Exploring the Bioleaching Potential of Indigenous Bacterial Isolate and *Chromobacterium violaceum*

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By

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To

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

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CERTIFICATE

This is to certify that the project titled "Exploring the Bioleaching Potential of Indigenous Bacterial Isolate and *Chromobacterium violaceum*" submitted by **Prarthna Kashyap (181803)** carried out under my supervision for the award of the degree of B. Tech in Biotechnology from Jaypee University of Information Technology. This work has not been submitted to any other university or institute for an award for this degree or any other degree.

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ABSTRACT

In the present study e-waste sample that has been growing exponentially, was analysed for its metallic constituents and exposed for its metals solubilization through bioleaching method. The indigenous bacterial strain habituated to metal contaminated soil was isolated, which possesses bioleaching potential for treatment of e-waste. The isolated bacterial strain was found to be Gram positive bacteria with e-waste toxicity tolerance up-to 625g/L pulp density. The bioleaching was performed with isolated strain and Chromobacterium violaceum and found that indigenous bacterial isolate solubilized higher copper and silver metal in comparison to *Chromobacterium violaceum.* Therefore, this bacterial strain could be a saving tool in biohydrometallurgy field against the growing concern of e-waste. The higher bioleaching ability of indigenous bacteria was contributed through its habituated environment as soil sample was also found to possess base metals as well as precious metals. Beside the exploration of bacterial bioleaching potential, the awareness for e-waste and its treatment was also observed, and found that informal recycling of e-waste is cause of environmental pollution and human health issues hence, formal recycling of e-waste is necessary for economical and eco-friendly treatment of e-waste.

Keywords: E-waste, indigenous, Gram positive, *Chromobacterium violaceum,* bioleaching

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CHAPTER 1

INTRODUCTION

Electronic garbage, or e-waste for short, is a broad phrase that refers to a variety of electric and electronic devices that have lost their utility to their owners. When we think of waste, we automatically think of garbage or solid/semi-solid waste. In the last ten years, e-waste has become a global issue. Over the last two decades, the global market for electrical and electronic equipment (EEE) has grown at an exponential rate, but product lifespans have shrunk [1]. The rapid rise of information technology and telecommunications, in particular, has resulted in an increase in computer capacity but a loss in product life time. Due to the rapid depletion of trash disposal capacity, this circumstance results in an increase in the number of outmoded products, posing environmental concerns [6]. The majority of e-waste is disposed of in landfills. However, due to their partial recyclability and the unavoidable restrictions in landfills, retrieval techniques for their recycling and re-use have been developed, highlighting the importance of e-waste recycling, not only from a waste management standpoint but also from a valuable materials retrieval standpoint [2]. The processing and disposal of abandoned EEE is usually unregulated in many low- and middle-income nations. E-waste recycling is critical for a developing country's long-term development because it is directly linked to environmental issues due to the presence of a huge unregulated industry [4] [5]. E-waste also contains toxic elements such as lead, mercury, and chromium, as well as some compounds in plastics and flame retardants, which raises safety concerns. Health impacts associated to contamination in air, soil, and water for persons working and living near informal e-waste processing operations are becoming more documented [3]. Because it contains both harmful and valuable metals, the safe handling of e-waste is becoming a big concern for governments and the general population, as well as trade opening [7][8]

OBJECTIVES OF THE STUDY

a) Understanding the flow cycle of e-waste and how it is being collected and disposed

b) Analyse the concentration of metals in soil obtained from an e-waste dumping site.

c) Isolating bacteria from the soil.

d) Toxicity assessment

e) Bioleaching e-waste through the isolated bacteria.

CHAPTER 2

REVIEW OF LITERATURE

2.1 What constitutes e-waste?

Computers and their peripherals (monitors, central processing units, printers, and keyboards), typewriters, mobile phones and chargers, headphones, remotes, batteries, compact discs, LCD/Plasma TVs, air conditioners, refrigerators, and fluorescent and other mercury-containing lamps are all examples of e-waste (Central Pollution Control Board, 2016).

Balde et al. (2015) divided the electronic waste into six distinct categories:

1.Refrigerators, freezers, air conditioners, and heat pumps are examples of temperature exchange equipment.

2. Televisions, monitors, laptops, notebooks, and tablets are examples of screens and monitors.

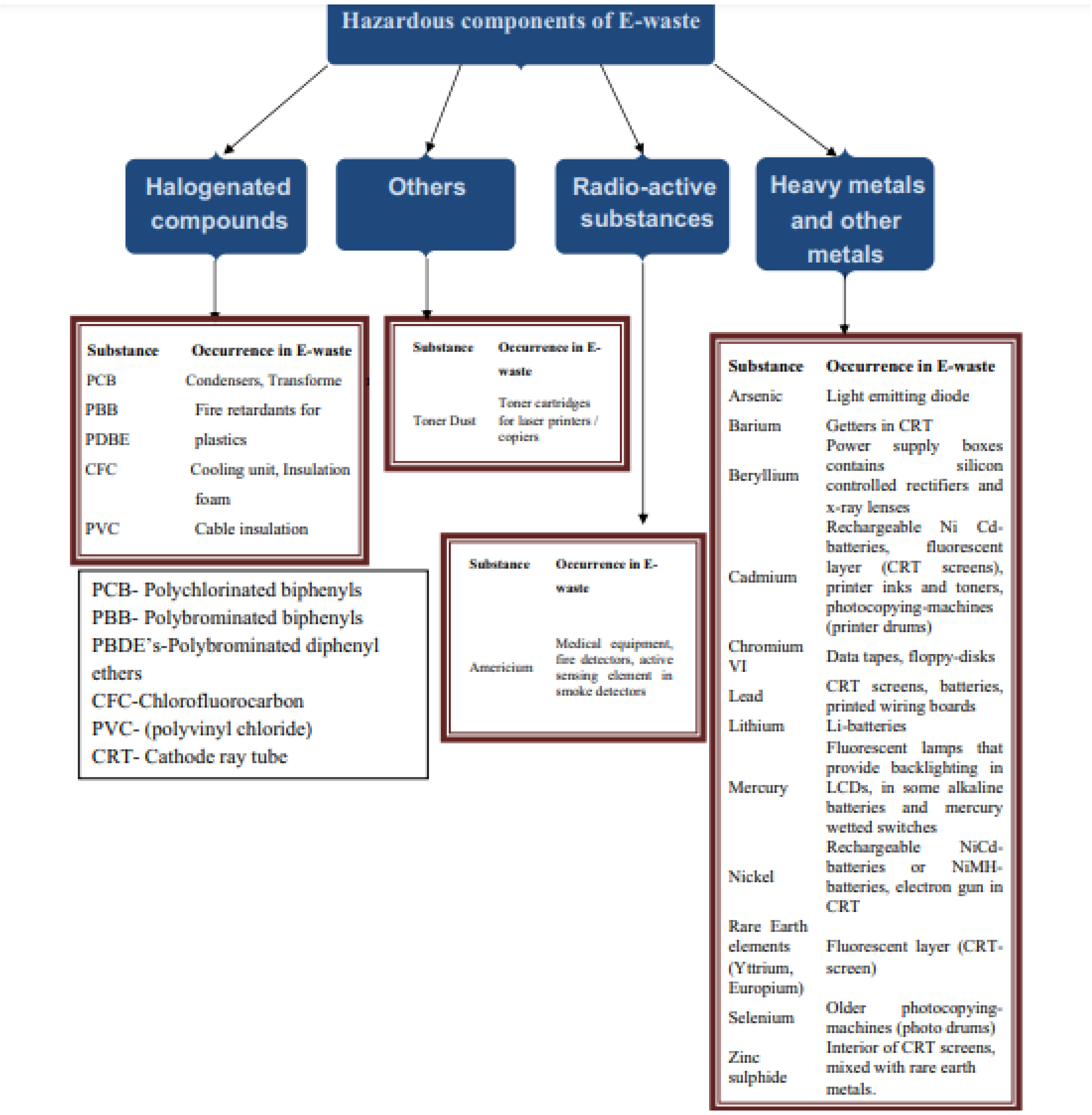
3. Fluorescent lamps, LED lamps, and high-intensity discharge lamps are all examples of lights.

4. Massive equipment: washing machines, dryers, electric stoves, large printing presses, copying presses, and photovoltaic panels;

5. Small equipment includes vacuum cleaners, toasters, microwaves, ventilation equipment, scales, calculators, radios, electric shavers, kettles, cameras, toys, electronic tools, medical devices, and small monitoring and control equipment.

6. Mobile phones, GPS, pocket calculators, routers, personal computers, printers, and telephones are all examples of small IT and telecommunications equipment.

E-waste contents vary depending on the manufactured goods and include over 1000 different compounds classified as 'hazardous' or 'non-hazardous. Fig.1 shows the various hazardous components of e-waste.



Substance	Occurrence in E-waste
PCB	Condensers, Transforme
PBB	Fire retardants for
PDBE	plastics
CFC	Cooling unit, Insulation foam
PVC	Cable insulation

PCB- Polychlorinated biphenyls
PBB- Polybrominated biphenyls
PBDE's-Polybrominated diphenyl ethers
CFC-Chlorofluorocarbon
PVC- (polyvinyl chloride)
CRT- Cathode ray tube

Substance	Occurrence in E-waste
Toner Dust	Toner cartridges for laser printers / copiers

Substance	Occurrence in E-waste
Americium	Medical equipment, fire detectors, active sensing element in smoke detectors

Substance	Occurrence in E-waste
Arsenic	Light emitting diode
Barium	Getters in CRT
Beryllium	Power supply boxes contains silicon controlled rectifiers and x-ray lenses
Cadmium	Rechargeable Ni Cd-batteries, fluorescent layer (CRT screens), printer inks and toners, photocopying-machines (printer drums)
Chromium VI	Data tapes, floppy-disks
Lead	CRT screens, batteries, printed wiring boards
Lithium	Li-batteries
Mercury	Fluorescent lamps that provide backlighting in LCDs, in some alkaline batteries and mercury wetted switches
Nickel	Rechargeable NiCd-batteries or NiMH-batteries, electron gun in CRT
Rare Earth elements (Yttrium, Europium)	Fluorescent layer (CRT-screen)
Selenium	Older photocopying-machines (photo drums)
Zinc sulphide	Interior of CRT screens, mixed with rare earth metals.

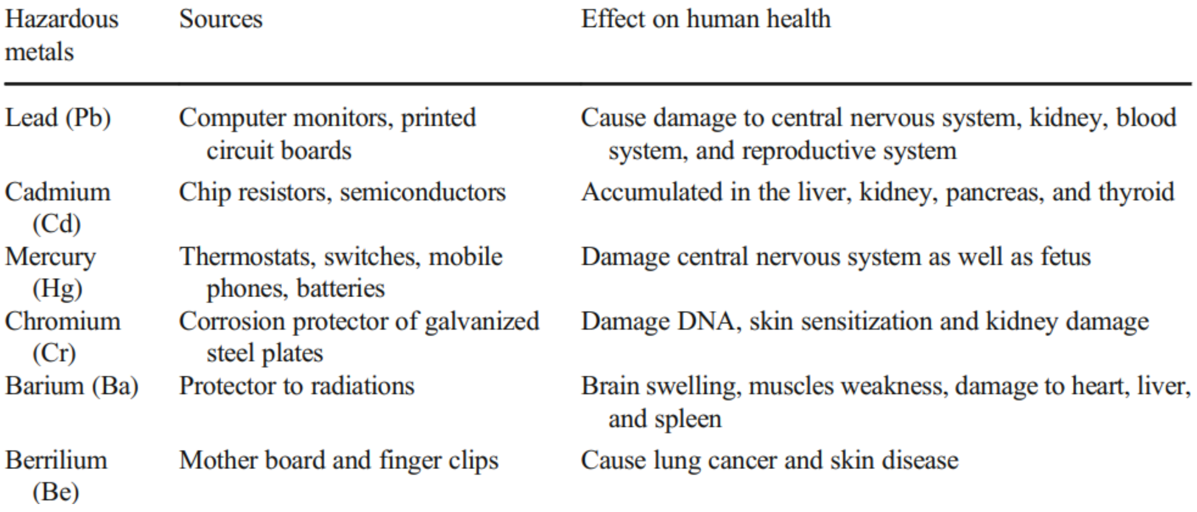
Figure 1 Hazardous components of E-waste (Source:Garlapati Vijay Kumar (2016).

Aluminum (Al), Copper (Cu), Cobalt (Co), Iron (Fe), Manganese (Mn), Tin (Sn), Nickel (Ni), Germanium (Gr), Gallium (Ga), Indium (In), Vanadium (Va) are present in Printed Circuit Boards (PCBs), cables, housing, heat sink, cooling fans, connectors, Cathode Ray Tube (CRT), and power supply, respectively.

2.2 Current scenario of e-waste recycling and disposal

E-waste handling is a very complex task as it involves handling of various toxins and other harmful materials that are present in the e-waste. The "Formal" and "Informal" sectors are the two main players in WEEE management and recycling. In the case of the former method, e-waste treatment is carried out with specially developed apparatus and facilities that ensure a safe working environment. In many underdeveloped nations, however, the exorbitant cost of equipment and infrastructure limits the use of formal processing. Despite the fact that official e-waste recycling plants have adequate facilities, toxicants can harm workers and the environment [14][15]. Metals are retrieved in an unskilled and unscientific manner without suitable equipment in most home-based informal recycling systems. E-waste disposal is a major issue that affects people all over the world. When WEEE is disposed of with other household waste, hazardous emissions result, posing a harm to ecosystem components. The traditional methods of e-waste disposal, such as landfilling and incineration, have a negative impact on the environment. E-waste that is dumped on the ground generates contaminated leachates into the groundwater. Acidification of land and poisoning of water supplies will result from the melting component of computer chips with acid [11]. During the land disposal process the Pb (lead) reaches the ground water which is then further biomagnified in the food chain causing serious danger to the ecosystem [12]. Also, the deposal of such hazardous materials causes harm to the soil surrounding the area as per the claims of Priya and Hait [13]. Various studies have also stated that traditional methods of informal recycling can cause to health problems such as cancer and neurological impairments. These evidences pose a significant occupational risk to personnel in formal recycling facilities, as well as to the environment. As a result, adequate safety management is required to avoid detrimental impacts of WEEE on humans and the environment. Some of the concerning human health effects are shown in table 1. [16]

Table 1 Effects of hazardous metals on human health [source: Thakur et al. (2020)]



Hazardous metals	Sources	Effect on human health
Lead (Pb)	Computer monitors, printed circuit boards	Cause damage to central nervous system, kidney, blood system, and reproductive system
Cadmium (Cd)	Chip resistors, semiconductors	Accumulated in the liver, kidney, pancreas, and thyroid
Mercury (Hg)	Thermostats, switches, mobile phones, batteries	Damage central nervous system as well as fetus
Chromium (Cr)	Corrosion protector of galvanized steel plates	Damage DNA, skin sensitization and kidney damage
Barium (Ba)	Protector to radiations	Brain swelling, muscles weakness, damage to heart, liver, and spleen
Berrilium (Be)	Mother board and finger clips	Cause lung cancer and skin disease

2.3 Recycling processes

As e-waste has been given the term of "urban mine" because of the various valuable and precious metals present in it. As a result, WEEE treatment for metals recovery would be beneficial from both an ecological and economic standpoint. The following are the major steps in recycling: (a) disassembly: selective disassembly, with dangerous or valuable components targeted and singled out for particular care; (b) upgrading: mechanical and/or metallurgical processing to increase the content of useful materials; (c) refining: chemical (metallurgical) processing to purify the recovered materials so that they are suitable for their original purpose [17]. There are various methods which are used in the treatment of recycling of e-waste as shown in figure 2.

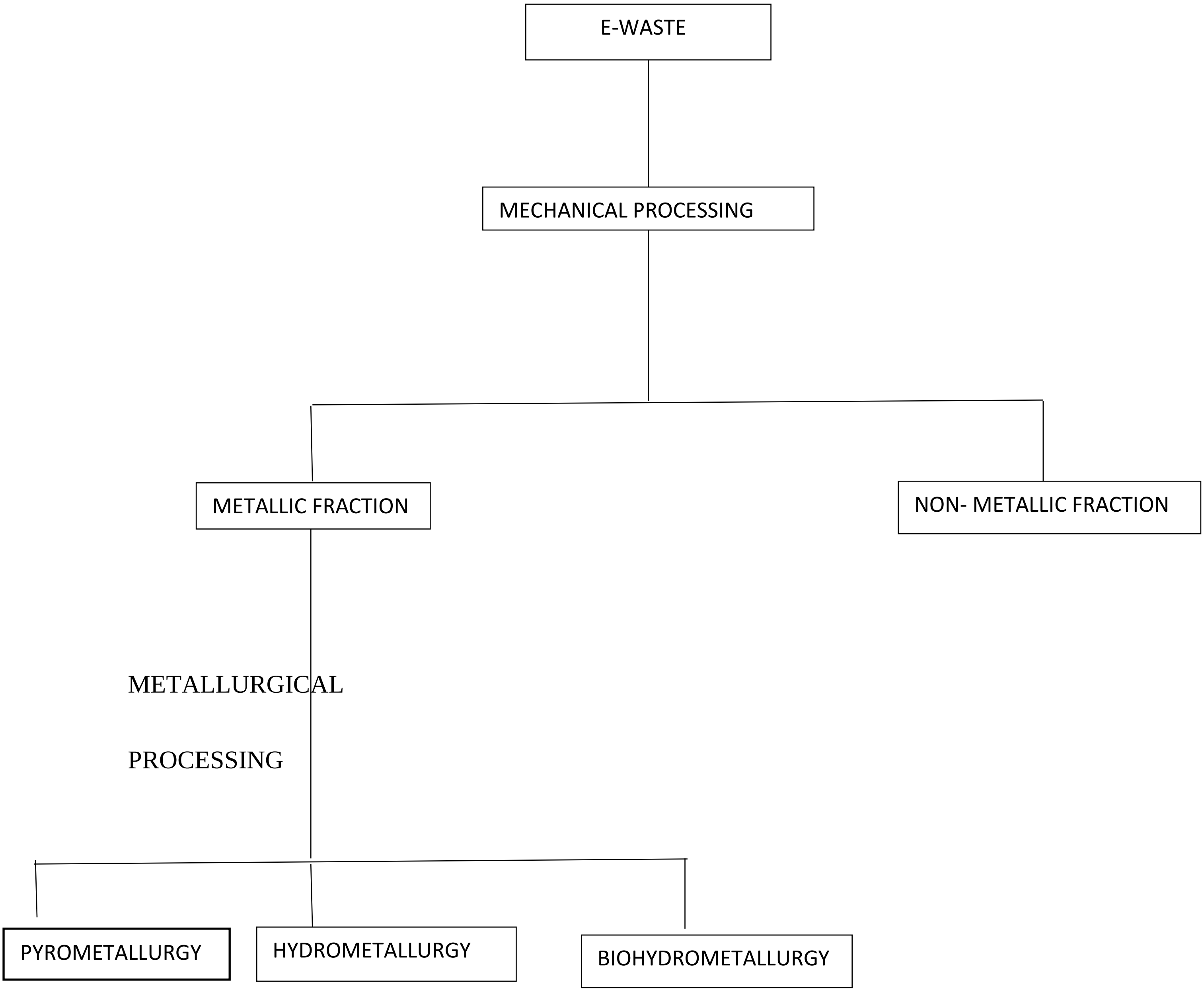


Figure 2 Treatment process of e-waste through various processes.

2.3.1 Mechanical process

Pre-treatment of e-waste for upgrading precious materials primarily involves mechanical processes, when the various metals and minerals present in e-waste are released and sorted through shredding or crushing. Cui and Forssberg researched mechanical recycling of electronic waste comprehensively in 2003, and several academics have looked into it. Following size reduction, the materials are separated into output fractions depending on physical attributes such as weight, size, form, density, and electrical and magnetic properties. Magnetic separation of ferrous parts (Magnetic separators are commonly used to separate ferromagnetic metals from nonferrous metals because of the differences in their magnetic characteristics.), Eddy current separation (electric conductivity) of aluminium, and gravity separation are examples of common sorting techniques (water or airflow tables, heavy media floating, sifting). The final product streams are normally components taken as a whole, a magnetic fraction (going to a steel mill for additional treatment), an aluminium fraction (to aluminium smelters), a copper fraction (to copper smelters), clean plastic fraction (sometimes), and garbage. Disassembly can be done in two different ways: selective or simultaneous. Despite its high efficiency rate, the usage of simultaneous disassembly processes is limited by the possibility of component destruction, additional sorting processes, and high operation costs, according to Chauhan's research in 2018 [18].

2.3.2 Pyrometallurgical processes

The most common pyrometallurgical techniques are incineration, smelting, and roasting. When metals are present in a complex matrix with various non-metals, ceramics, and other materials, it is typically difficult to recover them using physical recycling methods. The pyrometallurgical approach is an alternative in this instance [19]. Some of the thermal plants accessible for formal processing by pyrometallurgical techniques are Aurubis smelter, Noranda smelter, Ronnskar smelter, and Umicore. Smelting followed by electro-chemical refinement is a common pyro-metallurgical treatment procedure. Several precious metals are left over when this process is completed. As a result, smelting is regarded as one of the most effective ways for recovering pure metals. Copper and lead smelters are currently used as e-waste recyclers to recover Pb, Cu, and PMs. E-waste/copper/lead scrap is fed into a furnace in these pyrometallurgical processes, where metals are gathered in a molten bath and oxides form a slag phase [22].Xie et al. (2009) recovered Cu and Fe using innovative pyrometallurgy techniques and a cleaner ultrasonic approach, resulting in a better efficiency rate and less harmful by-products [20]. Some of the toxic compounds emitted by pyrometallurgical treatment include polybrominated dibenzodioxins (PBDDs), phenol, dibenzo-p-dioxin, biphenyl, anthracene, dibromobenzene, naphthalene, and polybrominated dibenzofurans (PBDFs), which are highly hazardous and may cause serious environmental issues [21]

2.3.3 Hydrometallurgical process

Traditional hydrometallurgical methods used for metal extraction from primary ores are modified hydrometallurgical methods used for metal recovery from E-waste. To leach out metals in the form of soluble salts, leaching is done with acid, alkali, or other solvents. Hydrometallurgical treatment ensures fortification of some precious metals recovery and boosts leaching efficiency rate when compared to traditional procedures like ion-exchange and electro winning [23]. Impurities are removed using gangue materials, and metals are isolated from the solution using techniques including adsorption and solvent extraction. Electro-refining or chemical reduction techniques are used to achieve the final forms of metals. Copper and other precious metals such as gold, silver, and platinum can be recovered using a hydrometallurgical process, according to Wu et al (2017) [24]. Through the co-processing of discarded PCBs and spent tin stripping solution created during PCB fabrication, a green hydrometallurgical technique for recovering tin from PCBs has been devised [25]. The use of hydrometallurgical technologies to process WEEE has grown in popularity in recent years due to decreased hazardous gas emissions, less dust formation, lower energy requirements, ease of implementation under laboratory conditions, lower capital and operating expenses, and significant metal recovery as cited by K. Ni et al.[26] and A.

Subramanian et al. [27].Figure 3 [28] depicts how trace metals included in E-waste can migrate from dump sites where they seep into groundwater to sufficient volumes of solution where they can be captured for further recovery using hydrometallurgy. Due to the high energy demands of pyrometallurgy, hydrometallurgical processing has been investigated as an alternative in the last decade. It is the most efficient technique of recovering metals because it can regulate various degrees of impurities at various stages, and it is the most environmentally friendly and economically viable process to utilise with chemicals for refinement at various stages [29]. For extracting PMs from e-waste, Park and Fray presented a hydrometallurgical technique. The leachate was aqua regia, and the metals and leachate were mixed in a constant ratio of 1/20. During the first stage, silver and palladium were extracted with 98 percent and 93 percent recovery, respectively. A liquid-liquid extraction process with toluene was used to extract gold, with a recovery rate of 97 percent recorded. Figure 3 shows a flow chart diagram for recovery of precious metals from PCB as proposed by Park and Fray.

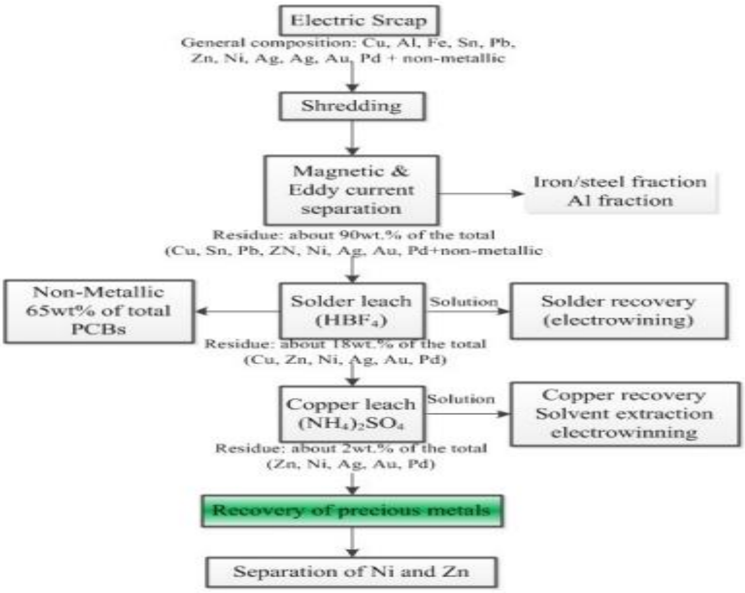


Figure 3 Example of hydrometallurgical recycling of PCBs for the recovery of PMs (source: Khaliq A et al. 2014).

2.3.4 Biohydrometallurgical Processes

Bio-metallurgy has the potential to be a major technological advance in the materials and minerals business, according to the high level of interest demonstrated by large worldwide corporations in this new technology [31]. A number of metals, including copper, nickel, cobalt, zinc, gold, and silver, are currently undergoing study and development [32]. Metal species are used by all microorganisms for structural and/or catalytic purposes. Microbes' interactions with metals are influenced by whether they are prokaryotic or eukaryotic. Figure 4 shows a table of all the microorganisms that have bio-leaching properties. Numerous studies have shown that moderate thermophiles have a greater bioleaching capability than mesophilic and extreme thermophiles, hence many Thiobacilli bacteria and thermophilic fungi (e.g., *A. niger and P. simplicissimum*) have been employed to extract metals from e-waste and low-grade metal reservoirs [33] [34].

Table 2 Various organisms able to produce different leachates and has been utilized for bioleaching applications.

Domain	Organism	Leaching agent
Archaea	*Sulpholobus sp.*	Ferric iron and sulphuric acid
	Acidianus sp.	Sulphuric acid
	Thermoplasmaacidophilum	-
Bacteria	*Acetobactermethanolicus*	Gluconate
	Acidiphilumcryptum	Organic acid
	Bacillus sp.	-
	Bacillus megaterium	Citrate
	Chromobacterium violaceum	Cyanide
	Leptospirilliumferrooxidians	Ferric iron

	Pseudomonas putida *Theobacillus sp.*	Citrate, gluconate Ferric iron and sulphuric acid
Fungi	*Aspergillus sp.* *Aspergillus niger* *Penicilliumfuniculosum* *Penicilliumsimplicissimum*	Citrate, oxalate Oxalate, citrate, succinate, malate Citrate Oxalate, citrate, gluconate
Yeast	*Candida lipolytica* *Saccharomyces cerevisiae*	- -

There are two types of bio-metallurgical processes: a) biosorption and b) bioleaching. Biosorption is the process of adsorbing metals using adsorbents made from waste biomass or plentiful biomass. Living and dead organisms can be employed in the biosorption process, which is a passive physico-chemical interaction between the charged surface groups of microorganisms and ions in solution. Biosorption has been used to recover metals from E-waste using algae (*Chlorella vulgaris*), fungi (*Aspergillus niger*), bacteria (*Peniciliumchrysogenum*), hen eggshell membrane, ovalbumin, and alfalfa, among other things. When opposed to traditional approaches, a biosorption-based technology has a number of advantages. Low operating expenses, a small volume of chemical and/or biological sludge to treat, and excellent effluent detoxification efficiency are among them.

Bioleaching is the extraction of metals from bacteria's metabolic activity (direct bioleaching) or metabolic products (indirect bioleaching) (indirect bioleaching). It can be used for metal extraction from low-grade ores, ore or coal beneficiation, hazardous metal removal, and metal recovery from waste materials. Sulfur and iron oxidising chemolithotrophicacidophiles like *Ferrobacillus, Acidithiobacillus*, and *Leptospirullum*, as well as heterotrophs like *Chromobacterium, Pseudomonas, Sulfolobus, and Bacillus*, are among the most often used bioleaching microorganisms [35]. Microorganisms use a variety of methods to solubilize metals from e-waste during the bioleaching process, including acidolysis, complexolysis, and redoxolysis. Cyanogenic bacteria (such as *C. violaceum, B. megaterium, P. aeruginosa, and P. fluorescens*) generate hydrogen cyanide as a secondary metabolite and build solubilized metal-cyanide complexes from metal-containing solids/wastes such lowgrade ore and e-waste [36] [37]. *C. violaceum* is a heterotrophic cyanogenic bacterium that has been frequently used to extract metals from e-waste. Despite the fact that *C. violaceum* has been extensively studied and has excellent bioleaching abilities, it is not suited for use in industrial bioleaching applications. *C. violaceum* is a pathogen that thrives in tropical and subtropical environments. The unique requirement for growth conditions limits its industrial application in the recovery of metals from e-waste [38][39].

2.4 Limitations of conventional methods of e-waste treatment

PMs from e-waste have been recovered successfully using hydrometallurgical techniques. However, these techniques are linked with a number of drawbacks that limit their practical applicability. Here are some of the most typical drawbacks of hydrometallurgical technologies for recovering PMs from e-waste [40][41]:

(i) Overall, hydrometallurgical routes are slow and time-consuming, and they have an adverse effect on the recycling economy. There are questions about the economics of hydrometallurgical approaches for extracting PMs from e-waste when compared to pyrometallurgical procedures.

(ii) Due to corrosive acids and oxidising circumstances, halide leaching is difficult to implement. For gold leaching utilising halide agents from e-waste, specialised equipment constructed of stainless steel and rubber is required.

(iii) Due to its high cost and consumption, the use of thiourea leachants in gold extraction is limited. Furthermore, improvements to the present thiourea-based gold leaching method are necessary.

(iv) Thiosulfate has a greater consumption rate and a slower overall process, which limits its use for gold extraction from ores and e-waste.

(v) There is a potential of PM loss during dissolution and following processes, which will impair the overall metal recovery.

Pyrometallurgical techniques are generally more cost-effective, environmentally friendly, and maximise PM recovery, but they do have some drawbacks, which are detailed here.[42][31]

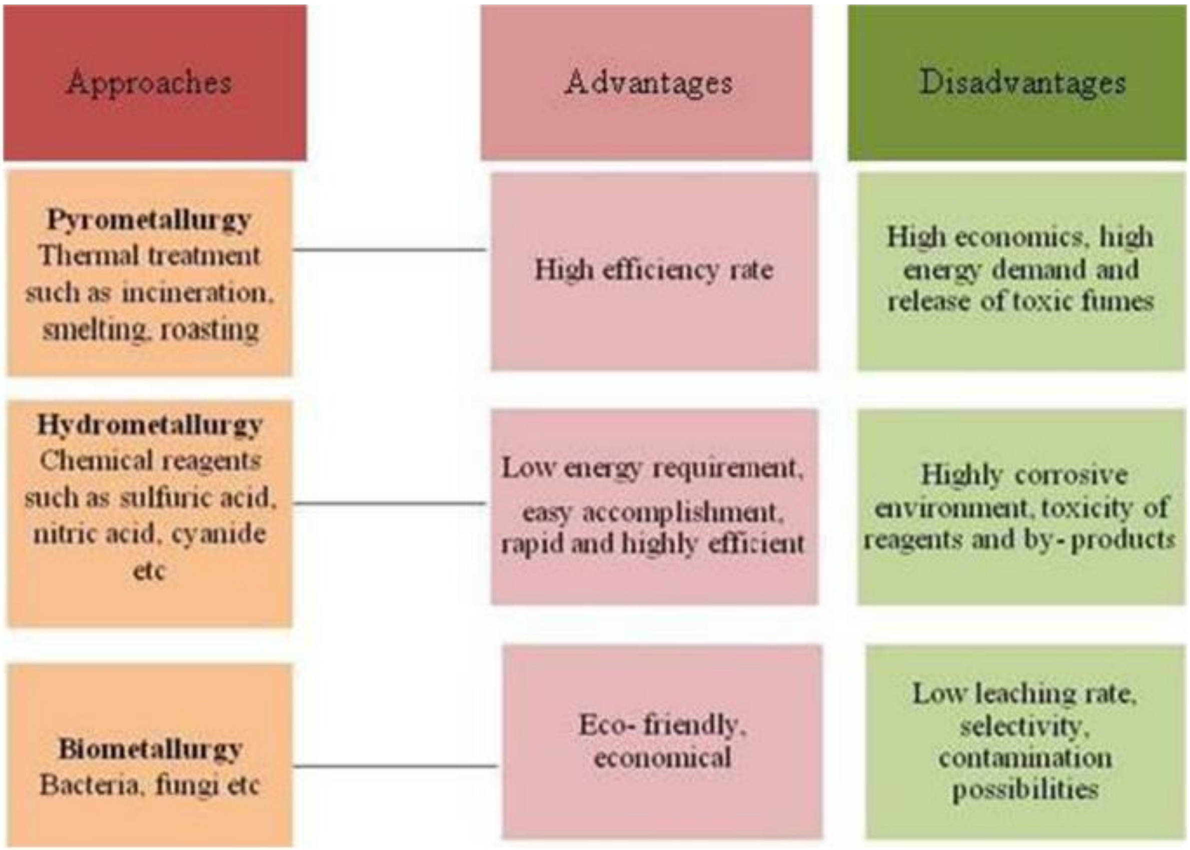
(i) Plastics cannot be recovered since they have replaced coke as a source of energy.

(ii) Recovery of iron and aluminium is difficult since they end up as oxides in the slag.

(iii) During the smelting of feed materials containing halogenated flame retardants, hazardous pollutants such as dioxins are produced. As a result, special installations are required to reduce pollution levels.

(iv) Pyrometallurgical approaches allow for partial recovery and purity of PMs. To extract pure metals from BMs, additional hydrometallurgical and electrochemical procedures are required.

(v) Due to the complexity of the input materials, managing the smelting and refining process is difficult. It will be challenging to obtain competence in process handling and the thermodynamics of probable reactions.



Approaches	Advantages	Disadvantages
Pyrometallurgy Thermal treatment such as incineration, smelting, roasting	High efficiency rate	High economics, high energy demand and release of toxic fumes
Hydrometallurgy Chemical reagents such as sulfuric acid, nitric acid, cyanide etc	Low energy requirement, easy accomplishment, rapid and highly efficient	Highly corrosive environment, toxicity of reagents and by-products
Biometallurgy Bacteria, fungi etc	Eco- friendly, economical	Low leaching rate, selectivity, contamination possibilities

Figure 4 Advantages and disadvantages of various metallurgical processes (Thakur et al. 2020)

2.5 Policies and legislations regarding e-waste

Many countries have launched measures and enacted regulations to address the ever-growing e-waste problem and to take advantage of this important secondary resource. To restrict the harmful effects of WEEEs, more than 2000 sections of law have been enacted in over 90 jurisdictions throughout the world [43]. Table 2 lists a number of worldwide organisations and initiatives that have taken steps toward appropriate monitoring and recycling. Producers are assigned major financial or physical responsibility for the treatment or disposal of post-consumer items under the Extended Producer Responsibility policy approach. Producers are responsible for collecting waste Electrical and Electronic Equipment from previously sold Electrical and Electronic Equipment, such as through dealers, collection centres, buy-back arrangements, exchange schemes, Deposit Refund Systems, and other methods, whether directly or through any authorised agency, and channelling the items collected to authorised recyclers. This EPR programme was originally compelled by a German directive on the avoidance of packaging waste (known as the "Green Dot" Program), which imposed a financial burden on manufacturers for assembling, discarding, and recycling (Chatterjee A et al. 2017) [44]. The EU commission passed the WEEE directive (2012/19/EU) for consistent regulation of e-waste management in the country. This directive establishes ten categories to guide comprehensive recovery and reprocessing methods for all WEEEs in order to increase the turnover of recyclable e-waste fractions. Later, they introduced the "Restriction of Harmful Compounds" (RoHS) directive 2012/ 95/EC, which aims to change product design and packaging to restrict the intake of hazardous substances such as lead, cadmium, hexavalent chromium, mercury, and many more.

The Indian government established Rules 2011, which control the use of hazardous compounds in electronic and electrical components and equipment. Rules 2011 is the equivalent of the European Union's WEEE e-waste laws, which govern the disposal of electronic and electrical equipment as well as hazardous materials limitations in electronic and electrical components, similar to the EU's RoHS directive. The legislation of India and the EU are strikingly similar. Batteries and radioactive waste are not included by the Rules 2011, as they are already governed by Indian legislation. There are 39 substance exemptions, just like the EU RoHS directive.

Table 3 Various initiatives taken by global leaders to end e-waste (Ilankoon et al.2018)

Initiatives	**Key features**
Basal convention	• Endorsed in 1992 to avoid exportation of hazardous waste from producer countries, I72

	nations stand by the agreement but US does notratify the treaty
Bamako convention	- Aimed to restrain the import of E-waste more stringently than Basel Convention, Applied in African Union nations from 1998 - In 2007 all the EU members adopted the system with initiation of takeback approach for 10 groups of electrical things
Solving the E-waste problem	- Initiated in 2007 by UN agencies to promote reusability of the recycled components to limits the waste generation
3R's (Reuse, Reduce, Recycle)	- Initiated by Japan. Work to prevent e-waste generation. Allows exportation to other countries for remanufacture and recycling. Conflicting the goal of Basal Convention treaty
National strategy for electronic stewardship	- Focus to limits the use old harmful substance Improve the handling and management strategies of e-waste in the US or reduce their harmful impact in other nations
International environmental technology centre	- Strengthen utilization of environmentally suitable technologies in developing nations on waste management

Solving the e-waste Problem (StEP), on the other hand, is a United Nations effort that focuses on the global recovery and reusability of materials. It is divided into five sections: tactics, reformation, recovery, reuse, and capacity building [45]. The efforts' distinguishing feature is the formulation of products that are simple to disassemble and recycle, with little use of harmful ingredients. At the 2004 G8 conference, Japan proposed 3Rs (reduce, recycle, and reuse) activities both nationally and worldwide. This effort targeted preventing e-waste generation by boosting technology reusability, pursuing the goal of removing barriers to intercontinental transit for reprocessing and recovery.

CHAPTER 3

MATERIALS AND METHODS

3.1 Collecting information to understand public point of view on e-waste and how it is collected and disposed

- The first step was to create different questionnaires for industries, rag pickers and general public.
- In total 40 people were surveyed using different methods to ask the questions.
- For the general public a google form created and sent them out to people of different age groups to get their opinion on the growing problem of e-waste.
- For the rag pickers and the people working in industries related to e-waste a simple method of interview was chosen to understand their viewpoint in a better way

The questionnaire for the public consisted of the following questions:

I. Do you use household electronic, namely computers and cell phones? if so, which ones do you use frequently?
II. Of the household electronics that you use, how many of each type have you purchased /replaced in the last 10 years?
III. The electronics that you have purchased in the last 10 years, how many do you still own and use?
IV. What was your reason for purchasing new cell phones and/or computers?
V. What have you done with the electronics that you no longer use?
VI. Do you consider your unused electronics to be a waste, or to have another purpose? please explain if you believe they have another purpose?
VII. Do you know someone who can collect your unused electronics for recycling or dismantling and refabricating or destroying?

VIII. Do you perceive any hazards or risks in e-waste or to the growing amount of e waste?
IX. Do you know any of the electronic waste management policies currently implemented in India? if so, what do you know of these policies?

The questionnaire for the rag pickers consisted of the following questions:

I. What is the amount of e-wasted you have collected in the last 5 years?
II. What are the metals you do or do not recycle?
III. Do you also accept the scrap metals coming from restricted materials?
IV. How do you deal with working leftovers?
V. What is your opinion on the growing amount of e-waste collected?
VI. What methods do you use for recycling or dismantling e-waste?
VII. Do you think the government should do anything to assist you in your work?
VIII. Who is responsible for the growing amount of e-waste in India?
IX. Do you perceive any health hazards in dealing with e-waste that you collect?
X. What components are the most valuable to you?
XI. From where and from whom do you collect the electronics?

The questionnaire for the industries consisted of the following questions:

I. Do your services comply with industry standards?
II. What services do you offer as a part of your disposal and recycling points?
III. What is the minimum and maximum volume of waste you will recycle?
IV. What types of devices do you accept?
V. How much of what you process is disposed of in the landfills? is it legal to dump computers in trash or landfills?

VI. What happens to the hazardous materials removed from electronics during the recycling process?

3.2 Source and compositional analysis of soil

The soil sample was collected from an e-waste dumping facility present in Solan, Himachal Pradesh. The soil sample was further taken to the laboratory of Jaypee University of Information Technology for metal concentration analysis. The metal content was checked for soil as well as the e-waste by following the acid digestion by aqua regia.; which is a protocol used by several researches in various studies [49][50][51]. In the aqua regia method HCl and HNO3 are taken in 3:1 ratio. 1gm of the soil sample and e-waste was dissolved separately into 100 ml aqua regia by refluxing the solution into a round bottom flask for 1 hour at 100ºC. the solution was then kept to cool and filtered further to obtain a particle less solution. The solution was used to create two dilutions of 100X and 10X to make the analysis easier and accurate. The metal analysis was done through atomic absorption spectroscopy.

3.3 Isolation of bacteria from soil sample

The soil sample collected was predicted to contain e-waste tolerant bacteria as it was found in an environment where metals and electronic items were disposed. The method used for isolation of bacteria is known as the "Direct incubation method" as used by P. Geetanjali et al. in 2016 [52]. In this method 1 gm of soil sample was added to 100 ml of Nutrient Broth under sterile conditions. The solution was left to incubate for 24hrs at 37ºC. After the incubation was complete the solution was serially diluted up to10^8 dilutions. Plating was performed for the 10^6 dilution by spread plate technique onto a nutrient agar plate to obtain isolated colonies of bacteria.

3.3 Biochemical and morphological characterization of the bacteria

The morphological features of the bacteria were studied through seeing the spread plate using the criteria's such as colour, size, margin, surface, opacity etc. for the biochemical characterization of the bacteria, gram staining method was used to know whether the bacterial colonies obtained were gram positive or negative. For, this method the bacteria were picked from the plate through an inoculating loop and spread on a sterile glass plate and heat fix the bacteria. Then crystal violet was added to the bacteria and left untouched for 60 seconds; washed off with water; added iodine to the glass slide so as to help the stain get absorbed. Washed the plate with ethanol. The last step was to add safranin stain for 10-20 seconds and then washed with water. The ability of the bacterial cell walls to retain the crystal violet dye after solvent treatment is the core principle of gram staining. Gram-positive microbes contain more peptidoglycan, while gram-negative organisms contain more lipid. All bacteria take up crystal violet dye at first, however with the addition of solvent, the lipid layer of gram-negative bacteria is dissolved. Gram negatives lose their principal stain as the lipid layer dissolves. Solvent, on the other hand, dehydrates gram-positive cell walls by closing pores, blocking violet-iodine complex diffusion and leaving bacteria marked. Hence if the bacteria are seen to retain the purple stain under microscope it is positive if not it would retain the colour of the counterstain that is safranin in this case and would be seen as pink. In gram staining, the period of decolourization is important since extended exposure to a decolorizing chemical can remove all stains from both types of bacteria.

3.4 Toxicity assessment of the bacteria

The isolated bacteria were tested for toxicity tolerance at 200,300, 400, 500 and 600 g/L pulp density of e-waste. As Anil Kumar et al. used the method of colony-forming unit (CFU) count method for the toxicity assessment in their study the same was used in the present study [53]. The experiment was performed with 3 flasks with 50 ml NB inoculated with bacteria along with various concentration of e-waste. A fourth flask was used as control which grew bacteria in the absence of e-waste. All the flasks were incubated at 37ºC for 24 hrs. Samples from each flask were taken, serially diluted to 10^8. The dilutions 10^2,10^4, 10^6 and 10^8 were plated from each sample. The colonies if bacteria were counted and then the CFU was counted by using the following equation;

CFU= No. of colonies x dilution factor / volume

The bacteria with the highest tolerance were the selected for bioleaching by calculating the %IR by the following formula [53]:

$$\%IR = \frac{Control - Test}{\mathrm{Control}}$$

where IR stands for inhibitory reaction, Control for bacterial growth without e-waste, and Test for bacterial growth with e-waste.

3.5 Bioleaching process

As the isolated bacteria bioleach ability is being compared to the bioreactivity of *Chromobacterium violaceum* which exhibited the highest bioleaching ability by leaching metals like Cu (79.3 percent w/w), Au (69.3 percent w/w), Zn (46.12 percent w/w), Fe (9.86 percent w/w), and Ag (7.08 percent w/w) as studies by J Pradhan and team in 2012 [54]. Two flasks were taken with nutrient broth and 0.5 g of glycine was added to the flasks. The isolated bacteria and *C. violaceum* were inoculated to the flasks. 1gm of e-waste was added to these flasks and then were kept for incubation at 37^0C for 8 days.

CHAPTER-4

RESULTS AND DISCUSSION

4.1 The survey of general public, rag pickers and industries

I had surveyed around 32 people while asking them the questions from the questionnaire for general public, there are a few things that came into light. The first and the foremost point was that everyone nowadays is surrounded by electronics and using them on a daily basis; which at the end obviously leads to the throwing away of the old and used e-waste. Second point that came up was that there are a lot of people who are not aware of the hazardous risks that the e-waste brings along with it; which means that people lack awareness or maybe there is not a lot of awareness spread about e-waste and its associated risks. The third and the last point was that the new e-waste rules issued by the government in 2016 are unknown to exactly 99% of the audience which again is the result of lack of awareness.

The rag pickers all had the same thought about the growing amount of the e-waste which was collected, they all agreed to it being a good thing as more and more people are getting the knowledge about throwing the e-waste at their rightful place. The government has not set any maximum or minimum amount of e-waste hat is to be collected which is why it is very difficult to get the estimate idea of the total e-waste collected in the last 5 years. They are known to the risks related to the disposal of e-waste which is why all the dismantling and recycling processes at their end are environment friendly.

Industries on the other hand have an altogether different set of steps to the collection of e-waste and then further their disposing/ recycling. Different companies like Samsung, Apple, HP, etc. have collecting points for their e-waste like faulty batteries, phones, cables and other accessories. These collecting points are only responsible for the collection of the waste and it is totally free of cost. The waste is then giving to the vendors who take on the processes like shredding, dismantling and recycling through machines or man power. The head in charge of the collecting point for Samsung had pointed out that in rural areas there are a lot of people who aren't aware of these points and hence their waste ends up in landfills which is a lot of harm to the environment. There was a massive audit by the government regarding e-waste that took place 2 years back which enhanced the knowledge of people about e-waste. For waste of bigger products like LG, Whirlpool who sell refrigerators, LED's etc. the consumer has to write a message to the customer care and their waste would be collected from their doorstep and then rightfully be sent to the vendors for recycling. There are a lot of campaigns and NGO's who take care of e-waste by having ties with the big companies like Lenovo, Sony, Apple, etc. and the proceed with the same

4.2 Metal concentration analysis

The metals that were present in the soil sample were copper, nickel, cobalt, silver and gold. The copper was present in the highest concentration i.e. at 0.6605 mg/g followed by nickel, gold, silver and the lowest concentration of cobalt. The concentration and percentage of the metals present has been given in the table below

Table Concentration in soil and e-waste sample

	Soil (mg/g)	e-waste (mg/g)
COPPER	0.6605	35
NICKEL	0.0978	0.061
GOLD	0.0348	1
SILVER	0.0318	0.35
COBALT	0.0142	0.0097

4.2 Isolation of bacteria

In order to utilize the advantages of an indigenous isolate bacteria that are inured to heavy metal concentration it is necessary to isolate them from their sites. In this study the plating of the bacteria, two different colonies of bacteria were obtained which had different features. Following were the results of the isolation and the colonies were distinct from each other.

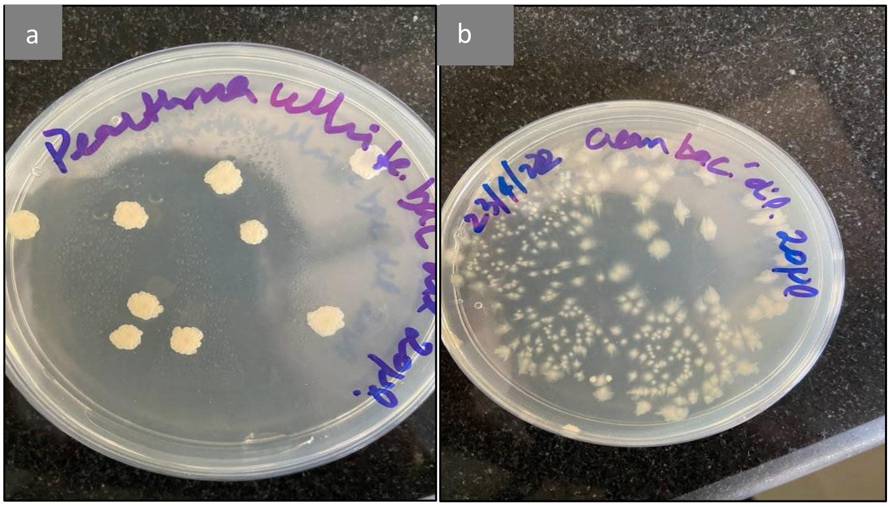


Figure 6 Isolated bacterial colonies on nutrient agar media(a) ISOA (b) ISOB

4.4 Characterization of the bacteria

The bacterial colonies obtained from the isolation was further inoculated in 100 ml NB which was further serially diluted up to 10^8 dilutions; the 10^6 dilution was plated onto the nutrient agar plate and clear separate colonies were obtained. The plate was further sub cultured onto an agar plate through streaking technique. The plated were incubated and further studied to check the morphological features of the bacteria. Picture 2 and 3 are the results from the plates of 2 colonies of bacteria obtained. The morphological features of the two strains are discussed in the table below.

Table 4 Morphological characteristics of the bacterial isolates obtained on nutrient agar.

CHARACTERISTICS	ISOLATE A	ISOLATE B
Colour	White	Yellow
Shape	Irregular	Circular
Size	Mid- large	Small
Margin	Undulate	Entire
Elevation	Umbonate	Flat
Opacity	Opaque	Translucent

The bacteria from the plates was picked and further underwent gram staining to know the type of bacteria.

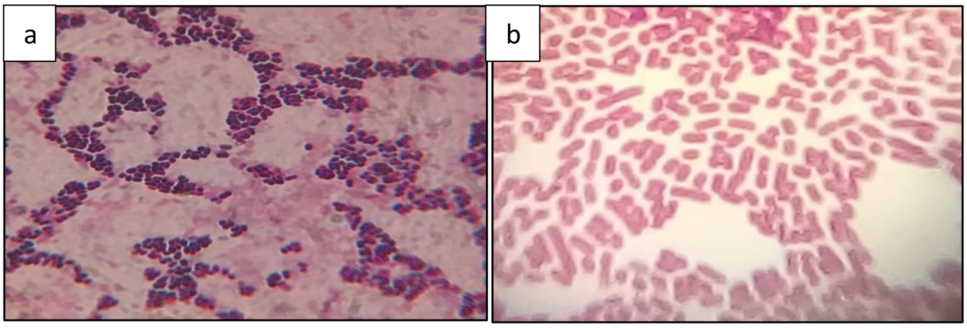


Figure 7 The morphological characterization of the indigenous bacterial isolates observed at 100X (a) Gram positive isolate A(b) Gram negative bacterial isolate B

4.4 Toxicity assessment

It is necessary to understand the tolerance of the bacteria with e-waste or heavy metal contamination so as to help understand the bioleaching properties in a better way. The bacteria with the most tolerance to e-waste could be of help when it comes to recycling e-waste or recovery of precious metals from the e-waste or soil that is found in a e-waste recycling facility. The two bacterial strains were subjected to e-waste tolerance assessment. The bacterial isolate A had a very low tolerance value. It reached its EC_{50} value at 295 g/L. hence its colonies could not grow above this concentration. The isolate B on the other hand was growing even at 500 g/L and had only reached EC_{45} value at this stage. Which is why this strain was selected for testing its bioleaching potential. The table below shows the calculated values of %IR of the bacterial strain at 200 g/L, 300 g/L, 400g/L, 500 g/L and 600 g/L. In a study conducted by Kumar et al. the EC_{50} value for *C. violaceum* was observed at 325.7 g/L whereas in the current study the EC_{50} value for the isolate A can be seen at 550 g/L which is much higher than the *C. violaceum* bacteria.

Table 5 The toxicity tolerance assessment in term of percent inhibition response

Pulp density	%IR (Isolate A)	% IR (Isolate B)
200	28	42
300	34	54
400	41	67
500	49	78
600	62	83

4.5 Bioleaching

Bioleaching is a process of extracting metals from various their ores through the use of microorganisms. The microorganisms involve are the ones that produce cyanide and are known as cyanogenic bacteria. This process is considered to be more environmentally friendly as compared to other conventional methods that are used or the treatment of e-waste. Two-step bioleaching of metals was done using indigenous bacterial isolate and *C. violaceum*. Both the microorganism exhibited cyanogenic mechanism as glycine was added as precursor compound for production of cyanide which make complex with various metals (e.g., Zn, Fe, Au, Ag, Ni and Cu but higher concentration of copper creates hindrance to leach out other metals. Therefore, leaching of Cu was found maximum with both microorganisms whereas other metals leached in very less amount. For the optimal production of cyanide, it is necessary to maintain some factors for e.g., the quantity of glycine. Glycine acts as a precursor molecule for production of cyanide and its addition can enhance the production of cyanide in a solution. Also, the temperature has to maintained at 15-35^{o}C. The pKa value of cyanide is 9.3 conducting the dissolution of metals at an alkaline condition would result in a better production of cyanide. Various experiments have shown maximum mobility of metals at a pH of 9. The bioleaching experiments were performed under optimized solutions of NB with pH set at 9 and 5g/L glycine. After 8 days of incubation of the bacterial isolate A and *C. violaceum* the said bacteria were diluted 100 times and then subjected to analyses in atomic absorption spectroscope for the metal concentration analysis. Out of all the metals analysed Cu and Ag were recovered at the highest percentage whereas others were recovered in a very small quantity. In the study 53% Cu was recovered through the isolated bacteria whereas only 36% was recovered by *Chromobacterium violaceum.*

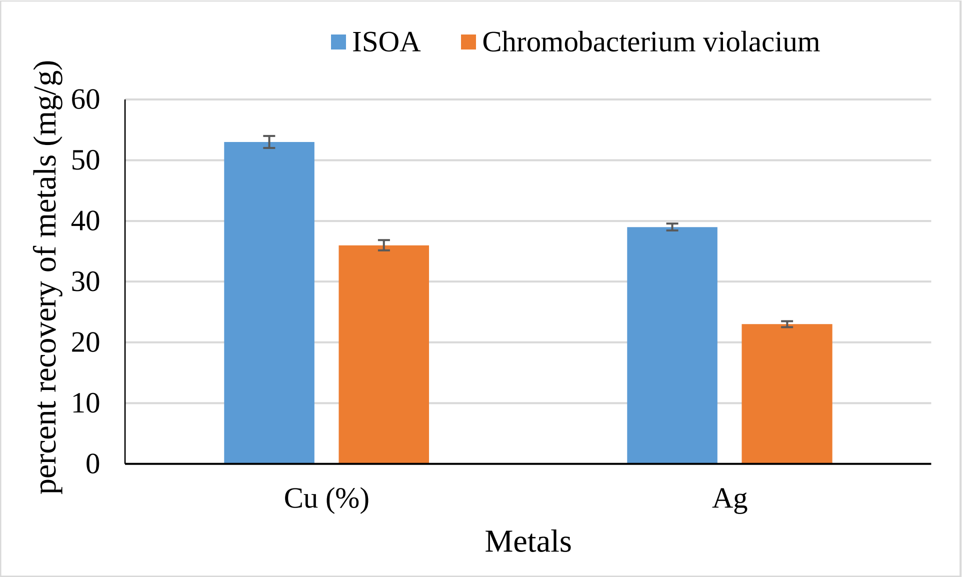


Figure 9 Bioleaching of Cu and Ag by indigenous isolate and *C. violaceum*

Kumar et al. claimed the recovery of Cu through various bacteria such as *B. megaterium, L. sphaericus*, *Bacillus sp.* and *C. violaceum*. The mentioned bacterial strains recovered 24.4%, 21.5%, 23.8% and 29.3 % respectively. Kumar et al. also recovered 33.8% Ag by *Pseudomonas sp.* On the other hand, the isolate A obtained from the present study has shown recovery of Cu (53%) and Ag (39%) higher than what was observed in the previous studies.

CHAPTER 5

CONCLUSION

The indigenous bacteria isolate A proved to be an efficient bioleaching agent. Isolating the same from an e-waste disposal facility, the bacteria showed the EC_{50} value at 550 g/L of e-waste which proves it have high tolerance to e-waste toxicity. The isolate A recovered high amounts of Cu and Ag as compared to *C. violaceum* and other bacteria used in studies by other researchers. The bacterial strain's better tolerance to e-waste toxicity, combined with its capacity to mobilise metals efficiently, validates its appropriateness for industrial bioleaching operations to recover precious metals from e-waste. These initiatives will undoubtedly pave the way for primary/natural resource conservation, environmental protection, and a significant contribution to the shift to a circular economy.

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