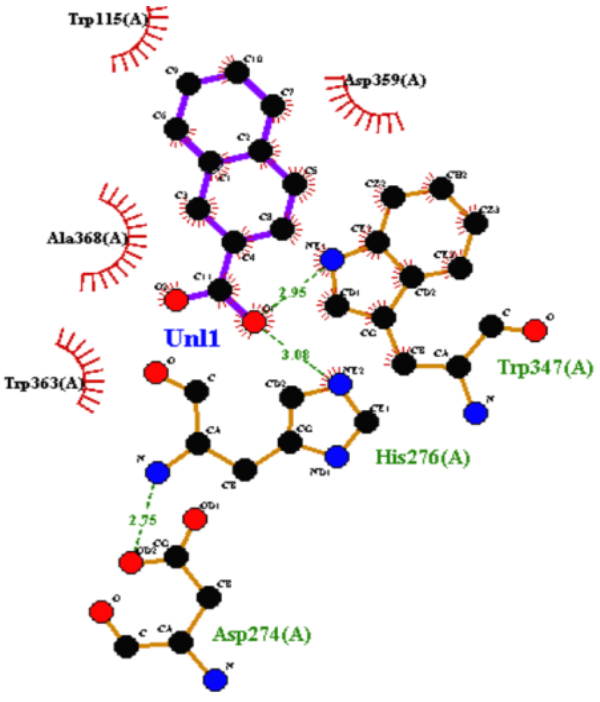
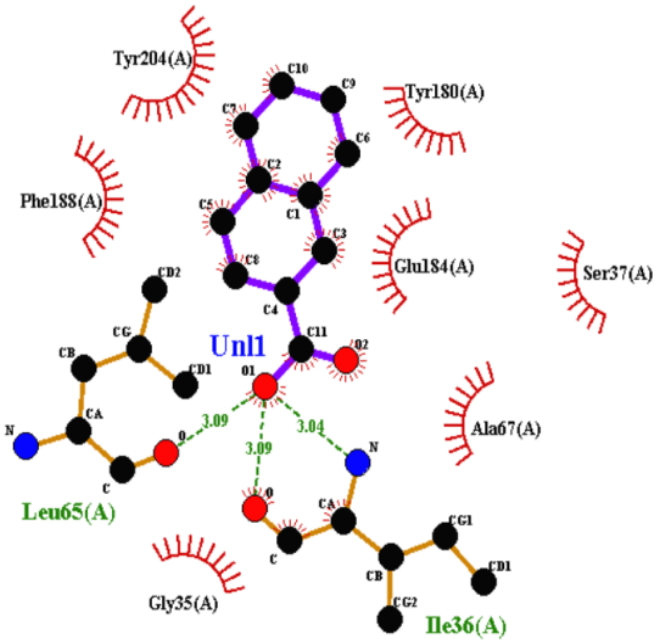
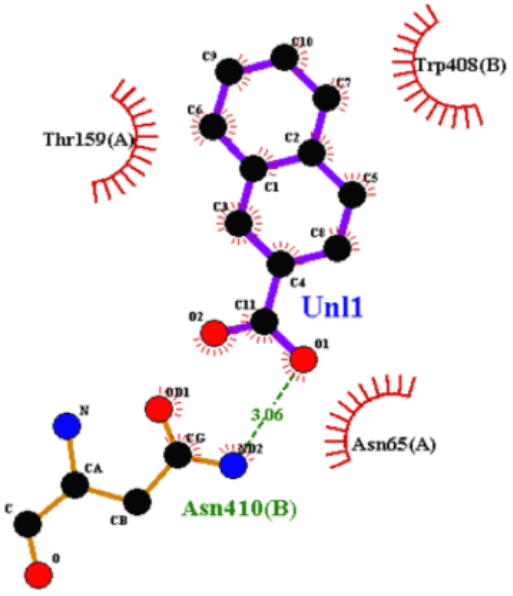


FIGURE 4.21: 2D representation of 2J74 protein with Naphthenic acids, lead salt.



receptor_LEADSALT_3oqi_out_2

FIGURE 4.22: 2D representation of 3OQI protein with Naphthenic acids, lead salt.



receptor_LEADSALT_4ott_out_4

FIGURE 4.23: 2D representation of 4OTT protein with Naphthenic acids, lead salt.

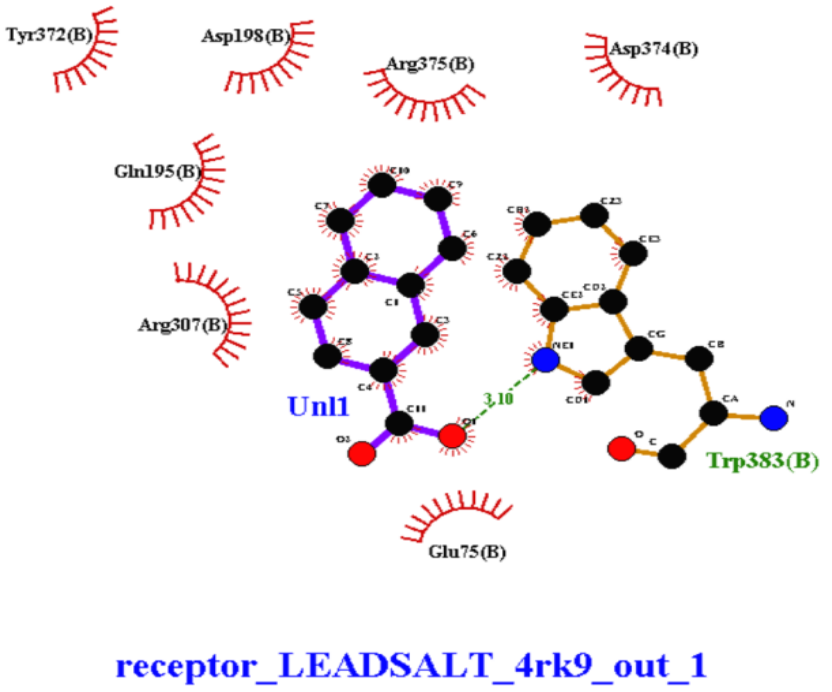


FIGURE 4.24: 2D representation of 4RK9 protein with Naphthenic acids, lead salt.

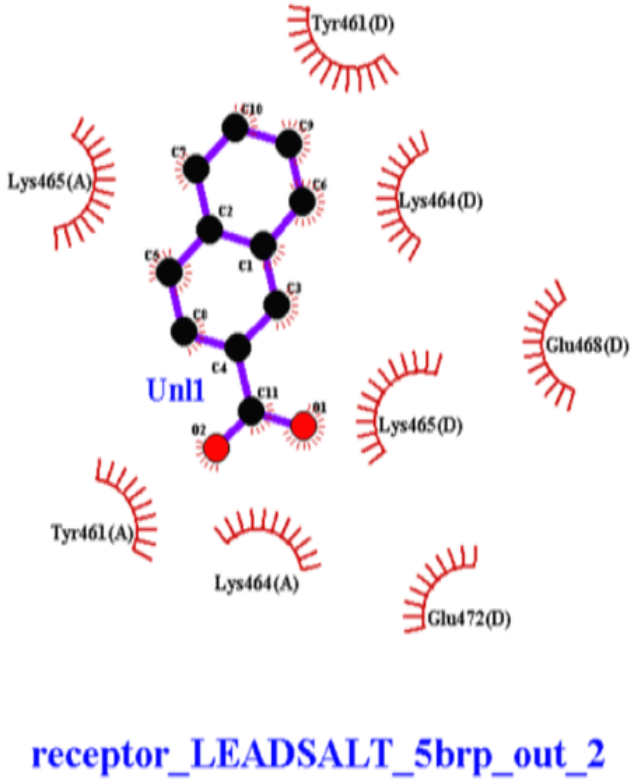


FIGURE 4.25: 2D representation of 5BRP protein with Naphthenic acids, lead salt.

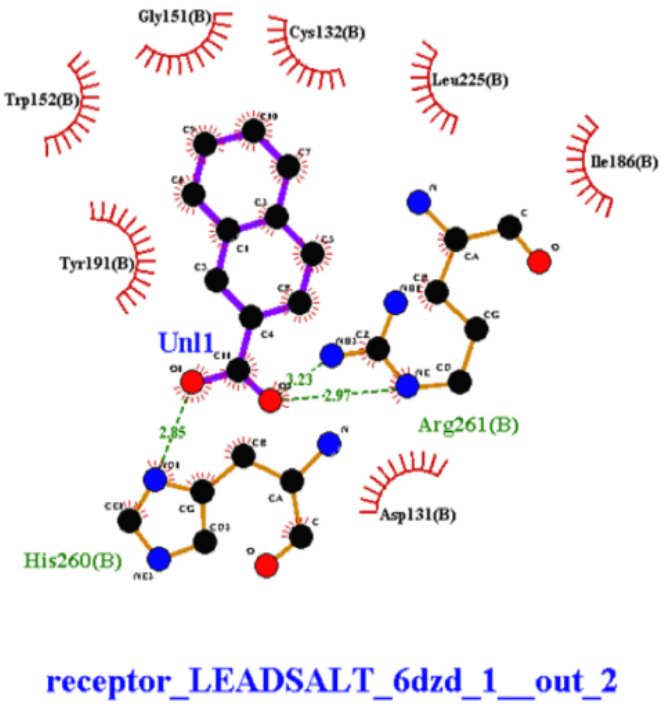


FIGURE 4.26: 2D representation of 6DZD protein with Naphthenic acids, lead salt

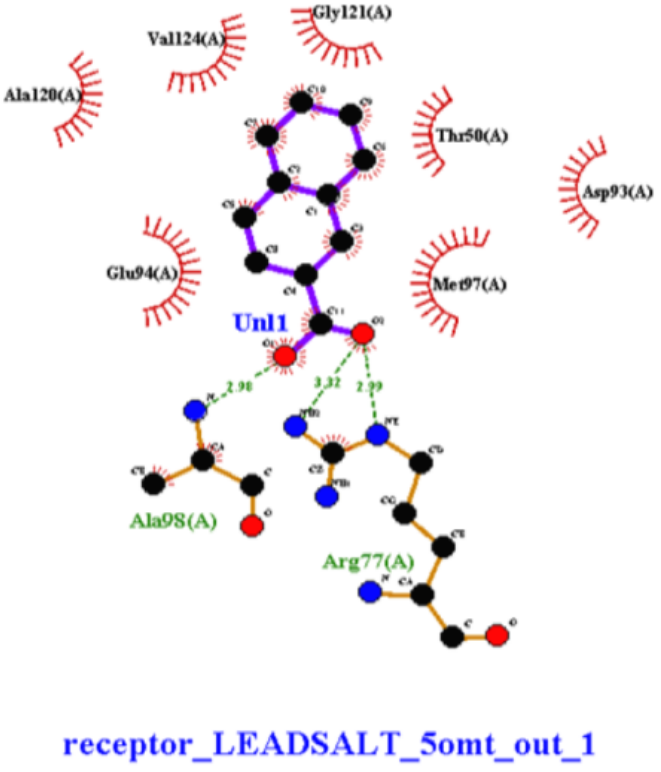


FIGURE 4.27: 2D representation of 5OMT protein with Naphthenic acids, lead salt.

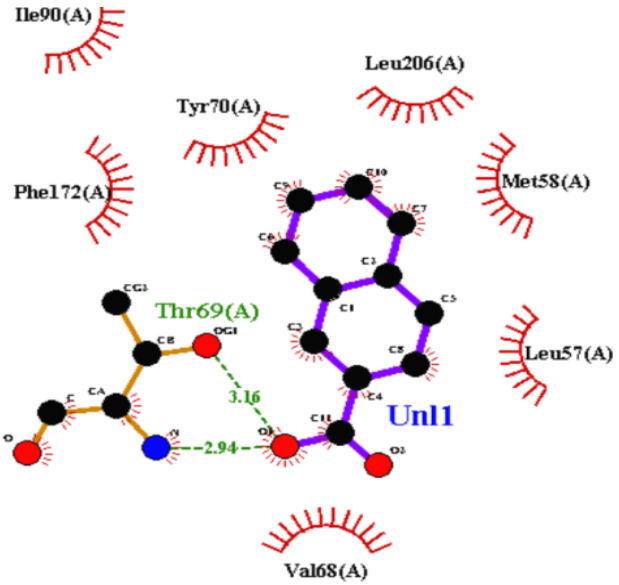


FIGURE 4.28: 2D representation of ATP cassette binding protein with Naphthenic acids, lead salt

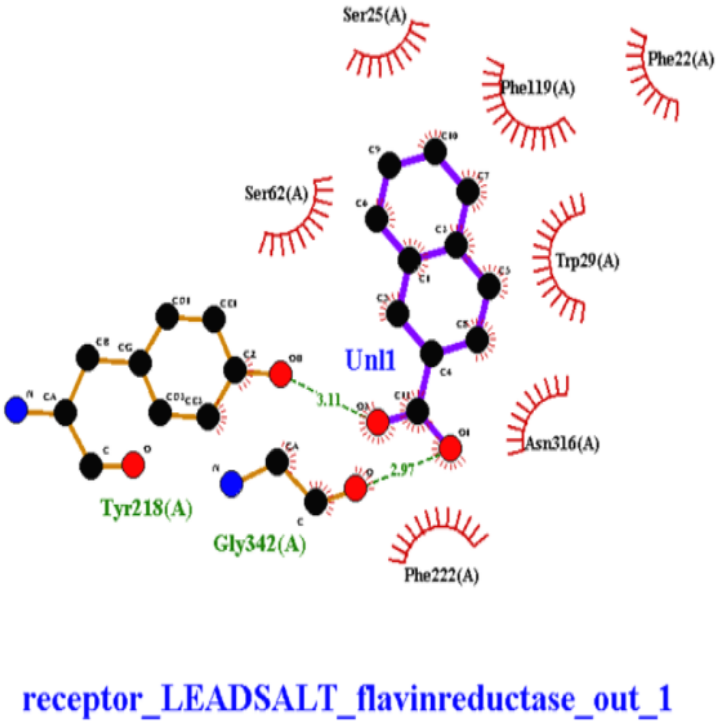


FIGURE 4.29: 2D representation of Flavin reductasefamily protein with Naphthenic acids, lead salt

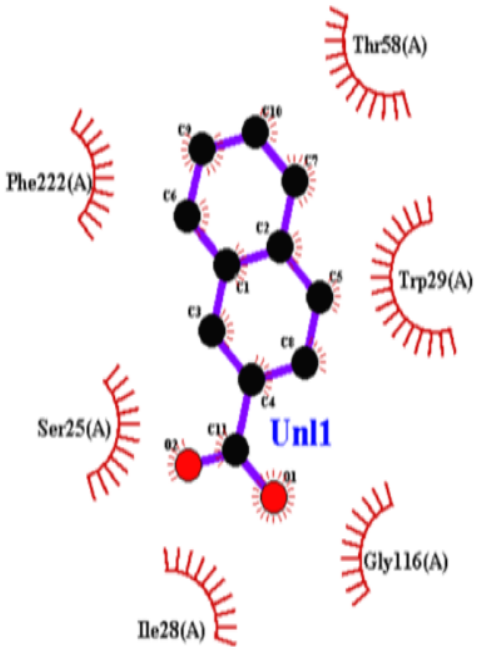


FIGURE 4.30: 2D representation of Nitrate transporter protein with Naphthenic acids, lead salt.

In Computational biology, LigPlot generates schematic 2D representations of protein ligand complex, which facilitates the rapid examination of many enzyme complexes and demonstrates an informative and simple representation of the intermolecular interactions.

These includes hydrophobic interactions, hydrogen bonds and atom accessibilities [48]. Ligplot interactions shows that both lead nitrate and naphthenic acid could interact with proteins of *bacillus spp*, means that bacterial specie have the ability to adsorb, absorb or transform the heavy metal Lead. This means that bacterial specie have the ability to adsorb, absorb or transform the heavy metal Lead.

H-bonding is shown by dotted lines only one protein i.e nitrate transporter protein show no hydrogen bonding with both of the ligands which reveals that bacterial strain under consideration has no role in transport of lead salts.

4.8 Analysis of Lead Tolerance Levels in Bacterial Colonies Using Naphthenic Acid Lead Salts

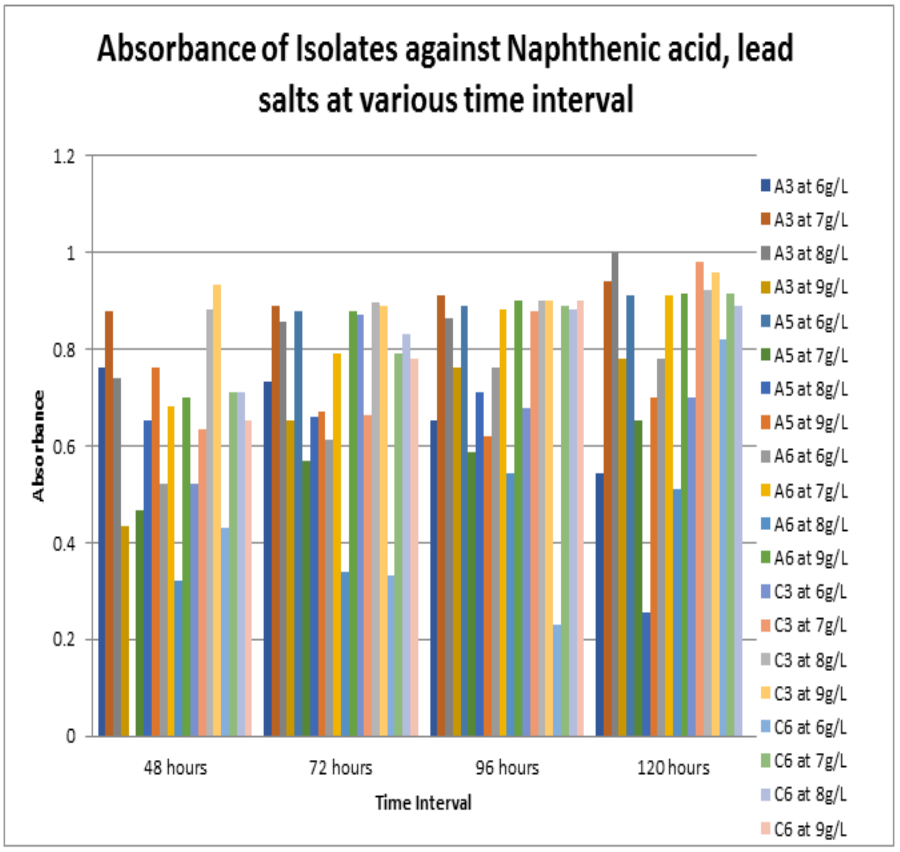


FIGURE 4.31: Absorbance of Isolates against Naphthenic acid, lead salts at various time interval.

We have noted that lead absorbance is more, when we used naphthenic acid lead salt as a source of lead salt and little bit low absorbance values were recorded when the salt added as lead source was lead nitrate.

This means when lead is in form of naphthenic acid lead salt, it is more bioavailablle and hence this form of lead could easily be up took by the bacterial strain present in sample. After performing a very keen estimation analysis it becomes clear that the strains when cultured on medium containg naphthenic acid lead salts showed more absorbance pattern.

TABLE 4.8: Absorbance of isolates against Naphthenic acid lead salts at 48,72,96 and 120 hour

Sample ID	**Incubation Time Intervals**	**Absorbance**			
		6g/L $C_{22}H_{14}O_4Pb$	7g/L $C_{22}H_{14}O_4Pb$	8g/L $C_{22}H_{14}O_4Pb$	9g/L $C_{22}H_{14}O_4Pb$
A3	48	0.761±0.04	0.876±0.20	0.741±0.01	0.435±0.07
	72	0.732±0.44	0.89±0.03	0.856±0.02	0.653±0.01
	96	0.654±0.45	0.91±0.06	0.864±0.04	0.761±0.03
	120	0.544±0.32	0.94±0.10	0.998±0.05	0.781±0.07
A5	48	0.732±0.65	0.466±0.07	0.654±0.71	0.761±0.03
	72	0.877±0.24	0.567±0.07	0.658±0.24	0.671±0.05
	96	0.889±0.56	0.588±0.02	0.711±0.27	0.618±0.02
	120	0.912±0.72	0.654±0.19	0.254±0.34	0.698±0.01
A6	48	0.522±0.02	0.680±0.30	0.321±0.11	0.698±0.03
	72	0.613±0.28	0.789±0.34	0.341±0.19	0.879±0.12
	96	0.762±0.10	0.881±0.36	0.543±0.21	0.899±0.19

Continue Table 4.8: Absorbance of isolates against Naphthenic acid lead salts at 48,72,96 and 120 hour.

Sample ID	**Incubation Time Intervals**	**Absorbance**			
		6g/L $C_{22}H_{14}O_4Pb$	7g/L $C_{22}H_{14}O_4Pb$	8g/L $C_{22}H_{14}O_4Pb$	9g/L $C_{22}H_{14}O_4Pb$
A6	120	0.781±0.19	0.912±0.54	0.551±0.22	0.913±0.12
C3	48	0.521±0.04	0.635±0.02	0.881±0.05	0.932±0.022
	72	0.872±0.26	0.662±0.10	0.895±0.02	0.890±0.023
	96	0.678±0.12	0.878±0.23	0.899±0.23	0.899±0.033
	120	0.699±0.25	0.978±0.21	0.921±0.21	0.956±0.04
C6	48	0.432±0.34	0.711±0.61	0.71±0.10	0.654±0.08
	72	0.333±0.44	0.792±0.53	0.83±0.02	0.781±0.02
	96	0.231±0.65	0.890±0.65	0.88±0.06	0.899±0.04
	120	0.821±0.28	0.913±0.21	0.89±0.26	0.981±0.12

4.9 Sequencing of the Final Strain

A5 which showed the highest amount of lead tolerance, was later sent for gene sequencing.

4.10 Phylogenetic Analysis of the Final Strain

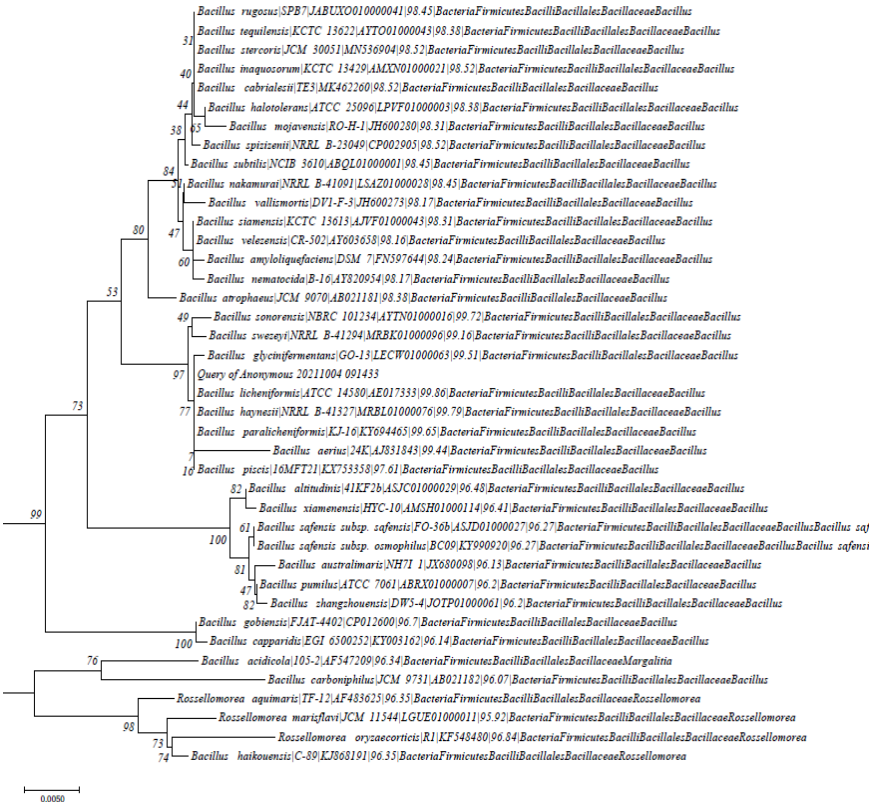


FIGURE 4.32: Phylogenetic analysis of final strain.

Sequence alignment was performed to analyze the obtained genetic sequence relative to the retrieved NCBI sequences. Phylogenetic Analsysis was performed using Neighbor Joining method [34]. The optimal tree with the sum of branch length = 0.149 is shown.The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [35]. The evolutionary distances were computed using the Kimura 2-parameter method. The analysis involved 40 nucleotide sequences. Evolutionary analyses were conducted in MEGA7.

Chapter 5

Conclusion

It's evident from the obtained results that the recent increase industrial activities in Faisalabad have severely affected the city's environment in terms of lead contamination. Majority of the area's soil microbiota, as a result, has developed a considerable tolerance against the toxic heavy metal contaminant. As such, there is strong potential to suggest that the identified bacterial species *Bacillus lichenformis* possess lead bioremediation properties (such as lead biosorption or lead bioaccumulation). All the bacterial proteins chosen for molecular docking are also present in *Bacillus lichenformis* hence this bacterial species could provide a substantial alternative for bio-treatment of Pb contaminated soil, considering the safety of the microorganism, its cost effective nature and the eco-friendly approach itself.

Bibliography

[1]. Abatenh, Endeshaw, Birhanu Gizaw, Zerihun Tsegaye, and Misganaw Wassie. "The role of microorganisms in bioremediation-A review." Open Journal of Environmental Biology 2, no. 1 (2017): 038-046.

[2]. O'Connor, D., Hou, D., Ok, Y. S., Song, Y., Sarmah, A. K., Li, X., & Tack, F. M. (2018). Sustainable in situ remediation of recalcitrant organic pollutants in groundwater with controlled release materials: A review. Journal of Controlled Release, 283, 200-213.

[3]. Azubuike, C. C., Chikere, C. B., & Okpokwasili, G. C. (2016). Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World Journal of Microbiology and Biotechnology, 32(11), 1-18.

[4]. Wani AL, Ara A, Usmani JA. Lead toxicity: a review. Interdiscip Toxicol. 2015 Jun;8(2):55-64. doi: 10.1515/intox-2015-0009. PMID: 27486361; PMCID: PMC4961898.

[5]. Manisalidis, I., Stavropoulou, E., Stavropoulos, A., & Bezirtzoglou, E. (2020). Environmental and health impacts of air pollution: a review. Frontiers in public health, 8.

[6]. Engwa, G. Azeh, P. Udoka Ferdinand, F. Nweke Nwalo, and Marian N. Unachukwu. "Mechanism and health effects of heavy metal toxicity in humans." Poisoning in the modern world-new tricks for an old dog 10 (2019).

[7]. Rehman, K., Fatima, F., Waheed, I., & Akash, M. S. H. (2018).Prevalence of exposure of heavy metals and their impact on health consequences. Journal of cellular biochemistry, 119(1), 157-184.

[8]. Chen, Z., Pan, X., Chen, H., Lin, Z., & Guan, X. (2015).Investigation of lead (II) uptake by Bacillus thuringiensis 016. World Journal of Microbiology and Biotechnology, 31(11), 1729-1736.

[9]. Boldyrev, M. (2018). Lead: properties, history, and applications. WikiJournal of Science, 1(2).

[10]. Chaudhary, S., Firdaus, U., Ali, S. M., & Mahdi, A. A. (2018). Factors associated with elevated blood lead levels in children. Indian pediatrics, 55(1), 38-40.

[11]. Yahaghi, Z., Shirvani, M., Nourbakhsh, F., De La Pena, T. C., Pueyo, J. J., & Talebi, M. (2018). Isolation and characterization of Pb-solubilizing bacteria and their effects on Pb uptake by Brassica juncea: implications for microbe-assisted phytoremediation. Journal of microbiology and biotechnology, 28(7), 1156-1167.

[12]. Riva, M. A., Lafranconi, A., D'orso, M. I., & Cesana, G. (2012). Lead poisoning: historical aspects of a paradigmatic "occupational and environmental disease". Safety and health at work, 3(1), 11-16.

[13]. Longman, J., Veres, D., Finsinger, W., & Ersek, V. (2018).Exceptionally high levels of lead pollution in the Balkans from the Early Bronze Age to the Industrial Revolution.Proceedings of the National Academy of Sciences, 115(25), E5661-E5668.

[14]. Mitra, P., Sharma, S., Purohit, P., & Sharma, P. (2017). Clinical and molecular aspects of lead toxicity: An update.Critical reviews in clinical laboratory sciences, 54(7-8), 506-528.

[15]. Shraideh, Z., Badran, D., Hunaiti, A., & Battah, A. (2018). Association between occupational lead exposure and plasma levels of selected oxidative stress

related parameters in Jordanian automobile workers. International journal of occupational medicine and environmental health, 31(4), 517-525.

[16]. Thayer, B. (2018).How to Fight Lead Exposure with Nutrition. Retrieved from Eat Right. Academy of Nutrition and Dietetics: https://www. eatright.org /health/wellness/preventing-illness/how-to-fight-lead-exposurewith-nutrition.

[17]. Vorvolakos, T., Arseniou, S., & Samakouri, M. (2016). There is no safe threshold for lead exposure: Alpha literature review. Psychiatriki, 27(3), 204-214.

[18]. Ara, A., & Usmani, J. A. (2015). Lead toxicity: a review. Interdisciplinary toxicology, 8(2), 55-64.

[19]. Charkiewicz, A. E., & Backstrand, J. R. (2020). Lead toxicity and pollution in Poland. International Journal of Environmental Research and Public Health, 17(12), 4385.

[20]. Liu, Y., Mou, H., Chen, L., Mirza, Z. A., & Liu, L. (2015). Cr (VI)-contaminated groundwater remediation with simulated permeable reactive barrier (PRB) filled with natural pyrite as reactive material: environmental factors and effectiveness. Journal of hazardous materials, 298, 83-90.

[21]. Thijs, S., Sillen, W., Rineau, F., Weyens, N., & Vangronsveld, J. (2016). Towards an enhanced understanding of plant–microbiome interactions to improve phytoremediation: engineering the metaorganism. Frontiers in microbiology, 7, 341.

[22]. Dodbiba, G., Ponou, J., & Fujita, T. (2015).Biosorption of heavy metals.In Materials for minerals, metals and materials and the environment (pp. 409-426). CRC Press.

[23]. Jacob, J. M., Karthik, C., Saratale, R. G., Kumar, S. S., Prabakar, D., Kadirvelu, K., & Pugazhendhi, A. (2018). Biological approaches to tackle heavy metal pollution: a survey of literature. Journal of environmental management, 217, 56-70.

[24]. Salehizadeh, H., & Shojaosadati, S. A. (2003). Removal of metal ions from aqueous solution by polysaccharide produced from Bacillus firmus. Water research, 37(17), 4231-4235.

[25]. Kang, C. H., Oh, S. J., Shin, Y., Han, S. H., Nam, I. H., & So, J. S. (2015). Bioremediation of lead by ureolytic bacteria isolated from soil at abandoned metal mines in South Korea. Ecological Engineering, 74, 402-407.

[26]. Igiri, B. E., Okoduwa, S. I., Idoko, G. O., Akabuogu, E. P., Adeyi, A. O., & Ejiogu, I. K. (2018). Toxicity and bioremediation of heavy metals contaminated ecosystem from tannery wastewater: a review. Journal of toxicology, 2018.

[27]. Jin, Y., Yu, S., Teng, C., Song, T., Dong, L., Liang, J., ... & Qu, J. (2017). Biosorption characteristic of Alcaligenes sp. BAPb. 1 for removal of lead (II) from aqueous solution. 3 Biotech, 7(2), 123.

[28]. Gauthier, P. T., Norwood, W. P., Prepas, E. E., & Pyle, G. G. (2014). Metal–PAH mixtures in the aquatic environment: a review of co-toxic mechanisms leading to more-than-additive outcomes. Aquatic toxicology, 154, 253-269.

[29]. Olaniran, A. O., Balgobind, A., & Pillay, B. (2013). Bioavailability of heavy metals in soil: impact on microbial biodegradation of organic compounds and possible improvement strategies. International journal of molecular sciences, 14(5), 10197-10228.

[30]. Syed, S., & Chinthala, P. (2015). Heavy Metal Detoxification by Different Bacillus Species Isolated from Solar Salterns. Scientifica, 2015, 319760. https://doi.org/10.1155/2015/319760

[31]. Shameer, Syed. "Biosorption of lead, copper and cadmium using the extracellular polysaccharides (EPS) of Bacillus sp., from solar salterns." 3 Biotech 6, no. 2 (2016): 1-10;from solar salterns." 3 Biotech 6, no. 2 (2016): 1-10.

[32]. Aslam, A., Jabeen, F., & Salman, M. (2017). Concentration level of Lead (Pb) in plants and soil of Faisalabad irrigated with waste water. Applied Science Reports, 20(3).

[33]. Bonnet, M., Lagier, J. C., Raoult, D., & Khelaifia, S. (2020). Bacterial culture through selective and non-selective conditions: The evolution of culture media in clinical microbiology. New microbes and new infections, 34, 100622.

[34]. Nath, S., Deb, B., & Sharma, I. (2012). Isolation and characterization of cadmium and lead resistant bacteria. Global Advanced Research Journal of Microbiology, 1(11), 194-198.

[35]. Masoumi, F., Khadivinia, E., Alidoust, L., Mansourinejad, Z., Shahryari, S., Safaei, M., ... & Noghabi, K. A. (2016). Nickel and lead biosorption by Curtobacterium sp. FM01, an indigenous bacterium isolated from farmland soils of northeast Iran. Journal of Environmental Chemical Engineering, 4(1), 950-957.

[36]. Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., ... & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nature communications, 10(1), 1-11.

[37]. Rehman, Z. U., Khan, S., Brusseau, M. L., & Shah, M. T. (2017). Lead and cadmium contamination and exposure risk assessment via consumption of vegetables grown in agricultural soils of five-selected regions of Pakistan. Chemosphere, 168, 1589-1596.

[38]. Salmaso, V., & Moro, S. (2018). Bridging molecular docking to molecular dynamics in exploring ligand-protein recognition process: An overview. Frontiers in pharmacology, 9, 923.

[39]. Wang, L., Liu, Q., Wu, X., Huang, Y., Wise, M. J., Liu, Z., ... & Wang, C. (2019). Bioinformatics analysis of metabolism pathways of archaeal energy reserves. Scientific reports, 9(1), 1-12;9, no. 1 (2019): 1-12

[40]. Tanchuk, V. Y., Tanin, V. O., Vovk, A. I., & Poda, G. (2016). A new, improved hybrid scoring function for molecular docking and scoring based on AutoDock and AutoDock Vina. Chemical biology & drug design, 87(4), 618-625.

[41]. Liu, Y., Grimm, M., Dai, W. T., Hou, M. C., Xiao, Z. X., & Cao, Y. (2020). CB-Dock: a web server for cavity detection-guided protein–ligand blind docking. Acta Pharmacologica Sinica, 41(1), 138-144.

[42]. Blaszczyk, M., Ciemny, M. P., Kolinski, A., Kurcinski, M., & Kmiecik, S. (2019). Protein–peptide docking using CABS-dock and contact information. Briefings in bioinformatics, 20(6), 2299-2305.

[43]. Leitão, A. L., & Enguita, F. J. (2021). Structural insights into carboxylic polyester-degrading enzymes and their functional depolymerizing neighbors. International journal of molecular sciences, 22(5), 2332.

[44]. Zolfaghari Emameh, R., Kazokaitė, J., & Yakhchali, B. (2021). Bioinformatics analysis of extracellular subtilisin E from Bacillus subtilis. Journal of Biomolecular Structure and Dynamics, 1-8.

[45]. Feng, X., Hu, Y., Zheng, Y., Zhu, W., Li, K., Huang, C. H., ... & Oldfield, E. (2014). Structural and functional analysis of Bacillus subtilis YisP reveals a role of its product in biofilm production. Chemistry & biology, 21(11), 1557-1563.

[46]. Siraj, F. M., Natarajan, S., Huq, M. A., Kim, γ. J., & Yang, D. C. (2015). Structural investigation of ginsenoside Rf with PPARγ major transcriptional factor of adipogenesis and its impact on adipocyte. Journal of ginseng research, 39(2), 141-147.

[47]. Yuan, Shuguang, HC Stephen Chan, and Zhenquan Hu. "Using PyMOL as a platform for computational drug design." Wiley Interdisciplinary Reviews: Computational Molecular Science 7, no. 2 (2017): e1298.

[48]. Laskowski, R. A., & Swindells, M. B. (2011). LigPlot+: multiple ligand–protein interaction diagrams for drug discovery.