"Impact Of Mercury Heavy Metal On The Growth Of *Escherichia coli (E.coli)*"

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Submitted By

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CANDIDATE'S DECLARATION

I, VIDHI, Roll No. 2K21/MSCBIO/59, student of M.Sc. Biotechnology, hereby declare that the Project Dissertation titled **"Impact Of Mercury Heavy Metal On The Growth Of *Escherichia coli (E.coli)*"** which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi, in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma, Associate ship, Fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that the Project Dissertation **"Impact Of Mercury Heavy Metal On The Growth Of *Escherichia coli (E.coli)*"**which is submitted by VIDHI (2K21/MSCBIO/59), Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is a recorded for the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any degree or any diploma to this university or elsewhere.

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ABSTRACT

The utilization of heavy metals in several agricultural and industrial processes is expanding dramatically, posing a severe hazard to human and ecosystem health from heavy metal contamination from natural and anthropogenic sources. A significant exposure source that results in the buildup of hazardous heavy metals in people, plants, and livestock is contaminated water. Mercury, lead, copper, cadmium, chromium, and copper are among the key metals of public health concern. Mercury is a heavy element that pollutes marine environments due to both natural and human-caused activities that release waste products into the sea. Mercury waste can be produced by the cosmetic sector, electronics, paint industry, dental industry, gold extraction sector, also many more. Humans are exposed to mercury not just through environmental pollution but also through the use of cosmetic chemicals, consumption of foods and beverages that contain mercury, or by direct contact with mercury-containing items. Due to exposure of mercury, bacteria strive to protect themselves by withdrawing the metal so that they can survive in conditions where mercury is present. The genes of bacteria that attempt to protect themselves from environmental mercury exposure alter, making them mercury-resistant bacteria. In this study,the bacteria was allowed to grow in the media containing mercury and was observed that with the subsequent days, it was thriving. The growth curve was made in the presence of mercury using UV spectrophotometry. It was also observed that at minute concentrations, mercury seems to be an inducer for the bacteria E.coli. It was analyzed by HPLC, where the bacteria was found to produce high amounts of citric acid at lower concentrations so as to thrive in the environment containing mercury. Total enzyme activity was analyzed by an FDA assay.

Keywords: *Heavy metal, Environmental pollution, Mercury, E.coli, HPLC, FDA, UV spectrophotometry*

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LIST OF SYMBOLS, ABBREVIATIONS

ºC - Degree Celsius

HPLC - High Performance Liquid Chromatography

HM - Heavy Metals

Hg - Mercury

Cu^{2+} - Copper ion

Mt - Metallothioneins

MeHg - Methylmercury

GSH - Glutathione

FDA - Fluorescein Diacetate

CHAPTER 1 : INTRODUCTION

Unusual quantities of hazardous heavy metals like Pb, Cu, Co, Mn, Cd, Hg, Ag, Sn, and Zn have been released into the surroundings over the past century as a result of unregulated mining, vast industrialization, contemporary agricultural practises, and poor waste disposal techniques.These sources release a range of poisonous chemicals that are detrimental to biological processes. Heavy metals can harm cell membranes, change enzyme activity and disrupt DNA's structural integrity[1] . They are exceedingly hazardous to people, animals, microorganisms, and plants.

Many metals are deleterious at greater doses yet necessary for microbial growth at lower ones. A desirable strategy is biosorption, which is the adhesion of heavy metals to dead cells or living microbes. Numerous microbes are capable of selectively accumulating metals[2]. The analysis determined that the amount of metal used from the initial concentration is still left in the media after the organism had adsorbed the remainder, is the basis for the current study. Elimination of harmful heavy metals is essential and relevant for a safe environment, especially in consideration of the rising environmental awareness. Various distinct microorganisms have the ability to absorb or adsorb metals. Rates of absorption may be influenced by the physiological state of the cells, the environmental conditions, and the constituents of growth medium. Based on their respective tolerance levels, different microorganisms have different capacities for absorbing metal in varied concentrations.

Both natural occurrences and human actions result in the release of mercury into the environment. Mercury in its different forms like metallic and ionic can build up in precipitates, where it could be metabolized by bacteria in the extremely deadly methyl mercury[3]. If mercury is further biomagnified through the trophic layers, eating seafood can make people sick.In order to increase heavy metal resistance and accumulation, heavy metal scavenging molecules including polyphosphates and metallothionein have been expressed in bacteria[4,5]. The mt genes encode metallothioneins, which are, less-molecular-weight, cysteine-rich, metal-binding proteins that could trap metal ions in a physiologically unreactive form. Negatively charged polyphosphates are orthophosphate polymers that have the ability to bind metal ions[6]. The enzyme polyphosphate kinase, which is in charge of bacteria's production of polyphosphates, is encoded by the ppk gene.

Also, one method for purifying the environment with the aid of microorganisms is bioremediation. This method is basic, economical, and most importantly, environmentally

beneficial. Some bacteria are able to acquire metals and tolerate high amounts. Some microbes develop plasmid-encoded resistance mechanisms that are typically specific for certain metals. Therefore, *E.coli* is a desirable choice for in situ bioremediation due to its capacity in such a harsh environment.

CHAPTER 2 : LITERATURE REVIEW

2.1. Sources of heavy metals

Heavy metals are released into the environment via industrial processes such mining, smelting, metal processing, and manufacturing. The application of fertilizers, pesticides, and sewage sludge can contaminate water sources by introducing heavy metals into the soil. When home and municipal waste are disposed of improperly, heavy metals may leak into the environment [7].

Heavy metals can be released into the atmosphere through emissions from vehicles, power plants, and other combustion activities. These metals subsequently settle on land and ocean surfaces. Also they can contaminate water sources due to industrial discharges, poor waste management practices, and the weathering of rocks [8].

2.2. Hazards of heavy metal pollution [9]

Effects on human health: Heavy metals including lead, mercury, cadmium, and arsenic can contribute to a variety of health difficulties, including neurological diseases, renal damage, respiratory problems, aberrant development, and even cancer.
Environmental impact: Pollution from heavy metals can harm ecosystems. It can destroy aquatic life, diminish biodiversity, upend ecosystem equilibrium, and taint soil, water, and plants.
Degradation of the soil: Over time, heavy metals build up in the soil, diminishing its fertility and hindering plant growth. This might have a big effect on farming and food production.
Water contamination: Heavy metal poisoning of water bodies can harm aquatic ecosystems and render the water unsafe for human consumption.
Bioaccumulation and biomagnification: Heavy metals can accumulate in the tissues of organisms over time . Heavy metal concentrations rise as prey is consumed by predators, offering a greater risk to species farther up the food chain, such as humans.

Toxic metals	Organ toxicity	Mechanism of action
Mercury (Hg)	● Nervous system disorders ● kidney dysfunction	● Enzyme inhibition ● GSH conjugation ● Aquaporin mRNA reduction
Copper (Cu)	● hemolytic anemia ● vomiting, malaise ● azotemia, anuria	● Causes cellular injury ● Lipid peroxidation ● DNA damage ● Chromosomal breakage in plants
Cadmium (Cd)	● Degenerative bones ● Gastrointestinal disorders ● Kidney dysfunction	● Induces cell death ● miRNA expression dysregulation
Chromium (Cr)	● Skin diseases ● Cause cancers in different organs	● Damages DNA ● Genomic instability
Lead (Pb)	● Causes anemia ● Damages liver	● Increases inflammatory cytokines ● Damages hematopoietic system ● Inhibition of heme biosynthesis

Harmful effects of Heavy metal [57-61]

2.3. Types of remediation of heavy metals

To minimize or remove heavy metals from contaminated locations, heavy metal pollution remediation uses a variety of methods and strategies. Here are a few typical corrective measures:

2.3.1. Soil remediation

Phytoremediation: It is possible to remove and store contaminants in the tissues of plants that have the capacity to accumulate heavy metals, such as some kinds of ferns.

Soil washing: This method uses solvents or chelating agents to physically or chemically separate the heavy metals from the soil.

Soil stabilization: Immobilizing heavy metals and lowering their bioavailability by adding chemicals or amendments to the soil.

2.3.2. Water Remediation

Precipitation/flocculation: Chemicals are added to contaminated water to create precipitates or flocs, which are insoluble and may be filtered or sedimented out of the water.

Ion exchange: To remove the impurities from the water, materials having a high affinity for heavy metals, such as activated carbon or zeolites, can be synthetic or natural.
Reverse osmosis: This technique applies pressure-driven filtration to a semi-permeable membrane to remove heavy metals from water.

2.3.3. Containment

Capping: To stop the spread of heavy metals, the polluted region is "capped" with a layer of clean soil or impermeable materials.
Landfilling: To prevent the discharge of contaminated materials into the environment, properly built and managed landfills can separate and contain them.

2.3.4. Biological treatment

Bioremediation: Utilizing microorganisms or plants, bioremediation converts hazardous heavy metals into less harmful ones. Processes like bioleaching or microbial-assisted immobilization can be used to accomplish this.

2.3.5. Chemical treatment [10]

Chemical precipitation: It is the process of adding substances that react with heavy metals to produce insoluble molecules that are simple to remove from water or soil.
Electrochemical techniques: By applying an electric current, heavy metals can be removed using processes like electrocoagulation and electrokinetics.

It's crucial to remember that choosing the best remediation strategy relies on a no. of variables, which includes the kind and quantity of heavy metals, site features, and legal requirements. To properly remediate heavy metal pollution, a number of alternative remediation strategies may be used.

2.4. Mechanism of Remediation

2.4.1. Biosorption

Heavy metals can be concentrated by microbes through either absorption or adsorption. Adsorption is different from absorption in that the absorbent dissolves a fluid the absorbate [11]. Adsorption occurs at the outer surfaces, while absorption affects the total amount of the substance. In order for heavy metals to be taken into cells through adsorption, they must first complex on the cell surface[12]. Heavy metals can be absorbed or adsorbed easily due to the structure of the cell surface, specifically the mucus layer and the cell wall. Several metal ions and functional group ions form complexes as coordination atom[13]. Also, the cell wall of the microbes contains negatively charged phosphoric acid anions and carboxyl anionic groups, while the majority of heavy metal surfaces contain a positively charged group that associate with the cell wall and allows the ions to adhere to or cross the cell membrane[14]. In general, heavy metal ions are quickly absorbed by microorganisms in high quantities. Bacillus have Cu^{2+} adsorbing capacity of 60% at pH 7.2 in one minute and acquire adsorption equilibrium in 10 minutes[15].

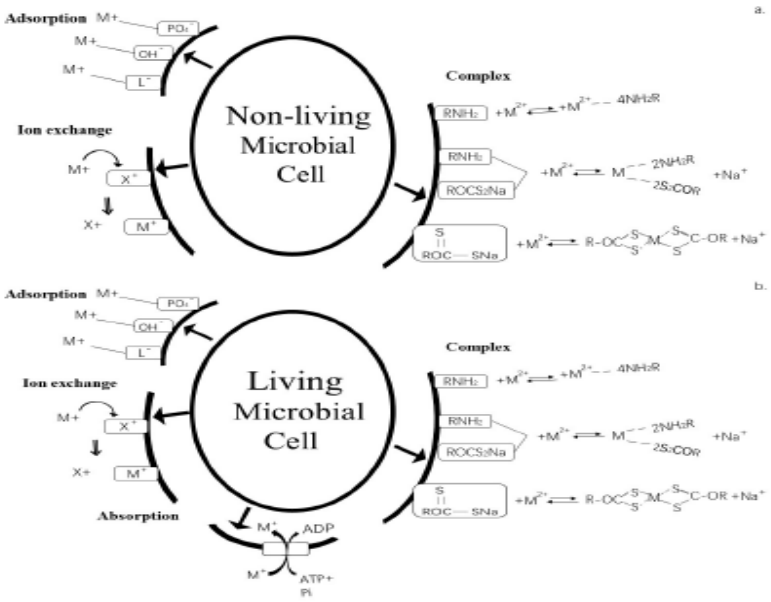


Figure 1. Biosorption mechanisms of microorganisms.

[16]

2.4.2. Bioleaching

The broad phrase "biomining" also known as bioleaching, is the process of releasing cationic heavy metal from insoluble ores, frequently by forming biological complex processes [17,18], and bio-oxidation [19]. Secretions from metabolism of microbes, such as organic acids with low molecular weight, have the ability to dissolve soil particles containing heavy metal minerals and heavy metals. It demonstrated how, in nutrient-rich environments, microorganisms can efficiently take on nutrients and energy to release organic acids and encourage the leaching of Cd. For instance, the leaching rate was determined to be 9.1 % without the presence of nutrients and 36% with the presence nutrients [20]. Studies have also revealed that many microorganisms, such as Citrobacter, could produce free inorganic phosphates, which can result in the creation of an insoluble metal PO4 coat that can sequester a significant amount of hazardous metals[21]. Prokaryotic microorganisms are involved in redox processes which alter the electrons in the outer most shell of heavy metals, modifying their reactivity and perhaps affecting the mobility or toxicity of the metals[22].

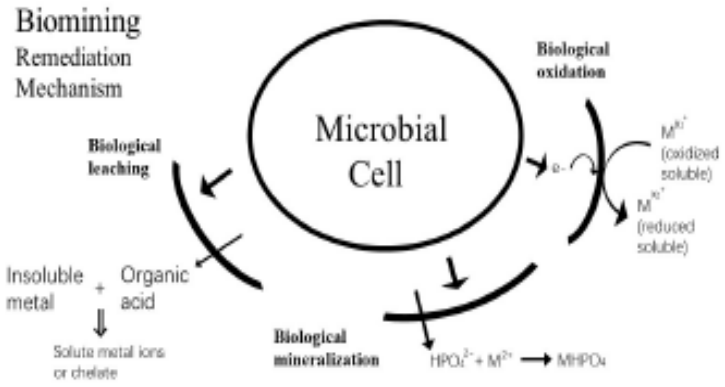


Figure 2. Biomining mechanism of microbes.

[16]

2.5. Principle of remediation

Bioremediation is a process by which organic wastes degrade biologically under maintained conditions to a harmless substance or to less concentrated levels which are permitted by official agencies. Because they contain enzymes that enable the microbes to consume toxins present in the environment as food, they are well adapted for this task of contaminant eradication. By giving them the adequate amount of nutrients and other chemicals that are necessary for metabolic activity, bioremediation aims to motivate them

to work and encourage the degradation or detoxification of pollutants that are detrimental to the environment and living organisms. Enzymes carry out every process of metabolism. These are a part of the ligases, hydrolases, lyases, transferases, isomerases, and oxidoreductases groups. Due to their specific and non specific substrate affinities, many enzymes have an astonishingly broad capacity for degradation. Microorganisms should attack the contaminants enzymatically and modify them into harmless substances and make bioremediation successful. As bioremediation is only effective in the environments which support the growth of microbes and enhance their activity, its application frequently demands for changing the environmental conditions to promote growth of the microbes and to degrade at a much faster rate [23]. Natural bioremediation occurs and is aided by the addition of living creatures and fertilizers. Technology used in bioremediation is mostly based on biodegradation. It refers to the complete conversion of harmful or naturally occurring organic contaminants into substances like carbon dioxide, water, and inorganic chemicals that are secure for use by people, animals, plants, and aquatic life [24].

2.6. Uses of bacteria in remediation

A simple and effective method for removing contaminants from wastewater, including non-biodegradable substances like heavy metals, is biosorption by bacteria. Cells that make up bacterial biomass might be alive or dead. For their survival, bacteria have evolved mechanisms for resistance to remediation and metal ions [25]. Numerous research has been conducted for the bioremediation of heavy metal ions by microbes. Metals including Cu, Zn, Pb, Cd, and Cr can be quickly removed using bacterial biomass. Because different bacterial species have distinct cellular structures like peptidoglycans such as poly-N-acetylglucosamine and N-acetylmuramic acid, the efficacy of biosorption is dependent on both heavy metal ions and bacterial species[26-31]. The actual physical interface between the bacterial biomass and metal ions is the cell wall of the bacterium. The ability to bind metals to or within the cell wall is conferred by the complete negative charges caused by anionic functional groups (such as OH^-, amine, carboxyl, PO_4 and sulphate) present in Gram positive bacteria and in Gram negative bacteria [32].

2.7. Bioremediation of Mercury by E.coli

Particularly the hazardous heavy metals that affects the environment is mercury. Battery operations, mercury switches, chloralkali plants, and medical waste facilities are the main causes of contamination in wastewater[33]. Hg^{2+} in sediment is methylated in an aquatic environment, producing more dangerous methylmercury[34]. The primary molecules that sequester the metals employed by these cells to immobilize the metal ions are

metal-binding peptides, such as phytochelatins and metallothioneins (MTs), which provide specific, high-affinity binding sites[35]. The creation of microbe-based biosorbents for the elimination and retrieval of Hg^{2+} from impure soil and water presents a viable approach as overexpression of these metal-binding proteins, such as MTs, in bacteria led to higher Hg^{2+} accumulation[36].

2.8. Impact of Mercury

The International Programme of Chemical Safety has mercury (Hg) on its list of most hazardous substances. The hazard of Hg to human health has drawn a lot of attention because of its capacity for methylation, accumulation, and biomagnification in food chains[37,38]. Hg is hazardous to the CNS, kidneys, cardiovascular, GI tract, immunological network and can build up in the human body[39-41] Hg's toxicity to tissue is greatly influenced by its chemical forms. Specifically, exposure to Hg during pregnancy, that can cross the blood brain barrier, has a negative impact on the neurobehavioral development of the offspring. Hg's high affinity for the thiols found in proteins is thought to be the cause of its cellular toxicity[42]. The covalent interactions that Hg2+ and methylmercury (MeHg) can form with glutathione (GSH) or the cysteine, cystine, methionine, and taurine residues of proteins can disrupt GSH metabolism, inactivate proteins, and harm cells[43]. Hg can also cause lipid, protein, or DNA oxidation as well as free radical production in cells[44-49]. Although Hg has been the subject of intensive research over the past few decades, the precise mechanisms underlying its cellular toxicity are still poorly known, making more study in this field very desirable [50].

CHAPTER 3 : MATERIALS AND METHOD

3.1. UV spectroscopy

Ultraviolet/visible (UV-Vis) spectroscopy works at multiwavelength and is a quantitative, flexible, quick, and reliable analytical instrument that can be used as a biosensor for the enumeration, recognition and detecting the cells and microorganisms[51]. Cell size, chemical constituents, and shape are examples of sample information in a spectrum. The spectroscopic study of a material measured across a wide wavelength range (200-900 nm) and with the scattered light detected at various distinct directions provide this information. A bacterial population's growth curve typically comprises four phases: lag phase, exponential phase, stationary phase, and death phase. Changes in cell number, size, form, chemical composition, and internal structure can all be categorized as changes in the cell population. In theory, multiwavelength spectroscopic measurements can find all these discrepancies.

Experimental procedure

E.coli was allowed to grow overnight in a shaker incubator at 35^{o}C after suspending a colony-forming unit in 4 mL Nutrient Broth (NB). From this overnight culture (10^{9} colony-forming units per ml), 0.5 ml of volume was added to 100 mL of clean and freshly prepared broth. This culture was placed in a shaker incubator at 35^{o}C. At certain intervals, duplicate samples were removed from the incubator, cleaned (in sterile deionized water), and then subjected to spectroscopic analysis. The samples were spun in a centrifuge for 3 minutes at a 13,000 rotation per minute. The test tubes were taken out, and the supernatant was carefully taken out and was discarded using 1.0 ml pipette. A small amount of supernatant was left in each tube to avoid unsettling of the pellet. After being briefly vortexed, the leftover pellets were resuspended in sterile deionized water. Three times were done with the washing step. The pellets formed with fresh cells were resuspended into sterile deionized water after the final washing, which was also utilized to dilute the samples. A 1-cm pathlength cuvette was used for every measurement, which was done at room temperature at 600 nm [52].

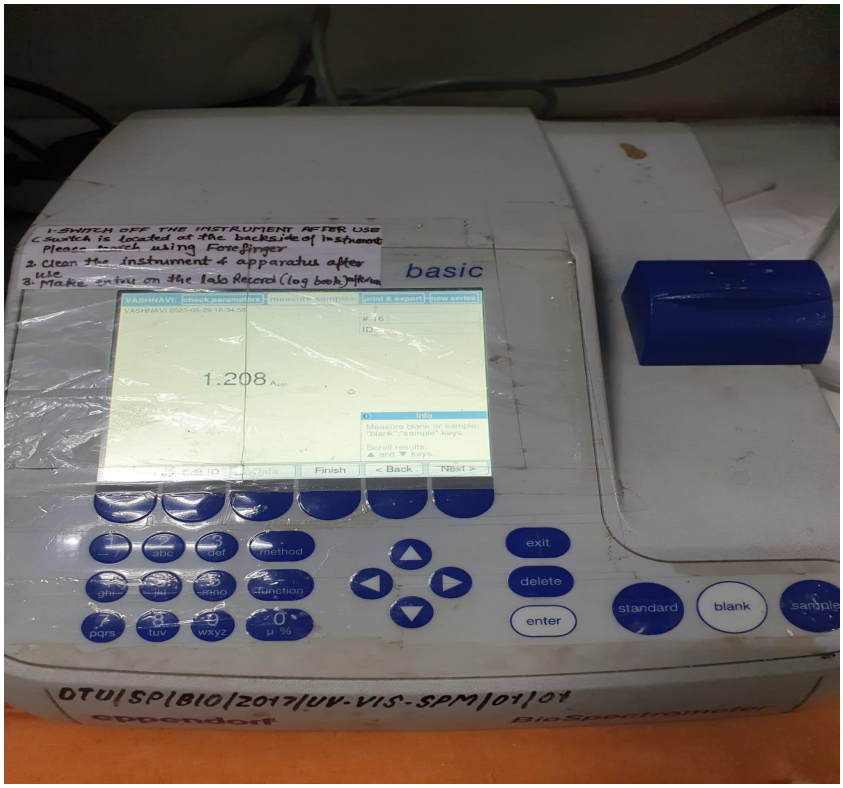


Figure 3. UV Spectrophotometer

3.2. Total enzyme activity (FDA assay)

Many distinct enzymes, including proteases, lipases, and esterases, hydrolyze fluorescein diacetate (3',6'-diacetylfluorescein [FDA]). This enzymatic reaction results in fluorescein, which may be seen inside cells using fluorescence microscopy. Fluorometry and spectrophotometry are other methods for measuring fluorescein[53].

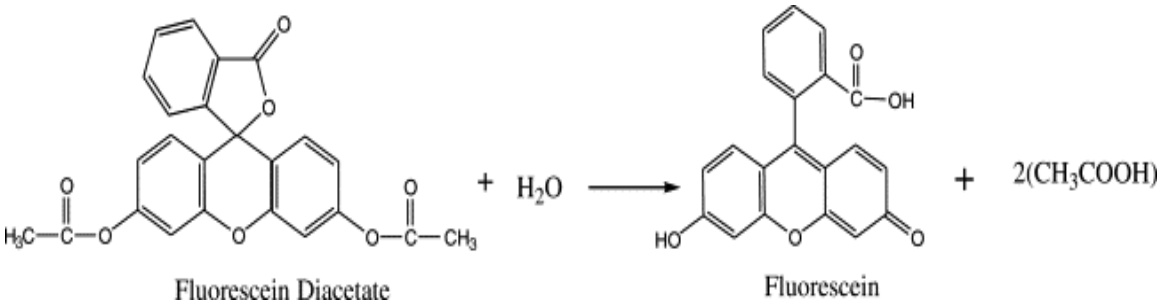


Figure 4. Fluorescein diacetate hydrolytic reaction [62]

Experimental procedure

A 50 ml conical flask containing 2 ml of sample was filled upto 15 ml of a 60 mM potassium phosphate buffer with a pH of 7.66. The reaction mixture was prepared by adding 0.2 ml of 1000 g/l FDA stock solution. A reasonable number of sample replicates and blanks without the FDA substrate were also generated. The flasks' contents were manually shaken after being stopped. After that, the flasks were placed in a shaker Incubator (110 rev/min) at 32 °C for 15 min. The procedure, containing methanol and chloroform, was completed in a fume hood. 10 ml of chloroform/methanol (2:1 v/v) was added right away to terminate the reaction when the incubator was turned off. The flasks' stoppers were replaced, and the solutions were vigorously shaken with the help of hand. The conical flasks' contents were then transferred to centrifuge tubes of 50 ml, where they were centrifuged at 6000 rev min-1 for around 7 minutes. After filtering (Whatman, No 2) the supernatant from each sample into 50 ml conical flasks, the filtrates were analyzed at 490 nm using a spectrophotometer[54].

3.3. HPLC

The HPLC separation principle relies on the distribution of the sample between a stationary phase (material inside the column) and a mobile phase (liquid moving through the column). The analyte molecules move at different speeds through the stationary phase, depending on their chemical composition. The time a sample spends on the column is determined by the interactions between its molecules and the packing material, resulting in the separation of different components. After exiting the column, the analytes are detected using equipment like a UV detector. Signals are converted, recorded, and displayed in a chromatogram using computer software. The mobile phase can undergo additional detection units, a fraction collector, or be disposed of after passing the detection unit. An HPLC system typically consists of a solvent reservoir, pump, injection valve, column, detector unit, and data processing unit. The pump maintains a high-pressure, steady flow of the solvent throughout the system. It is crucial for the pump to provide a continuous and pulseless flow to minimize detector signal drift and noise. The injection valve introduces the sample into the mobile phase.

Figure 5. HPLC Ultimate 3000

Experimental procedure

After every 5 days of mercury treatment, the quantity of organic acids like oxalic acid, citric acid, and malic acid in the bacterial culture was calculated. To get cell free metabolites, 1 ml of the culture was removed and filtered using 0.2 m polytetrafluorethylene Millex filters. High Performance Liquid Chromatography with PDA detector that operates at 210 nm was used to assess the presence of organic acids. The Organic Acid 5 m Analytical Column, measuring 250 micrometer 4.6 micrometer, used with the injection volume of 10 mL having an ideal temp. of 25 °C.

Because the mobile phase with the flow rate at 1 ml/min, methanol and potassium dihydrogen phosphate {10 mM, pH 2.8} were combined in the ratio of 10:90. [55,56]

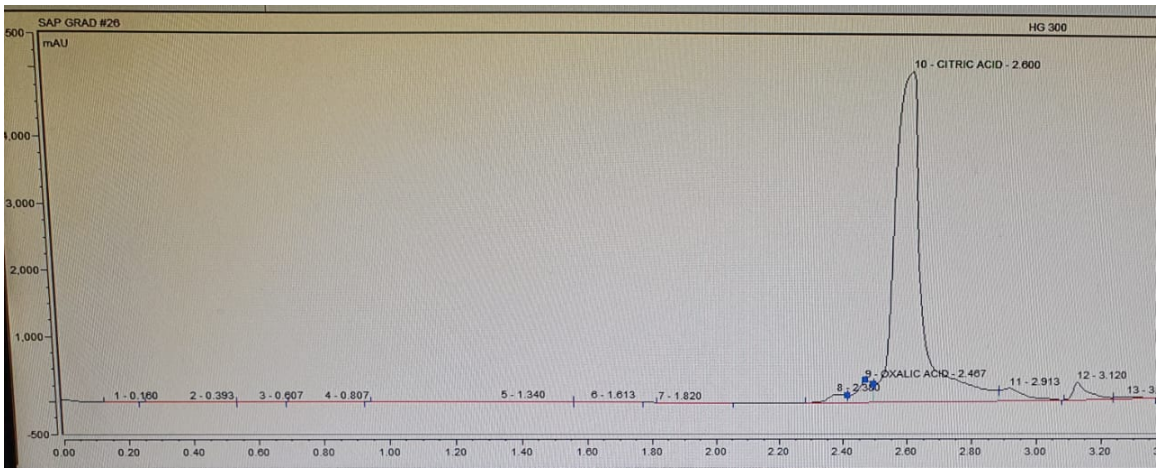


Figure 6. Peak of citric acid at 300 ppm Mercury

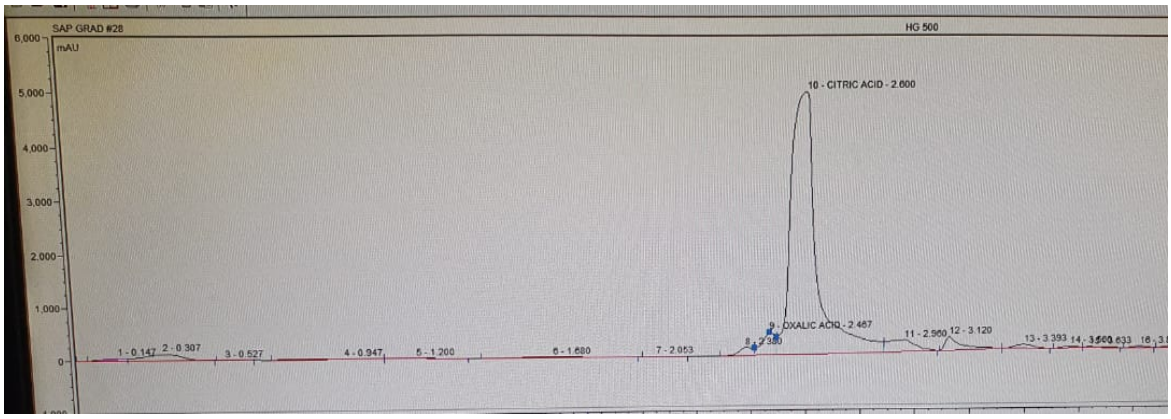
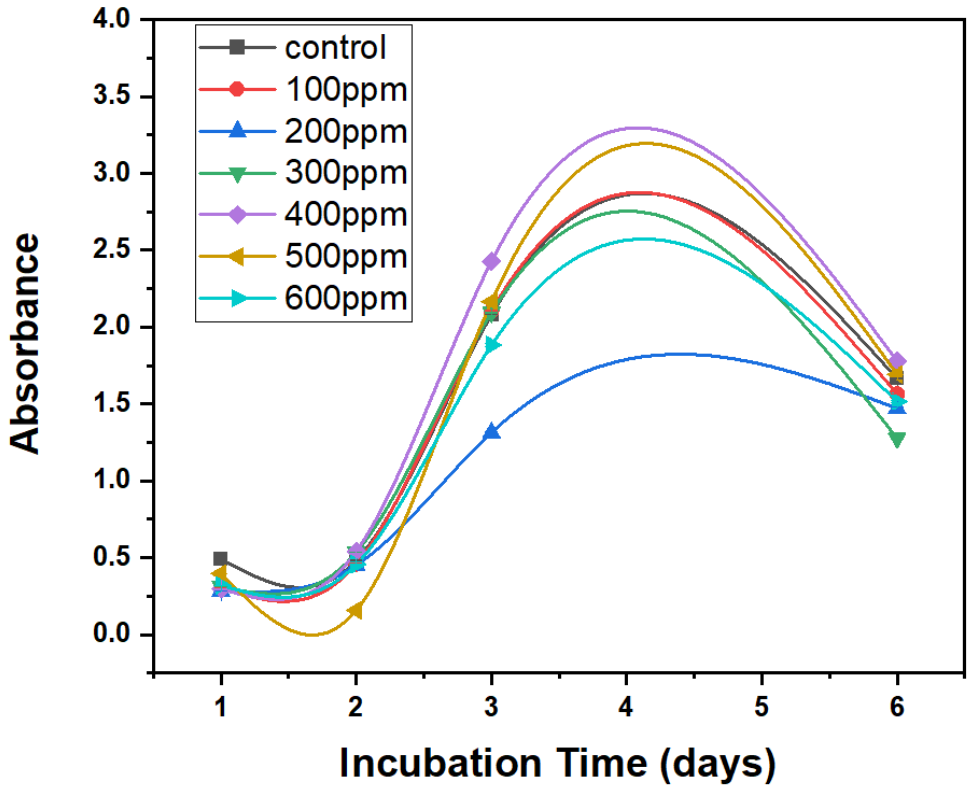


Figure 7. Peak of citric acid at 500 ppm Mercury

CHAPTER 4 : RESULT AND DISCUSSION

4.1. Growth curve of *E.coli* in the presence of Mercury

To check the heavy metal resistivity of bacteria, the standard growth curve of the *E.coli* was plotted in the presence of different concentrations of mercury (100-600 ppm).



Graph 1. Growth curve of *E.coli* in the presence of Mercury

After plotting the standard growth curve in the presence of mercury, it was recorded that the maximum growth was seen at the concentration 400 ppm. The minimum growth was observed at 200 ppm.

4.2. Total enzyme activity by FDA analysis

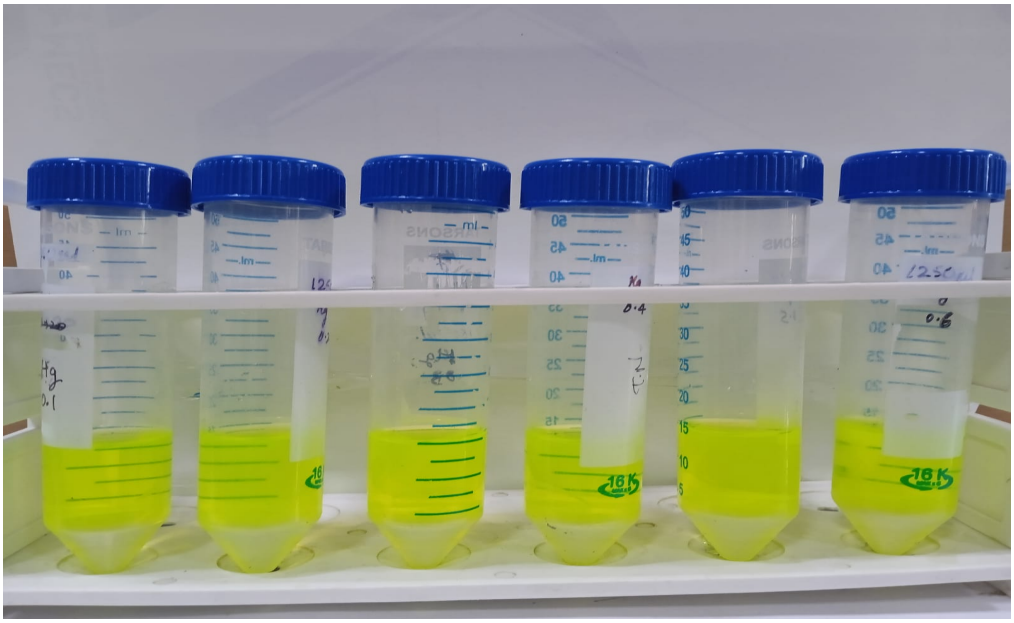
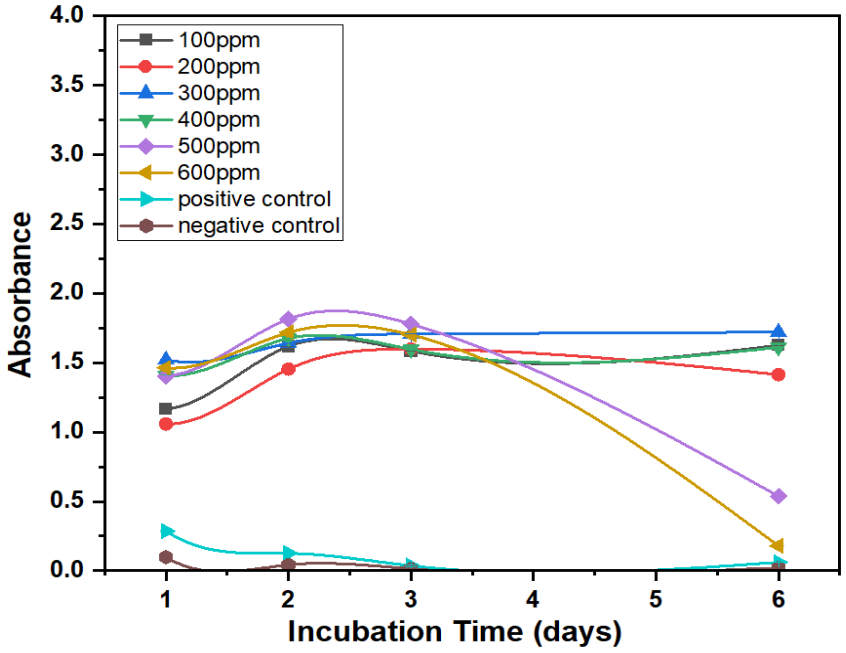


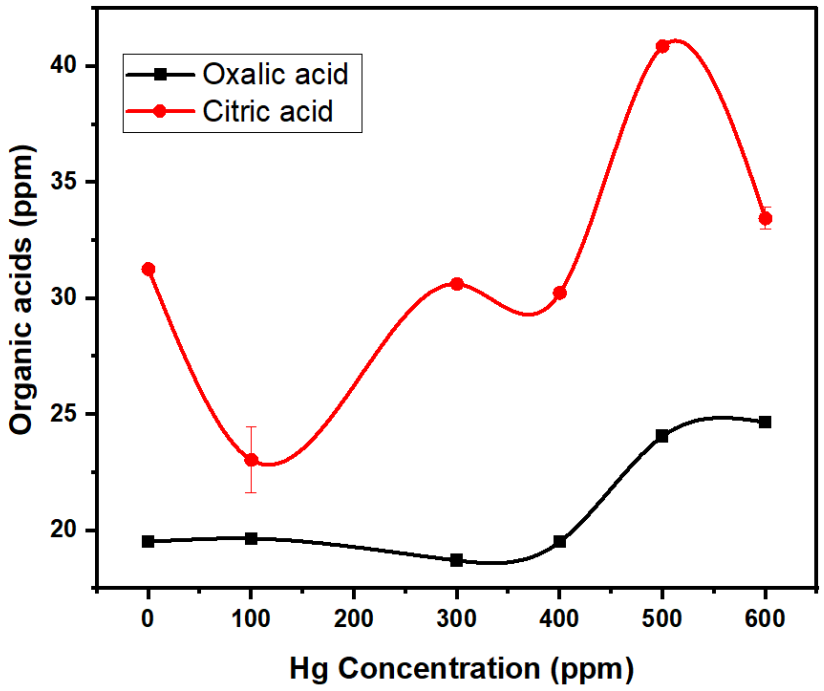
Figure 8. Hydrolysis of FDA into Fluorescein



Graph 2. FDA analysis of E.coli in the presence of Mercury

At the concentrations 500 ppm and 600 ppm of mercury, the total enzyme activity was found to be the highest on the second day of incubation. But later the curve gradually declines.

4.3. Organic acids analysis by HPLC



Graph 3. Organic acid analysis by HPLC

The citric acid was found to be the highest at 300 ppm and 500 ppm of mercury and hence, the highest peak was observed at 500 ppm because in the presence of heavy metal mercury the bacteria is thriving and thus producing high amounts of citric acid. This concludes as there is a high metabolic rate due the presence of mercury.

CHAPTER 5 : CONCLUSION

In this work, it was studied that the presence of heavy metal impacts the growth of bacteria E.coli. Unusual quantities of hazardous heavy metals such as Zn, Pb, Sn, Co, Cu, Mn, Hg, Cd and Ag, have been released in the surrounding environment over the past century as a result of unregulated mining, vast industrialization, contemporary agricultural practises, and poor waste disposal techniques. These sources release a range of poisonous chemicals that are detrimental to biological processes. The hazard of Hg to human health has drawn a lot of attention because of its capacity for methylation, accumulation, and biomagnification in food chains. Hg is hazardous to the CNS, kidneys, cardiovascular, GI tract, immunological network and can build up in the human body.

It was analyzed that at different concentrations of mercury there were variable effects seen on the bacteria. At higher concentrations the growth of bacteria was found to be increased during initial phases of incubation.

Upon further testing, it was found that the total enzymatic activity was highest at the same concentrations as it was seen in the growth curve. Later, the samples were run in HPLC and the presence of organic acids was analyzed. It was found that the amount of citric acid was highest at 300 ppm and 500 ppm, which confirms that the metabolic rate in the presence of mercury has increased so as to maintain the viability of the bacterial cells in the medium.

Therefore, mercury acts as an inducer and inhibitor at various concentrations thereby increasing the metabolic rate.

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