Isolation of metal tolerant microorganisms from natural environment and to analyze their metal bioleaching capabilities.

Project report submitted in partial fulfilment of the requirement for the Degree of

Bachelor of Technology

In Biotechnology Submitted by

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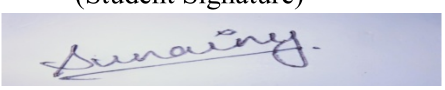
CERTIFICATE

Candidate's Declaration

I hereby declare that the work presented in this report entitled **"Isolation of metal tolerant microorganisms from natural environment and to analyze their metal bioleaching capabilities**" in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in biotechnology submitted in the department of biotechnology and bioinformatics, Jaypee University of Information Technology Waknaghat is an original record of my own work carried out over a period from July 2019 to May 2020 under the supervision of Dr Sudhir Kumar (HOD, Department of biotechnology and bioinformatics).

The matter expressed in the report has not been submitted for the award of any other degree or diploma.

(Student Signature)



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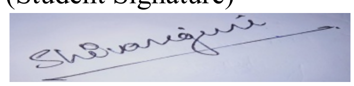

Shivangini Singh (161811)

This is to certify that the above statement made by the candidates is true to the best of my knowledge.

(Supervisor Signature)

Name: Dr Sudhir Kumar

Department name: Department of Biotechnology and Bioinformatics
Date: July 16th, 2020

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Abstract

E-waste is taken into consideration due to presence of feasible amount of metal content in it which could be extracted by using bacterial strains with bioleaching capabilities. E-waste recycling is limited due to heterogeneity in e-waste. Bacterial strains were isolated from natural environment and tested for e-waste toxicity tolerance. E-waste toxicity tolerance of bacteria was analyzed using powdered printed circuit boards (PCBs), which was procured from Exigo Recycling Pvt. Ltd., India. The EC_{50} value of isolate 1 and isolate 2 was found to be 250 g/l of ewaste pulp density. Further, two step bioleaching was carried out for maximum metal mobilization and to estimate leaching capabilities of the isolates.

1. Introduction

The project entitled "Isolation of metal tolerant microorganisms from natural environment and to analyze their metal bioleaching capabilities from e-waste" is mainly concerned with the objective of isolating bacteria from the natural environment which can tolerate high e-waste toxicity and also concerned with the determination of the cyanide lixiviate and optimization of different parameters like pH, temperature and pulp density. Different samples were collected from the natural environment and their isolation was done by applying the enrichment strategy. Aqua regia digestion was performed to determine the composition of e-waste of particle size **≤ .15mm** used in the experiment and the results were compared with the previous studies. The comparison showed a significant difference in the metal composition of the e-waste which was procured from storehouse of **Exigo Recycling Pvt. Ltd., Panipat, Haryana, India**. EC_{50} was calculated for both the strains to determine their toxicity tolerance level by calculating their percent inhibition response (%IR).Further, two step bioleaching was carried out to isolate the metal tolerant bacterial strains which have the capability of leaching metals at higher concentration.

There has been an exponential increase in the generation of e-waste due to the high consumption of electronic devices and their early obsolescence. E-waste contains various toxic and hazardous metals (lead, cadmium, mercury, beryllium etc.) which has raised various health and environmental concerns and to their disposal also [1,2]. Management of e-waste is a matter of great concern because the crude and unscientific methods can cause a huge damage. Recycling of e-waste should be encouraged to preserve our natural resources [3]. The spectacular development in the technology has undoubtedly enhanced our lives but at the same time, it has led to manifold problems which include the generation of the massive amount of hazardous waste. E-waste comes from the various types of goods and it has been stated that the waste from grey goods is more toxic than white and brown goods. White goods include household appliances, brown goods include TVs, camcorders and cameras and grey goods include computers, printers, fax machines, scanners etc. These wastes are generally dumped in the nearby soil and water which results in the significant contamination of the soil and ground water. Obsolete PCs are attractive to the informal recyclers in our country due to the presence of high content of precious metals [4]. A survey of the UN suggests that e-waste is expected to exceed 40 million tons per year globally. Recycling of precious metals from e-waste like Copper and Gold is the major source of income mostly in the informal sector of the developing countries. Around 80000 people are involved in the recycling sectors in India. It is also estimated that about 80% of the electronic items are stored because of the doubt of how to manage it [5].

There are 65 cities in India which generates more than 60% of the total e-waste. Mumbai followed by Delhi, Bangalore, Chennai, Kolkata, Ahmadabad, Hyderabad, Pane, Seurat and Nagpur are among the top ten cities in the generation of e-waste. In India, e-waste is usually given to the rag pickers who pay some amount to the people from whom they are collecting. After the collection, further activities include segregation, dismantling, disposal etc., which are mostly carried out by the informal sectors. It is a great source of income for rag pickers as well as for middlemen and scrap dealers. But most of this work is done by unskilled labors and they do not take proper safety measures. Lack of appropriate technology is the main reason behind not properly doing the disposal. Hence, implementation of proper e-waste management system is required in India. [6]

There are various recycling techniques which are used for its proper disposal which includes hydrometallurgy, hydrometallurgy and biohydrometallurgy (bioleaching). Hydrometallurgy and hydrometallurgy are considered to be the fastest method as they consume less time and their metal extraction is also rapid as compared to the bioleaching. But these two methods do have some disadvantages which make bioleaching more preferable and their disadvantages include high toxicity, high metal loss, extensive energy requirement and high investment cost. In comparison to them, bioleaching is an eco-friendly and cost-effective method which has been employed by various researchers [1].

2. Review of literature

2.1 What is E-waste? E-waste is considered as an issue of great concern due to its complex composition and increasing volumes. Therefore, e-waste recycling is an important subject for both- waste management as well as recovery of valuable metals [7]. Electrical appliances like computers and television sets are composed of more than 1000 materials, most of which are highly toxic in nature due to the presence of the chlorinated and brominates substances, toxic metals, photo-active and biologically active materials etc [8]. Most of the hazardous waste comes from the discarded electrical and electronic equipments (EEE). Along with the hazardous products, many costly components are also found in them which have high economic value if recycled. Increasing value of this complex is of great concern because e-waste is considered to be the fastest growing source of waste worldwide [9].

2.2 Why is e-waste harmful?

E-waste may contain harmful components such as mercury, lead, hexavalent chromium which is present in the batteries as well as the circuit boards and in color cathode ray tubes (CRTs) also. On average, four pounds of lead has been found in the CRT monitors and telivisions. In municipal waste, electronics are the main source of mercury. Also, brominates flame retardants are usually added to plastic part of electronics which is toxic and persistent in nature. And, hence, is banned by Europe. These toxics need to be handled and disposed carefully as it can release in the environment and cause potential health problems and risks to our resources [10].

Health effects of some common components in e-waste:

2.2.1 Lead

Lead is used in computer monitors, printed circuit boards and in other parts of electronics. It affects the kidneys, blood system, and reproductive system in humans and causes a huge damage to the nervous system and specifically on its peripheral and central part. In children, lead causes hindrance in brain development. Adverse effects are seen in plants, animals and microorganisms due to accumulation of lead in the environment [10].

2.2.2 Mercury

According to studies, 22% of the annual world consumption of mercury is constituted in electrical and electronic appliances such as lamps, mobile phones, sensors, thermostats, switches, medical equipment, relays and batteries.

It causes damage to the central nervous system. The growing fetus is highly vulnerable and can get affected on exposure to mercury. Mercury gets accumulated in the living organisms and travels to the entire food chain, specifically via fishes. This happens when inorganic mercury spreads through water and gets transformed to ethylated mercury [10].

2.2.3 Cadmium

Cadmium is present in semiconductor chips, surface mounted devices (SMD) and infra-red detectors. These are toxic compounds which get accumulated in human body parts, specifically liver, pancreas, kidney and thyroid [10].

2.2.4 Plastics

The largest amount of plastic e-waste comes from poly vinyl chloride (PVC) which is present in the computer housings as well as cables. It gets accumulated in the environment and the living organisms. A considerable concentration of dioxins is released in the environment when PVC is burnt [10].

2.2.5 Brominated flame retardants (BFRs)

Brominated flame retardants are generally used in the plastic housings and in printed circuit boards in order to prevent flammability. They also show bioaccumulation and their persistent toxicity in a potential concern [10].

2.2.6 Where did this begin?

The term e-waste is not a very old concept. Higher technology production started after the half of 20th century. There were very less technological products before the 1970s, which often constitute substances which can be toxic to environment and cause pollution on its disposal [10].

Every year, technology advances and new devices are launched and old ones are disposed off in a large number. This occurred in countries like Japan and Unites States and along with technological revolution, came along the electronic pollution revolution [10].

2.3 Laws to management e-waste in India

MoEFCC- The ministry of environment, forest and climate change primarily holds the responsibility for the proper regulation regarding e-waste. And the SPCB (State Pollution Control Board) and the CPCB (Central Pollution Control Board) are responsible for implementing the procedures which ensures the proper regulation of the rules given by MoEFCC.

2.3.1 E-waste Management Rules 2016- This law of 2011 came into effect in May 2012. According to this law, all the importers and manufacturers of electrical appliances were required to come up with an idea to manage the e-waste. This law also mandated that the sellers of the goods must provide the information to the consumers on how to dispose those electronic products properly [11].

There were specific responsibilities given to the producers, consumers, dismantlers and recyclers.

2.3.1.1 Responsibilities of the producers-

- It is the responsibility of the producers to collect all the electronic equipment produced during its manufacturing and channel it towards the recycling process.
- Collection centers should be setup either individually or collectively.
- Contact details like address, telephone number of the authorized collection centers should be provided to the consumers [11].
- They should try to create awareness through the posters, advertisements, publications or by any other means of communication [11].

2.3.1.2Responsibilities of the consumers

- It is the responsibility of the consumers to properly channelize the e-waste to their collection centers and also, they can contact the registered recyclers or dismantlers.

- They should maintain a record of e-waste generated by them in Form 2 [11].

2.3.1.3 Responsibilities of the dismantlers-

- Every dismantler should have authorization and registration from the SPCB.
- They should ensure that no harm should be done to the natural environment during its transportation and storage.
- They should also ensure that there should be no adverse effects on the health and environment during the dismantling process.
- Dismantling process should be done in accordance with the guidelines provided by CPCB (Central Pollution Control Board) [11].

2.3.1.4 Responsibilities of the recyclers-

- Every recycler should have authorization and registration from the SPCB.
- Recycling process should be done according to the guidelines provided by CPCB (Central Pollution Control Board) [11].

2.3.2 E-waste management and Handling rules, 2011

Under this rule, CPCB has been given the responsibility to draw up various guidelines for Extended Producer Responsibility (EPR). The rules under this law are applied to every consumer, producer and to all those consumers who are in to the practice of manufacturing, selling and purchasing of the electrical equipments.

Amendments in e-waste management rules 2016-Various amendments are done in this rule whose only objective is to channelizing the e-waste to the authorized dismantlers and recyclers.
The amendments in this rule were properly imposed on March 22, 2018.

2.4 Status of e-waste in India

In India, electronic market is growing rapidly. Due to increasing demand and innovation in electronic sector a wide range of electronic equipments are available in market including a range of mobile phones, computers, laptops, printers, televisions, etc. In Asia, India has taken the second place in producing e-waste. As stated by Ministry of Environment and Forest (MoEF), report of 2012 tells that e-waste production has increased by 8 times in last seven years i.e. 8 lakh tones [8, 13].

In 2005, the Central Pollution Control Board (CPCB) estimated that generation of e-waste in India is 1.47 lakh tones or 0.572 MT per day. Seventy five percent of total electronic waste generated was from top ten states including Tamil Nadu, Maharashtra, Uttar Pradesh, Delhi, Karnataka, Madhya Pradesh, West Bengal, Andhra Pradesh, Punjab and Gujarat. Increased amount of generation of e-waste was due to increased demand of electronics such as computers, televisions and telephones since last 5-10 years as shown in Fig (1) [8,14].

As stated in a survey by Singh and Kumar, 24% of total e-waste generated in India comes from Mumbai. Mumbai generates the maximum e-waste followed by other cities such as Chennai and Kolkata.

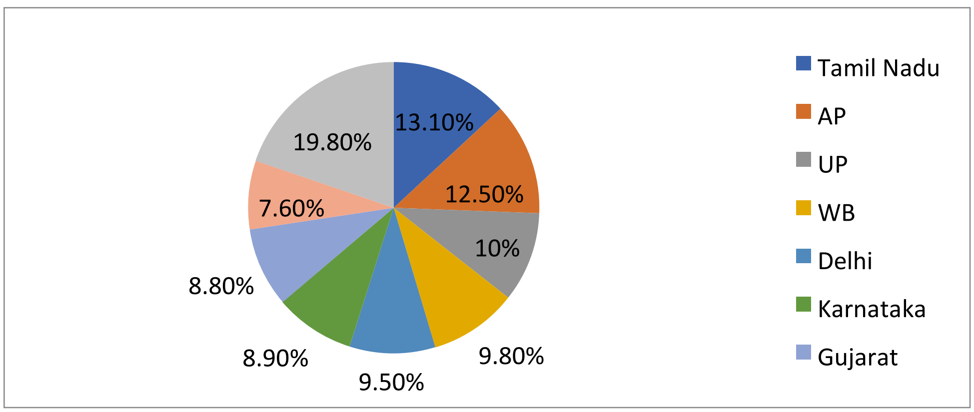

Fig. 1- generation of e-waste in India

2.5Status of e-waste globally

E-waste generation is increasing by 3 times more than municipal waste generation annually (Lepawsky and McNabb 2010). According to stats by United Nations, 90% of the ewaste is dumped illegally every year. This is done by many countries of Asia including India, China, Pakistan and Vietnam and in Africa [15].

United Nations University reported that in year 2014, 41.8 million tons of e-wastes were generated globally. As reported by United States Environmental Protection Agency (USEPA), in

1997, 3.2 million tons of e-waste was deposited in USA landfills and in year 2000 it had risen to 4.6 million tons. So, this shows the increasing rate of e-waste is the fastest growing waste in USA. After USA, China is the second largest e-waste producer with a rate of 2.3 million tons. The main reason of this large e-waste generation is due to high demand of computer and electronic appliances in households and offices. Also, due to illegal waste transport, the overall e-waste percentage of China is high. 80% of e-waste from USA is imported to Asia out of which 90% is imported to China. African countries such as Ghana and Nigeria are the biggest e-waste continent though use of electronics in these countries is not much but due to large imports from other countries, overall e-waste content in country is high. In 2003, Switzerland was stated as biggest e-waste generating country with 68,000 tones of collection [15].

Fig. 2 shows global generation of e-waste in China, South Africa, Brazil, France, UK, Japan, Australia, Germany and USA.

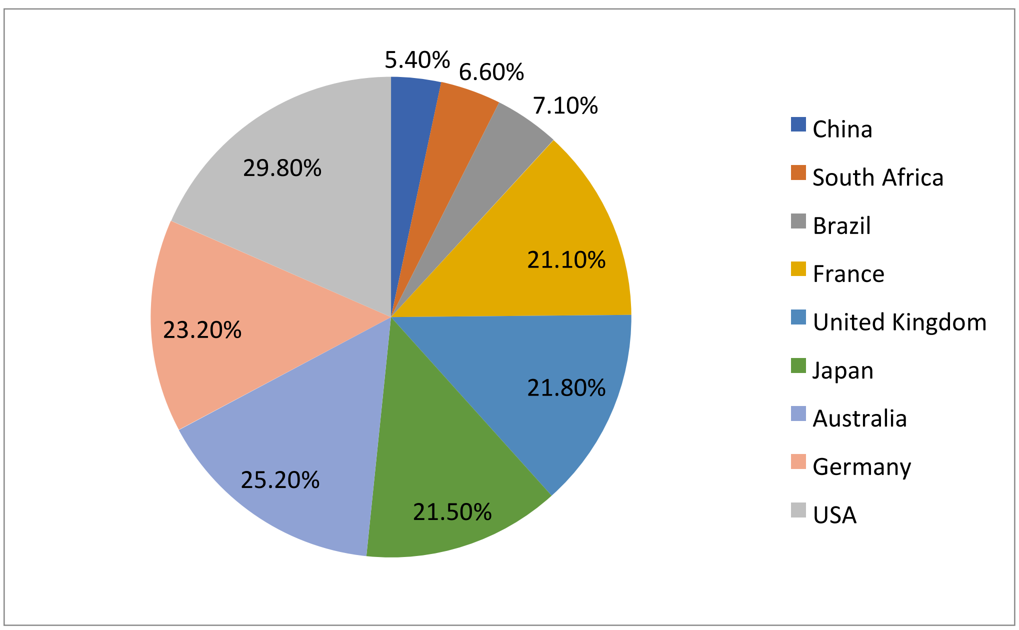

Fig.2-Generation of e-waste on a global level.

2.6 Metal composition in e-waste- E-waste generally contains all the three major categories of metals- [1]

- Precious metals- Ag, Au, Pt.

- Base metals - Cu, Al, Ni, Si, Zn, Fe.
- Toxic metals- Hg, Be, Cd, Cr, As.

Halogens and combustible substances are also present in them along with these metals [1].

Table (1) - Metal constituents and their sources in E-waste [17]

CONSTITUENTS	**SOURCES**
Lead	Solder in PCBs, glass panels and gaskets in computer monitors.
Cadmium	Chip resistors and semi- conductors.
Mercury	Relays and switches, PCBs.
Hexavalent Chromium	Corrosion protection of untreated galvanized steel plates, decorator or hardener for steel housing.
Plastics including PVCs	Cablings and computer housing.
Brominated flame retardants	Plastic housing of electronic equipment and circuit boards.
Barium, Phosphorus and heavy metals	Front Panel of CRTs.
Beryllium	Motherboard.

E-waste composition and their recycling potential for each appliances is highly specific and to handle their complexity, they are divided into various categories like-

- Iron and Steel.
- Non-ferrous metals like Copper and Aluminum.
- Glass.
- Plastics used in cables and circuit boards.
- Electrical components.
- Others (wood, ceramic etc.) [10]

2.6 Recycling of e-waste-

Recycling of e-waste is basically an alternative of its disposal which reduces the hazardous and toxic substances which may enter the environment by simply disposing it [18].

Recycling in India is of two types i.e. formal recycling and informal recycling. Formal recycling is mainly concerned with the disposing and dismantling of e-waste so that an eco-friendly and healthy environment can be maintained. However, in informal recycling, the e-waste is being sold to the scrap dealers. The main reason behind recycling of e-waste is the recovery of valuable materials present in that waste as well as waste removal. Countries like USA and UK have several strict guidelines to manage the increasing amount of e-waste whereas India still needs to have some strict laws for the management of e-waste. The percentage of recycling done in formal and informal sector in India is shown in the graph given below i.e. Fig-3.

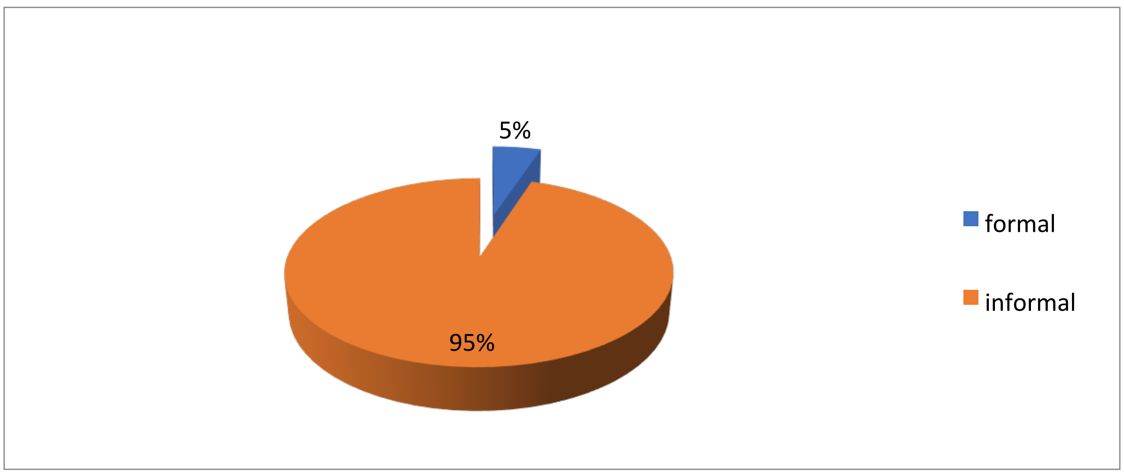

Fig 3- Percentage of recycling practices followed in India.

Countries with formal e-waste recycling- India, Vietnam, China, Pakistan, Malaysia, the Philippines, Singapore, Kenya, Sri Lanka and Thailand.

The informal recycling of e-waste is the largely unknown and well supplied method but mostly unregulated. It is a type of "hidden flow" of e-waste which is really difficult to manage and costly too. And this recycling is carried out with very few or no protective equipments. This is often run by the families and home-based. India and China are among the top most countries where informal e-waste recycling is carried out. There are many other countries also like- Ghana, Nigeria, the Philippines and Vietnam. Around 25000 workers are employed in Delhi at unregulated scrap yards where 10000-20000 tons of e-waste is recycled annually. These two recycling sectors i.e. formal and informal sectors are independent of each other. The informal ewaste recycling not only causes the harm to the human health and environment but also the deficiency of supply in the formal sectors. According to the present scenario, e-waste recycling is a source of livelihood for people in the developing countries.

2.7 Techniques to recycle e-waste

2.7.1 Pyrometallurgy- Pyrometallurgy is a traditional approach for the recovery of metals which includes blast furnace, smelting in plasma arc furnace, incineration and roasting at high temperature. This process has various limitations also like high demand of energy, high economy and release of toxic fumes which makes it less efficient in comparison to the other methods. This method is used in recovering for both precious metals and the non-ferrous metals. This process usually takes place in the presence of high temperature. This process has various steps and it begins with the collection of e-waste, dismantling them, shredding and then the end process to get the desired metals. It is basically a chemical technique which is extensively used for synthetic polymers mixed with glass fibers [23].

2.7.2 Hydrometallurgy- The use of hydrometallurgy method has been increased in the recent years because of its low emission of the toxic gas in comparison to the hydrometallurgical method. It is a common process for the extraction of copper, Lead and Zinc. It is a method in which the contents of metal are dissolved into the leaching solutions like alkali and strong acids and then the electro refining of the desired metals are done. It is considered to be one of the flexible and energy saving method and hence cost effective. Cyanide solutions, nitric acid, sulfuric acid and aqua regia are the widely used leachants. The major drawback of this method is the poisonous and corrosive nature of the liquid which can cause harm to the environment. Recovery of metals through this method began in the late 1960s in the developed countries.

Metals like Gold and Silver are obtained with a very high quality by this technique. The most important advantage of this method is its low risk of air pollution that usually happens by pyrometallurgy.

It has various disadvantages also-

- Solution is needed in the large volumes.
- High standards of safety should be maintained while working with cyanide.
- High level of contamination can be caused which can lead to the serious health risk.

2.7.3 Biohydrometallurgy- It is basically a branch of hydrometallurgy which is used for the recovery of metals by utilizing the activity of microbes. It is considered as one of the promising technique for the recovery of metals and especially for low grade and complex ores. Copper followed by Zinc, Gold, Cobalt, Nickel and Uranium are the most widely used commodity in this technique. Bioleaching. And those techniques include vats, reactors, heap dumps as well as in situ leaching[r]. Bioleaching is basically a solubilization process and occurs in the natural environment wherever growth conditions are suitable. [21]

The main advantage of this method is that this is an environmental friendly technique with a high potential to lower energy requirements and operational cost. The most widely used group of microorganisms for bioleaching is the Chemo-lithoautotrophs. The rate of bioleaching of metals is demonstrated to be high by moderates thermophiles in comparison to the mesophiles and extreme thermophiles. Biological techniques are considered as a more suitable process in comparison to the other technologies for the treatment of heavy metals. Extraction of metals through biosorption and bioaccumulation has gained a significant importance. These are basically an ion-exchange process where the metal ion is exchanged for a counter ion. Bioleaching is also similar to these processes in which metals present in the e-waste are dissolved in the soluble form with the help of the microbes. Generally, these processes occur in the acidic medium. Acidophilic microorganisms play a very major role in the process of bioleaching. The major bacterial groups that are involved are Chemolithotrophic acidophiles *Acidithiobacillus penicillium, Acidithiobacillus ferrooxidans* and *Leptospirrilum Ferrooxidans* [21].

Chromo bacterium Violaceum is a mesophile, gram negative and facultative anaerobe and has the ability to produce CN^- which has the ability to dissolve gold from the metallic particles of printed circuit boards (PCBs). It is considered to be non pathogenic for humans and are found in tropical and subtropical areas. They can live in both aerobic and anaerobic conditions. This bacterium is found to be the most effective for the bio-dissolution of gold due to its cyanide-associated activities. They also have an inherent activity to degrade cyanide and moreover they produce cyanide as a secondary metabolite [21].

2.7.3.1 Advantages of bioleaching over conventional methods-

- Energy requirement and investment cost in conventional methods is much higher than bioleaching.
- Metal loss and toxicity is also high in the conventional techniques whereas bioleaching is a cost effective method with high extraction of metals and less toxicity.
- Metal extraction rates are also higher in bioleaching and also lowers the demand for various resources like ores, energy and landfill space.[22]

2.8 Role of microbes in recycling-

Biotechnology is the most trusted technology to carry out metallurgical processes. The technique used for extraction or recovery of precious and toxic metals from electronic waste is the 'bioleaching'. Organisms involved in the recovery process are mainly autotrophic bacteria, heterotrophic bacteria and fungi. In this whole process, microbial cells need energy for metabolic processes which they obtain through aerobic oxidation of reduced sulphur compounds. The most commonly used strains of fungi, as reported by several researchers, for bioleaching processes are *Aspergillus* sp. and *Penicillium* sp. [6].

Acidophilic bacteria take part in metal dissolution as they grow in inorganic medium with low pH value and tolerate high metal ion concentrations. Main role of these bacteria is to oxidize Fe (II) to Fe (III) and S to H_2SO_4. Reaction kinetics is mainly controlled by these two reactions [5].

Acidophilic bacteria are categorized into three groups, based on their temperature tolerance, i.e. mesophiles, moderate thermophiles and extreme thermophiles.

2.8.1 Mesophiles

Mesophiles are the type of organisms which grow around room temperature i.e. 28-37˚C. *Acidithiobacillus ferrooxidans* is the most popular and widely used species of mesophilic bacteria. The optimum growth conditions observed for most strains of *Acidithiobacillus ferrooxidans* are at pH 1.5-2.5 and temperature range of 28-37˚C. As *Acidithiobacillus* *ferrooxidans* is a lithotroph, it obtains energy for metabolic processes by oxidation of Fe (II) to Fe (III) and sulphur and its different oxyanions to sulphate [5, 11].

Leptospirillium ferrooxidans is another acidophilic microorganism but has a major drawback i.e. it cannot oxidize sulphur to sulphate. Therefore, it is used along with *Acidithiobacillus ferrooxidans* as a mixture [5, 12].

2.8.2 Moderate thermophiles

Moderate thermophiles grow at a temperature of 50˚C. Lot kinds of thermophilic strains are obtained from number of geothermal environment and mining sites [13]. *Sulfobacillus thermosulfidooxidans* is a popular moderate thermophile which has the ability to oxidize sulphur and iron [14]. Moderate thermophiles show higher bioleaching kinetics than mesophiles as the experiments are performed at high temperature. Occurrence of moderate thermophiles is usually observed inside the core of the dump as the temperature there is 10-15˚C [5].

2.8.3Extreme thermophiles

Bacteria which can at a very high temperature of 80˚C are known as extreme thermophiles. The genus *Sulfolobus* comprise of a number of extreme thermophiles such as S. acidocaldarius, S. solfataricus, S. brierley, S. ambioalous [15]. Properties of these organisms are:

I. Anaerobic growth along with reduction of elemental sulphur
II. Aerobic growth along with oxidation of sulphur
III. Oxidizes both Fe(II) to Fe(III) and sulphur to sulphate

As these organisms grow at a very high temperature, they show higher bioleaching kinetics than mesophiles and moderate thermophiles [15] [5].

2.9Factors influencing bioleaching

Bioleaching depends upon the type of microorganism used and the metal composition of the ore to be leached. Maximum yield can be obtained only when the growth conditions for bacteria are optimum. Factors which influence bioleaching are:

2.9.1 Nutrients

Metal extraction from sulphide materials requires chemolithoautotrophic bacteria which needs only inorganic compounds for its growth. Nutrients are obtained from the environment and from the ore to be leached. Iron and sulphur compounds are supplemented with other compounds such as ammonium, phosphate and magnesium salts, for optimum growth [7]

2.9.2 O_2 and CO_2

A good growth and leaching activity require an adequate supply of oxygen. This could be achieved by aeration, stirring or shaking. But in case of dump or heap leaching, sufficient oxygen supply can be problematic. Carbon dioxide acts as a carbon source, but is not really needed [7].

2.9.3 pH

Correct value of pH is important for good growth of leaching bacteria and metal solubilisation. pH range of 2-2.5 is optimum for oxidizing ferrous iron and sulphide. Low Ph, i.e. below 2, causes inhibition in growth of T. ferrooxidans but by addition of acid, it could be made adaptive to even lower pH [7] [16].

2.9.4 Temperature

For oxidation of ferrous iron and sulphide by *T. ferrooxidans*, optimum temperature required is 28-30˚C [17, 18]. Though at low temperature metal extraction decreases, bacterial solubilisation of copper, cobalt, nickel and zinc still occurred at 4˚C. thermophilic bacteria can be used for leaching at higher temperatures i.e. 50-80˚C [7].

2.9.5 Heavy metals

Usually the leaching organism has a higher tolerance to heavy metals like *thiobacilli.* Different strains show variation in sensitivity to toxicity tolerance. But it is possible to make a strain adaptive to higher concentrations of metals or substrates. This could be done by gradually increasing metal concentrations [7].

2.9.6Surfactants and organic extractants

Surfactants and organic extractants are used in solvent extraction. They show inhibitory effect towards the leaching bacteria. This happens because of the surface tension and reduction in oxygen mass transfer. Solvent extraction is a technique used to concentrate and recover metals from pregnant solutions. When bacterial leaching and solvent extraction are carried out together the solvents get enriched in aqueous phase and need to be separated before the left out solution is recirculated [7].

3. Materials and methods

3.1Isolation of bacteria from metals consisting natural environment

Rust samples were selected from the JUIT Campus, Waknaghat. Three different samples were selected from different locations. The rust samples procured from the natural environment comprise of wide range of microorganisms including bacteria which we want to isolate. The samples were added to 100 ml water separately and used to inoculate medium broth prepared to grow bacteria in it. Two kinds of media were selected i.e. nutrient broth and Bushnell haas broth. Media were optimized at a pH value of 9.0. This was done to provide an alkaline environment to bacterial strains and allow the growth of only those bacteria which grow at alkaline pH.

3.2Source and metal composition analysis of e-waste

Powdered computer printed circuit e-waste was procured from storehouse of Exigo Recycling Pvt. Ltd., Panipat, Haryana, India. Particle size of e-waste is greater than 0.15mm. Waste PCBs collected from different states of India were segregated, dismantled, pulverized and recycled by the company [1]. This powdered e-waste was then used to recover precious metals such as silver, platinum and gold. and base metals such as nickel, iron, copper etc. The metal content analysis was done by aqua regia digestion ($HNO3$: HCL= 1:3) [1].

3.3 Isolation of e-waste tolerant bacteria

For isolation of e-waste tolerant bacteria from rust sample, 1 gm of sample and 50 g/l e-waste was added to 100 ml nutrient broth media in 250 ml Erlenmeyer flask. All the flasks were kept for incubation at 30˚C and 150 rpm for 24 hours. Controls were maintained along, with 5 gm ewaste in 100 ml nutrient broth. 1 ml of sample from previous flask was inoculated in next flask with 100 ml nutrient broth and 50 g\l e-wastes and incubated for 24 hours at 30˚C and 150 rpm. This sub culturing was carried out for 4 consecutive days and then sample from last flask was spread on nutrient agar plates. Sub culturing was done in order to obtain separate and countable colonies of bacterial strain. Two types of colonies were obtained which were further streaked on nutrient agar plates separately.

3.4 Morphology characterization of isolates

For identification of bacterial strains, two different strategies were applied. One is the gram staining and other is the KOH string test. Both the tests are to identify if the bacteria are gram negative or gram positive. In KOH string test, bacterial colonies were collected with a loop and added to a sterilized glass slide under aseptic conditions. 3% KOH was added and mixed well with the bacterial colonies. After mixing when loop was pulled up and string was seen to form, that depicted that the bacteria are gram negative. Similarly, when no string was formed, it showed that bacteria are gram positive.

Gram staining was carried out using stain i.e. crystal violet, safranin, gram's iodine and 70% ethanol. After than bacterial strain was visualized under compound microscope. Pink colonies depict that bacteria are gram negative and purple colonies depict bacteria are gram positive.

3.5 Determination of e-waste toxicity tolerance levels

Both of the bacterial isolates were tested for their toxicity tolerance at 200, 250, 300, 350 and 400 g\l pulp density of e-waste. Toxicity tolerance assessment was done by the bacterial growth at respective pulp density in terms of colony forming unit (CFU) counting method [1]. 100 ml of nutrient broth was inoculated with the first isolate in a 250 ml Erlenmeyer flask. Same was done for the second isolate and both flasks were incubated overnight at 30˚C and 150 rpm. After the incubation, samples from each flask were taken following enumeration of bacteria by serial dilution and spread plate method. CFU counting was done on basis of which inhibition response was calculated and the EC_{50} values as compared with the control. EC_{50} value is the value which shows the estimate of the substance concentration which results in the reduction of the growth of bacteria by 50% in a time period. [1].

$$\%IR = \frac{(control - test)}{control} \times 100$$

Where,

IR=Inhibition response.

Control= the flask with no e-waste and the bacterial growth is carried out.

Test= the flask with e-waste.

3.6 Two-step bioleaching

Two step bioleaching was done for the solubilization of metal from e-waste. Loop full of bacterial colonies were inoculated in 100 ml of sterile nutrient broth media and incubated for next 48 hours. Optical density was measured in the spectrophotometer in order to estimate the bacterial growth after 48 hours. Sample was serially diluted and spread on nutrient agar plates. Numbers of colonies were estimated by CFU counting method. Selected dilution was inoculated in 5 flasks of sterile nutrient broth medium and incubated for 4, 8, 12 and 16 days respectively. Control was maintained along. After 4 days, sample was taken from one flask and filtered using Whatman filter paper and then centrifuged at 7000 rpm for 10 mins to remove solid particles and cell biomass. Then, again filtration was done with 0.45 micrometer micro filter paper. Same was done for another flask on 8^{th}, 12^{th} and 16^{th} day respectively. These filtered samples will be used to analyze bioleaching capability of bacteria using atomic absorption spectrophotometer.

3.7 Parameter optimization

3.7.1 Temperature- Optimization of temperature is one of the necessary steps in the process of bioleaching in order to proceed the metal extraction fastly. A great variation in the temperature range is shown for the different bacteria which completely depend on the bacterial category being used in the bioleaching. But in most of the studies it is carried out at 30°C. Bioleaching rate is usually affected by suboptimal temperature. Therefore, in every study it has been well stated that the temperature range should support the metabolism of the bacteria for its effective growth and to achieve the required metal dissolution [24].

In the present study the isolated bacteria were kept for two-step bioleaching at various temperatures ranges (25, 35, 40, 45°C) with the pH (9.0) and pulp density (1gm) constant. After the completion of this experiment the cultures were filtered with **Whattman filter paper** (pore size- 0.45µm) and kept for the observation of results.

3.7.2 pH

pH is also an important parameter in the growth of the microorganisms and also considered to be the decisive parameter in the metal dissolution. Most of the bacterial strains grow at the ph between 7.0-8.0 as well as the production of cyanide also takes place between this range but in this ph range, the gaseous form of cyanide is present i.e. Hydrogen Cyanide (HCN) and may lost through volatilization [24]. But in this study, we could not optimize this parameter due to the sudden National lockdown because of increasing COVID-19 cases.

4. Results and discussions

4.1. Compositional analysis of e-waste-

Analysis of metal composition was done using aqua regia digestion at 100°C (HCl: HNO3, 3:1) and the results are shown in the Table-2. It was observed that concentration of Ag and Cu was in bulk i.e. 10.34 mg/g and 8.38 mg/g respectively. Concentrations of other metals were also determined which were- Au (0.0404 mg/g), Fe (2.46 mg/g) and Ni (0.14 mg/g) as shown in the table (13.1.1). Concentration of silver was observed to be higher in our studies when compared to the previous studies given in Table-3 and Table-4, respectively [1,24].

In one of the previous research work, it was found that Cu was present in the highest concentration using e-waste from PCBs with particle size ≤ 150µm [1]. In other studies, also the concentration of Cu was found to be the highest i.e. 12.06 mg/g and Ag was only 0.06 mg/g [24]. The comparison with the previous studies show that there is a great variation in the metal composition of PCBs. Heterogeneous nature of the e-waste can the main reason behind this which can be attributed to the nature, origin, analytical methods and processing of the waste.

Table-2 - E-waste (PCBs) metal content analysis using aqua regia (HNO_3; HCl, 1:3)

Metals	Concentration (mg/g)
Cu	8.38
Au	0.040
Ag	10.34
Fe	2.46
Ni	0.14

Table-3 – Analysis of metal composition using aqua regia digestion (HNO_3: HCl, 1:3), [1].

Metals	Composition (mg/g)
Cu	23.4
Au	0.4
Ag	0.08
Fe	22.2
Ni	2.0

Table-4 –Analysis of metal composition using aqua regia digestion (HNO_3: HCl, 1:3), [24].

Metals	Composition (mg/g)
Cu	12.06
Au	0.08

Ag	0.06
Fe	2.08
Ni	0.03

4.2 Morphology characterization of the isolates

After the multiple sub-culturing of all the samples, two different strains were characterized on the basis of their morphological characteristics. There was no growth in the control flask which contained only sterilized e-waste. Gram staining was performed for the characterization of the isolates. Morphology characterization of the isolates is shown in the Table-5. KOH string test was also done and it was used as the confirmation test of the Gram's nature of isolates.

Table-5 - Morphology of the isolates.

Characteristics	**Isolate 1**	**Isolate 2**
Shape	Rod	Rod
Color	White	Yellow
Strain	Gram negative	Gram positive

4.3 Toxicity tolerance assessment of the isolates

The toxicity for both the isolates was measured in terms of CFU/ml for a range of pulp densities (200g/l, 250g/l, 300g/l, 350g/l, and 400g/l) including control as shown in the Table (13.3.1). The percent inhibition response (%IR) was calculated for each pulp density is shown in the Table. It was observed that the isolate 1 inhibited its growth by more than 50% at the pulp density of 250gm/l. Isolate 2 was still able to grow at 250gm/l pulp density but its growth was completely inhibited at 300g/l. Therefore, 250g/l pulp density was considered to be the EC_{50} for both the isolates. The data is shown in the Table-6 given below.

Table-6 - EC_{50} of the isolates at different pulp densities.

Concentration of e-waste (g/l)	Isolate 1 (%IR)	Isolate 2 (%IR)
200	14.94	22.34
250	58.96	33.33
300	99.97	99.5
350	99.99	99.99
400	99.99	100

4.4 Parameter optimization-

In order to identify the influence of temperature on cyanide production using the isolated strains, temperature optimization was carried out. The goal was to obtain the suitable temperature for maximum metal solubilization. But after the filtrations with whatman filter paper, further samples could not be analyzed in the atomic absorption spectrophotometer due to the sudden national lockdown because of COVID-19 pandemic.

5. Conclusions-

Results of the present study indicate that there is a huge variation in metal composition of the waste from PCBs when compared with the previous studies [1,24]. It may be due to the heterogeneity of the e-waste.

For our research, we procured the bacterial samples from the natural environment. We could have taken the previously available isolates but our main objective was to discover an unknown bacterial strain which was not known to be active in bioleaching processes.

Also, as we know from the previous studies, fungi help in bioleaching [6]. It secretes organic acids which help in the process of bioleaching. However, as bacteria can recover metals available in very low concentration, fungi cannot do that. Therefore, we preferred bacteria over fungi to carry out bioleaching.

We obtained the EC_{50} value of 250 g\l which is less as compared to the previous studies (Kumar A.). However, we cannot conclude anything from this as the bacterial strains may not be the same as we isolated and other factors such as pH were also variable.

Further no more conclusions can be made based on our research as the work stopped due to the national lockdown condition because of the COVID-19 pandemic.

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