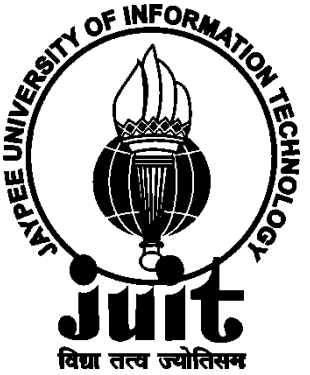
BIOLEACHING OF METALS (Cu, Au, and Ag) FROM WASTE COMPUTER PRINTED CIRCUIT BOARDS USING CYANOGENIC MICROORGANISMS

Thesis submitted in fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

ANIL KUMAR



Department of Biotechnology & Bioinformatics
JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT, DISTRICT SOLAN, H.P., INDIA

September 2018

Ph.D. ANIL KUMAR JUIT, WAKNAGHAT 2018

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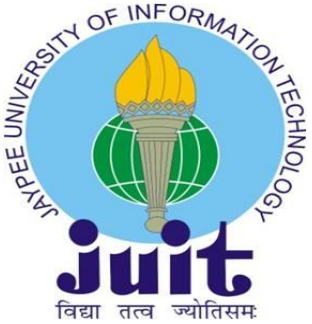

Department of Biotechnology & Bioinformatics

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September 2018

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DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Bioleaching of metals (Cu, Au, and Ag) from waste computer circuit boards using cyanogenic microorganisms"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Sudhir Kumar.** I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. Thesis.

Anil Kumar **Date:**

Enrollment No. 136566

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat, Solan, H.P., India - 173234

CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Bioleaching of metals (Cu, Au, and Ag) from waste computer circuit boards using cyanogenic microorganisms"** submitted by **Mr. Anil Kumar** at **Jaypee University of Information Technology, Waknaghat**, **India,** is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

Supervisor:

Dr. Sudhir Kumar **Date:**

Professor and Head

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat, Solan, H.P., India - 173234

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Needless to say, errors and omissions are solely mine.

Anil Kumar

ABSTRACT

The present work has addressed an important global issue the electronic waste (e-waste). The bioleaching process was applied to treat waste computer printed circuited boards (CPCBs) for recovery of Cu, Au, and Ag using indigenous bacterial strains isolated from an abandoned gold mine and e-waste recycling facility. The tolerance levels of bacterial strains towards e-waste toxicity was examined using pulverized (particle size ≤150 μm) waste CPCBs per liter (L) of the culture medium. The toxicity assessment and dose-response analysis of waste CPCBs showed EC_{50} values of 325.7, 128.9, 98.7, and 90.8 g/L for *Pseudomonas balearica*, *Bacillus* sp. SAG3, *Bacillus megaterium* SAG1 and *Lysinibacillus sphaericus* SAG2, respectively, whereas, for *Chromobacterium violaceum* EC_{50} was 83.70 g/L. To maximize precious metals dissolution; optimization was conducted using both one-factor-at-a-time (OFAT) and central composite design of response surface methodology (CCD-RSM). The process parameters such as initial pH, e-waste pulp density, temperature, and precursor molecule (glycine) were optimized to enhance metal mobilization. The maximum metals recovery under OFAT optimized conditions occurred at 10 g/L pulp density, 9.0 pH, 5 g/L glycine concentration, and 30°C temperature for 7 days by *C. violaceum*; was 87.5% and 73.6% of Cu and Au, respectively. Whereas, Ag (33.8%) mobilization was maximum by *P. balearica*. The kinetic modeling results showed that bioleaching using cyanogenic microorganisms followed the first-order reaction kinetics, where the rate of metal solubilization from CPCBs depends upon microbial lixiviant production. The CCD-RSM optimization extracted 81.7, 73.9 and 41.6% of Cu, Au, and Ag by *P. balearica* at pulp density 5 g/L, glycine concentration 6.8 g/L, initial pH 8.6, and temperature 31.2°C, respectively. The CCD-RSM proposed three polynomial quadratic models which can be used as an effective tool to predict bioleaching of Cu, Au, and Ag from e-waste using cyanogenic microorganisms. The bioleaching of unprocessed/virgin waste CPCBs-P5 did not show significant metals mobilization. A combination of chemical and biological methods showed significant metals mobilization from the unprocessed waste CPCBs-P5. The residual CPCBs after bioleaching was sent back to the authorized recyclers i.e. Exigo Recycling Pvt. Ltd. for final disposal through TSDF (Treatment Storage and Disposal Facility). The novel findings of the study include: - i) indigenous bacterial strains *P. balearica* and *L. sphaericus* were first time employed for

metal bioleaching from e-waste, ii) e-waste toxicity assessment and dose-response analysis conducted on bacterial strains have not been reported elsewhere, iii) higher silver leaching by *P. balearica* and iv) abandoned goldmine Solan, Himachal Pradesh was first time explored for bacterial diversity. The present study has its importance in industrial e-waste recycling and safe disposal.

LIST OF ABBREVIATIONS

E-waste	Electronic waste
WEEE	Waste Electrical and Electronic Equipment
EEE	Electrical and Electronic Equipment
E-scrap	Electronic scrap
PCBs	Printed Circuit Boards
CPCBs	Computer Printed Circuit Boards
PBDEs	Poly Brominated Diphenyl Ethers
PCBEs	Poly Chlorinated Biphenyl Ethers
TSDF	Treatment Storage Disposal Facility
HCN	Hydrogen Cyanide
CN^-	Cyanide
OFAT	One Factor At a Time
RSM	Response Surface Methodology
CCD	Central Composite Design
HCl	Hydrochloric acid
HNO_3	Nitric acid
H_2SO_4	Sulphuric acid
REEs	Rare Earth Elements
Cu	Copper
Fe	Iron
Au	Gold

Ag	Silver
Zn	Zinc
Pb	Lead
Cr	Chromium
Cd	Cadmium
Ni	Nickle
Pd	Palladium
MTCC	Microbial Type Culture Collection
LB	Luria Broth
NA	Nutrient Agar
NaCl	Sodium Chloride
CFU	Colony Forming Unit
O.D.	Optical density
sp.	Species
psi	Per square inch
rpm	Rotation per minute
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
NCBI	National Centre for Biotechnology Information
ETBR	Ethidium Bromide
EC	Effective Concentration

EC_{50}	50% Effective Concentration
L	Liter
M	Molar
mL	Milliliter
mg	Milligram
mM	Millimolar
g	Gram
Kg	Kilogram
µ	Micro
ng	Nanograms
µg	Microgram
^{o}C	Degree centigrade
h	Hour
min	Minute
Mt	Million Metric Tons
%	Percent
ppm	Part Per Million
nm	Nanometer
Eq	Equation
UV	Ultra Violet
AAS	Atomic Absorption Spectrophotometer
SEM	Scanning Electron Microscopy

R^2	Coefficient of determination
p	Probability
w/v	Weight by volume
v/v	Volume by volume

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INTRODUCTION

E-waste (e-waste) consists of all the electrical and electronic equipment's (EEEs) that have been discarded by the end users and are intended to no more use. The innovation and progression in technology have turned the industries towards automation that augments the usage of electronic goods. Electronic goods are manufactured, developed, applied, sold and used at an exponential rate, globally. At the same time, high obsolescence rate, short life span, design for dump policy of manufacturers, consistent changes in technological applications and falling prices of electronic goods have forced the consumers to purchase of newer/current electronic devices by discarding the older ones [1, 2].

Worldwide, e-waste has become one of the rapidly growing solid waste streams [3]. In 2016, the world has produced 44.7 million metric tons (Mt) of e-waste per annum and is projected to increase up to 52.2 Mt by 2021. Surprisingly, only 20% of e-waste is documented to be collected, processed and properly recycled. The remaining 80% is not documented and is likely to be thrown into the residual waste/traded/recycled/dumped under substandard conditions [4]. E-waste contains metals and other hazardous components such as persistent organic pollutants (POPs), and those have significant negative impacts if treated/processed inadequately [5, 6]. On the positive side, e-waste contains the significant amount of precious metals like Cu, Au, Ag, Pd, and Pt, that on recycling will lower the burden of primary resources, save landfill space and can provide job opportunities.

In the e-waste, printed circuit boards (PCBs) bear the majority of metals (approximately 30% *w/w*) and comprises 3-5% weight of total e-waste stream. The purity of metals in the PCBs is 10 times higher than those of primary resources/natural ores [7, 8]. Due to rich metals content, e-waste is treated as "Urban mine or Secondary ore" under the concept of urban mining [9]. The presence of high amount of valuable metals makes it's recycling a business opportunity. The WEEE directives (2002/96/EC) and specific rules of different countries/states/regions set guidelines for mandatory e-waste recycling [10]. In the present research, we have selected printed circuit boards of waste computers for recycling and metal recovery.

E-waste recycling is carried globally in formal and informal sectors. But unfortunately, only 10% is processed by formal sector i.e. environment and workers friendly way to reduce the exposure of harmful metals/toxic compounds [11, 12]. The rest of world's e-waste is either recycled by informal sector or ends up in landfills [13]. The current practices of metals recovery from e-waste include physico-mechanical, hydrometallurgical and pyrometallurgical methods [14, 15]. However, these methods are energy intensive, costly, unsafe (to the environment as well as humans) and generate considerable quantities of secondary pollutants: for example, pyrometallurgical treatment generates a large amount of slag, heavy metals fumes, and mixed halogenated dioxins and furans, especially from the plastic part, contained brominated flame retardants (BFRs) [7, 16]. Whereas, hydrometallurgical methods generates spent acid, toxic gases, a large amount of sludge and liquid waste [17, 18].

In contrast, biological processes have shown promise compared to the traditional processes (physical/mechanical/thermal/chemical) regularly used in metallurgy [19]. Among the biological processes, bioleaching is the most widely employed method for metals extraction from e-waste, which exploits the natural abilities of microorganisms to convert the solid metallic portions into its aqueous form [20]. The process offers several advantages such as environmentally friendly, low energy consumption, minimum volume of chemical sludge generation, easy metals recovery as well as cost-effective compared to traditional metals recovery processes [21]. Additionally, during bioleaching the necessary lixiviant required to solubilize metals are biologically produced, thus eliminates the external supply of it to the recovery units [22]. We applied two-step bioleaching process to recover metals i.e. Cu, Au, and Ag from waste CPCBs in this study.

The microorganisms mostly exploited for bioleaching belong to three main groups: chemolithoautotrophic bacteria i.e. *Acidithiobacillus thiooxidans, Acidithiobacillus ferrooxidans*, and *Leptospirillum ferrooxidans*; heterotrophic bacteria i.e. *C. violaceum*, *Bacillus*, and *Pseudomonas* sp.; and heterotrophic fungi i.e. *Aspergillus niger* and *Penicillium* sp. [23-26]. The chemolithoautotrophic bacteria leach metals through a series of biooxidation reactions. In the reaction, ferric ions (Fe^{3+}) as oxidizing agents react with the sample and dissolve metals like Cu, Zn and Ni, thereby reduced to ferrous ions (Fe^{2+}). Further, Fe^{2+} are

taken by bacteria as an energy source and oxidate it to regenerate Fe^{3+}. Thus, forms a cycle between Fe^{2+} and Fe^{3+} to dissolve metals like Cu, Zn, and, Ni [27]. The fungal strains mobilize metals from e-waste through the production of organic acids (citrate, gluconate, oxalate) [24]. However, Au and Ag's bioleaching has been majorly conducted via heterotrophic cyanogenic bacteria such as *C. violaceum*, *B. megaterium* and *Pseudomonas* sp. [28-31]. The present study also used *C. violaceum* and other heterotrophic cyanogenic bacteria like species of *Bacillus* and *Pseudomonas* isolated from different sites to recover metals from waste CPCBs.

It was reported that cyanogenic bacteria produce hydrogen cyanide (HCN) as a secondary metabolite and form water-soluble metals-cyanide complexes when reacts with solid metallic fractions such as e-waste [28, 32, 33]. The dissolution of Au by biological cyanide is shown in Eq. (1.1):

$$4Au + 8CN^- + O_2 + 2H_2O = 4Au(CN)_2^- + 4OH^- \qquad (1.1)$$

The peak cyanide production by *C. violaceum* takes place at the early stationary phase of their growth [30]. One of the important features of the cyanogenic bacteria is their inherent capacity to degrade cyanide. Previous studies revealed that *C. violaceum* and *B. megaterium* convert cyanide into β-cyanoalanine by synthesizing an enzyme β-cyanoalanine synthase during the end of stationary phase and death phase [34, 35]. *P. fluorescens* converts cyanide to ammonia by utilizing hydrogen cyanide as nitrogen source [36]. Biogenic cyanide exists in an equilibrium reaction ensuing a reversible reaction as given in Eq. (1.2):

$$HCN \leftrightarrow H^+ + CN^- \qquad (1.2)$$

At physiological pH (7), cyanide is mainly present as HCN which is a gas and may be lost by volatilization. However, conducting metals recovery experiments under alkaline conditions (pH of ≥9) enhances the availability of cyanide ions (CN^-) because of the pKa (9.3) of HCN, hence increases the bioleaching efficiency [8].

Since e-waste is toxic to microorganisms and bioleaching is dependent on the metabolic status of cells [8, 21, 29], e-waste toxicity assessment should be conducted to ensure the viability of bioleaching process. In this context, microorganisms indigenous to contaminated

sites are the preferred choice over foreign microorganisms. The indigenous microorganisms have the metabolic and physiological machinery to resist/degrade the contaminant/pollutants due to the natural selection for the "survival of fittest". The toxicity assessment studies produce quantitative dose-response curves that provide estimates about EC_{100} (maximum treatment concentration), EC_{50} (50% effective concentration), and EC_0 the threshold concentration (maximal 'no-response' concentration) permissible for bioleaching of e-waste [21, 37]. The principle of toxicity assessment indicated that for every chemical/pollutant/contaminants toxic concentration and leads to microbial population extinction and metabolic failure i.e. the EC_{100}. Therefore, microorganisms indigenous to mines especially gold mine and e-waste recycling facility were isolated and assessed for their e-waste toxicity tolerance and bioleaching abilities.

Bioleaching like other biological processes is impacted by abiotic/biotic parameters which affect the microbial metabolic activity and subsequently metals recovery [10]. The abiotic parameters include temperature, pH, aeration rate, medium composition, a precursor for lixiviant, incubation time, particle size, and the concentration of e-waste, whereas, biotic factors include inoculum size, characteristics, and the type of microorganism used [25, 38]. The little change in these parameters may create a huge variance in the final yield of the process. Therefore process optimization is a vital step in this field [39]. According to literature, bioleaching process can be optimized using conventional OFAT and statistical approach the RSM [21, 40]. For example, Natarajan and Ting [8] studied the effect of pH and pulp density on gold dissolution by *C. violaceum* from e-waste. Shin et al. [41] evaluated the influence of glycine concentration and pH on bioleaching of Au using *C. violaceum*. They found the optimal conditions (pH-9 and glycine concentration 5 g/L) for maximum recovery of Au. Jujun et al. [42] investigated the influence of pH, temperature, aeration and rotation speed on cyanide producing ability of *Pseudomonas chlororaphis*. Arshadi et al. [40] optimized e-waste pulp density, pH, and glycine concentration for simultaneous recovery Cu and Au from mobile phone printed circuit boards (MPPCBs). They optimized the process using response surface methodology via a cyanogenic bacteria *B. megaterium*. In accordance with the available literature, we have chosen different factors like pH, pulp density, temperature, and precursor molecule (glycine) for optimization in the present study. Both OFAT and RSM based approaches were used to optimize bioleaching process using isolated bacterial strains.

Bioleaching has been widely accepted as a green technology for metals recovery [43]. Bioleaching has application in the extraction of metals as well as precious metals from battery wastes, metals contaminated sediments, mine wastes, electronic waste and the metallic waste of industry. Currently, bioleaching accounts for 25% worlds mined Cu and are in use in approximately 20 mines around the globe [44]. The current scenario of bioleaching is promising, however, research in this area is majorly conducted using autotrophic microorganisms with deviation in the process parameters. Thus there is a pressing need for intensive research on bioleaching focusing on the exploration of new microbial strains and consequently optimization of the process parameters to meet profitable metals recovery [10]. Keeping all these factors in view, the present work was carried out with an aim to explore new microbial communities capable of bioleaching of Cu, Au, and Ag from waste CPCBs, their toxicity assessment, dose-response analysis and optimization of process parameters to enhance the metals extraction. This study is first to 1) report on the isolation of bacterial strains from abandoned gold mine (Solan) and e-waste recycling facility (Haryana) with an aim to exploit them for bioleaching of metals from waste CPCBs and 2) dose-response assessment of the CPCBs toxicity on bacterial strains.

Rationale of the study

- E-waste comprises of valuable as well as precious metals such as Cu, Au, Ag, Pd, Al, and Fe, respectively, and the concentration is significantly higher than natural mines. According to United Nations University estimates, the resource perspectives of the secondary raw material of e-waste is worth 55 billion Euro (€) of raw material. Other than this, electronic industry has consumed 320 ton of gold and 7500 ton of silver every year. The recycling of e-waste under the concept of urban mining could generate $21 billion each year [2, 4]. This makes e-waste recycling worth economic as well as a business opportunity. Recycling of e-waste to recover metals will lower the burden on the natural mines/primary resources.
- Bioleaching is a cost-effective, energy efficient, eco-friendly process which can recover metals even from depleted or low-grade ores and complex sources like e-waste

compared to traditional processes. Bioleaching process also offers advantages of extracting metals that are not accessible by traditional processes [45].

Objectives: The present work was laid down with following objectives:

- To determine the heterogeneity in the metals content of waste computer printed circuit boards (CPCBs).
- Isolation, screening, and characterization of potent bacterial strains capable of bioleaching of metals (Cu, Au, and Ag) from waste CPCBs.
- E-waste toxicity assessment and dose-response analysis of potent bacterial strains.
- Optimization of the process for maximum recovery of metals (Cu, Au, and Ag) from waste CPCBs.

REVIEW OF LITERATURE

Today competition among electronic companies, innovation in technology, reduced lifespan, and dependency on e-gadgets has created a stream of waste known as e-waste. E-waste refers to all types of electrical and electronic equipment's (EEEs) which are at their end of life state and has been discarded by end users. The volume of e-waste is growing with a very high speed i.e. 3-5% every year globally. The rising volume of e-waste is a major threat to the whole world as it contains harmful metals and non-metals both of which have significant environmental and health impacts if processed improperly. On the positive side, e-waste is a source of valuable metals, for example, the metallic content of gold in e-waste is 17 times higher as compared to the natural ores and generally, it is treated as "artificial ore" under the concept of "urban mining". Other than this, the formation of country-specific stringent legislation and extended producer responsibility (EPR), safe e-waste recycling is mandatory. E-waste recycling provides opportunities for resource recovery, employability vis a vis waste management. Presently 18% of world's e-waste is recycled either formally or informally and rest goes to landfills, posing a threat to the environment and human's health. The informal sector is recycling e-waste by unscientific and unethical manners. Another sector i.e. formal sector is known for processing e-waste in a more ethical and scientific manner protecting the users from the hazards of e-waste. However, both formal and informal sector uses conventional methods of metals recovery which includes physical separation, manual dismantling, open burning, hydrometallurgical and pyrometallurgical methods. The methods are recommended eco-unfriendly, costly, and energy intensive. In contrast, bioleaching is recommended as green technology, which utilizes the natural ability of the microorganism to transform metals into their soluble forms from solid metallic fractions. The process is cost economic and can recycle the lowest concentration of metals which are not accessible by conventional techniques. Thus, the aim of the chapter is to understand the problem and to find out the research gaps so as to create a base for further study.

2.1 E-waste definition

Electronic-waste (e-waste) covers any kind of discarded EEEs. E-waste is also named as WEEE (waste electrical and electronic equipment's) or e-scraps in different parts of the

world. Solving the e-waste problem (SteP) is an international initiative provides the solution for e-waste issues across the globe [2]. Step Initiative (2014), defines e-waste as: *"E-waste is a term used to cover items of all types of electrical and electronic equipment (EEE) and its part that has been discarded by the owner as waste without the intention of re-use"*.

2.2 Categories of e-waste

According to United Nation University (UNU) reports, e-waste has been classified into six different categories [4]. The equipment's covered and quantity produced for each category are given in Table 2.1. Each category differs in its material composition, function, size, and weight, respectively. Previously e-waste was categorized into 10 different types as per European Union Directive, 2002/96/European Commission (EC), which also include toys, leisure, medical devices, sports equipment, and automatic dispensers. However, in the European Union Directives, 2012/19/ European Union (EU), these instruments (toys, leisure, medical devices, sports equipment, and automatic dispensers) are merged in the six categories [2, 46]. Globally, among the six categories of e-waste, the amount of small equipment's discarded was highest (16.8 Mt) whereas, the lowest amount of e-waste belongs to the category of lamps in all the continents (0.7 Mt) (Table 2.1).

2.3 Global e-waste statistics

E-waste is mounting at a very fast rate and its increasing volumes are the cause of concern. The amount of e-waste produced is increasing year by year for example, from 33.8 Mt in the year 2010 to 44.7 Mt in the year 2016 and is forecasted to reach approximately 52.2 Mt by the year 2021 (Fig. 2.1). The e-waste growth rate was estimated at 3-5% per year, which is three times faster as compared to other waste streams [47, 48]. The global e-waste management market was projected to grow at Compound Annual Growth Rate (CAGR) of 23.5% during 2014-2020. In India it is estimated at a CAGR of 26% during 2015-2019, while in China it grows at a CAGR of 19.41 percent over the period 2013-2018 [49]. Table 2.2 shows the list of the top countries majorly contributing to the e-waste generation. The e-waste growth rate in terms of kg/inhabitant is high for developed countries such as Great Britain (24.9 kg/inhabitant), Germany (22.8 kg/inhabitant), France (21.3 kg/inhabitant) and USA (19.4 kg/inhabitant).

Table 2.1: E-waste categories and the amount produced in 2016

Sr. No.	Waste category	Equipment's	Amount generated (Mt)
1	Temperature exchange equipment	Heating pumps, freezers, refrigerators, dehumidifying equipment, and air conditioners	7.6
2	Screens	Notebooks, LCD frames, Televisions, laptops, tablets, and monitors	6.6
3	Lamps	LED, fluorescent, Straight fluorescent, compact fluorescent, and high-intensity discharge lamps	0.7
4	Large apparatus	Electric stoves, washing & dishwashing machine, clothes dryers, printing machines, appliances for knitting & weaving, sound & image producing equipment, musical instrument, copying machine, coin slot equipment, mainframes computer, automatic money and product delivery appliances, large medical devices, heavy monitoring and control instruments, and photovoltaic panels.	9.1
5	Small apparatus	Microwaves, ventilation apparatus, toasters, vacuum cleaners, electric kettles, video cameras, calculators, electric shavers, radio sets, small electrical and electronic tools, small monitoring & control instruments, electrical & electronic toys, smoke detectors, heating	16.8

		regulators, thermostats, and small medical devices.	
6	Small IT & telecommunication equipments	GPS, pocket calculators, routers, personal computers, printers, telephones, and mobile phones.	3.9
Total (Mt)			44.7

Data retrieved from Balde et al. [4].

The high e-waste growth rate (Kg/inhabitant) of the countries corresponds to their high gross domestic product (GDP). Worldwide, China (7.2 Mt) is the top producer of e-waste followed by USA (6.3 Mt), Japan (2.1 Mt), India (2.0 Mt) and Germany (1.9 Mt) [4]. Exceptionally, few countries like India are ranked among the top 10 e-waste producers in 2016 despite its low GDP. According to Kumar et al. [2], the high e-waste generation is due to the large population of India, however low GDP is responsible for low per inhabitant e-waste generation. Further, they stated that it is not necessary that countries with high population will generate more e-waste when the GDP and purchasing power is lower. It is expected that with the increase in purchasing power of the inhabitant of the developing countries, the e-waste generation of these countries will exceed compared to developed countries [50]. This exactly happened in 2016, when China surpassed USA and India surpassed Germany in e-waste generation, globally.

The key factors for the high growth rate of e-waste are competition in the telecommunication market, technological advancements, short lifespan, and falling prices of both EEE services and devices. For example, the smartphones in the USA are on sale for less than 200 USD whereas, in India and China producers are committing even lower costs. The average lifespan of a smartphone in the countries like USA, China and EU economies is less than 2 years [51]. This is probably because of advancements in technology and to get benefits of latest updates and higher speeds etc. [52]. In addition to smartphones, businesses/traders/companies frequently replace their e-gadgets such as PCs, routers, laptops, and TV sets, etc. In the majority of cases, the older e-gadget is unnecessarily replaced though it is not obsolete/broken, hence the amount of e-waste continues to grow. For example, in a recent transformation, from analogue to digital TV; broadcasting had left a mountain of

Carbon-Ray-Tube TVs which, have important environmental impacts [53, 54]. Therefore, clear policies and efficient recycling technologies are needed.

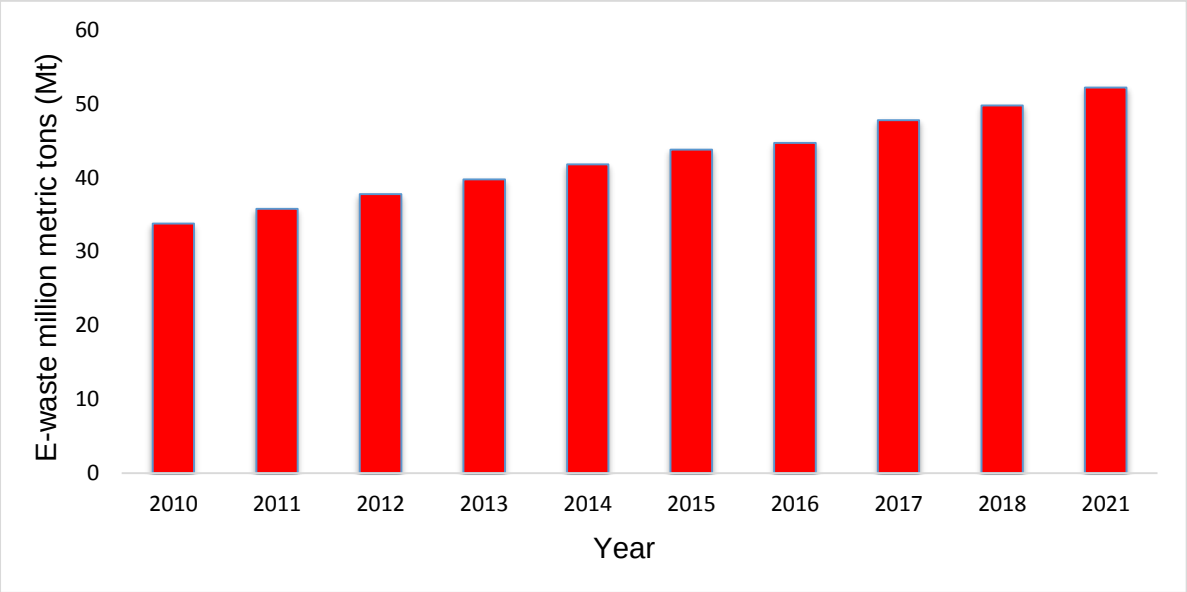


Figure 2.1: Representing year wise increase in global e-waste production (Forecasted data from 2017-2021).

2.4 Composition of E-waste

It is tough to provide a general composition for the e-waste stream because of a multitude of components present in it [13]. However, the majority of the studies have categorized e-waste into ferrous and non-ferrous metals, plastics, glass, and other materials [3, 13, 55]. Iron and steel are the most abundant components of e-waste i.e. approximately 50 wt.% whereas, plastic (10-30 wt.%) is the second most abundant material present in it. The plastics contain several polymers ranging from few to 21 (approx.) including polycarbonate blends, polystyrene, and polypropylene. E-waste contains 55 kinds of metals including base metals (Cu, Zn, Ni, and Fe), toxic metals (Pb, As, Hg, Cr, and Cd), and precious metals (Au, Ag, and Pd) [7, 18, 56]. The amount of Cu and Au in the e-waste is 40 and 17 times higher than the ores [3]. According to literature, almost 0.5-11% of e-waste is made of glass [57, 58]. The glass present in the e-waste is of two types: CRT glass used in the televisions and monitor screens and LCD glass comes from the mobile phones. Both of them, contain the varying

percentage of oxides of silicon, aluminium, sodium, calcium, and lead [47]. Over the time, the metallic fraction has remained dominant while the pollutants and toxic constituents steadily declined [13].

Table 2.2: The top ten countries majorly contributing to the e-waste stream

Sr. No.	Countries	E-waste generated (Kt/year)	E-waste Kg/inhabitant	GDP ($ billions)	Population (millions)
1.	China*	7211	5.2	10,380.3	1,378
2.	USA**	6295	19.4	17,418	323.9
3.	Japan**	2139	16.9	4,616.3	126
4.	India*	1975	1.5	2,049.5	1,309
5.	Germany**	1884	22.8	3,859.5	82.5
6.	United Kingdom**	1632	24.9	2,945.1	65.5
7.	Brazil*	1534	7.4	2,353.0	206
8.	Russia*	1392	9.7	1,857.4	143
9.	France**	1373	21.3	2,846.8	64.5
10.	Indonesia*	1274	4.9	2,147.9	258.8

** Developed Countries; * Developing Countries; (2016 data)

2.5 E-waste regulations

The United Nation's (UN) has designed Basal convention in 1992 to control the transboundary or cross border movement of hazardous wastes. The convention was signed by 186 countries [2]. Several international organizations were launched to manage the problem of e-waste which includes Solving the E-waste Problem (StEP), Partnership for Action on Computing Equipment (PACE), Mobile Phone Partnership Initiative (MPPI), National Electronics Product Stewardship Initiative (NEPSI), and WEEE Forum [50]. In the US, 25 states have e-waste legislation, however, most do not offer adequate infrastructure to enforce compliance and to encourage public participation. The Basel Convention in June 2008 adopted PACE whose objective is to provide new/innovative strategies to tackle evolving issues on

used and end-of-life computing equipment. Although the Basel Convention was enforced by international treaty, national legislation regarding WEEE remains different in the countries or regions of the world.

The majority of countries have adopted and enforced the national e-waste policies and laws to develop a sustainable and functional way of e-waste collection, transportation, and recycling. However, national e-waste regulation is completely absent in certain regions of the world such as in large parts of Africa, the Caribbean, Central Asia, Eastern Asia and Melanesia, Polynesia, and Micronesia [4].

Globally, Europe has well-developed e-waste legislation and the amount of WEEE collected and recycled formally is also highest in the Europe. The European Union (EU) have its directives i.e. WEEE Directive 2002. The directives were developed to improve the collection and efficiency of the recycling chain. The collection targets are defined as a fixed amount per inhabitant (currently 4 kg). However, the directives were recast in 2012. Starting in 2016, the regulations were defined with an aim to collect 45% of the EEE put on the market till 2019. From 2019 onwards, it will be enhanced to 65% of the EEE or alternatively, 85% of the WEEE generated [59]. The directive categorizes WEEE into distinct categories and defined target for recycling and recovery of each category of WEEE generated. [60]. Similarly, in Japan, Home Appliance Recycling Law (HARL) and Small Appliance Recycling Law (SARL) were applied to enhance recycling rates by enforcing responsibilities and costs on producers, retailers, and consumers [50].

The Restriction of Hazardous Substances (RoHS) Directive was created in 2003 which restricts the use of hazardous substances such as lead, mercury, cadmium, hexavalent chromium, PBB, and PBDE in the EEEs.

The most populous countries China and India have adopted the e-waste legislation. In India, E-waste (Management) Rules, 2016 are in place, enforced by Ministry of Environment Forest and Climate Change (MoEF&CC). These rules apply to every producer, consumer, manufacturer, bulk consumer, dealers, e-retailer, collection centers, refurbisher, dismantler and recycler involved in e-waste trade [61]. However, these rules/regulations do not necessarily

infer the effective execution of the sound e-waste management system. The Indian e-waste regulation is solely dependent on EPR, but in China, the "polluter pays" principle is strengthened by 3R's (reduce, reuse and recycle) as well [62].

The majority of policies and legislation have introduced the concept of "Extend Producer Responsibility"; emerged in the early 1990s [50]. EPR is a policy principle that impinges the concerns and responsibility of producers/manufacturer for all stages of their product till the end-of-life management of the product. Although, the policies and regulations are in place in different regions and countries around the world, yet there are difficulties in the successful enforcement of these regulations. Therefore, joint efforts of the state and national regulators, producers, recyclers, and the public are needed to handle the mounting volume of WEEE [48].

2.6 E-waste as "urban mine"

The EEEs contain a large amount of valuable material and plastics and can be technically recoverable. The United Nation University estimated the values of secondary raw material in e-waste as 55 billion € (Table 2.3). The volume and value of e-waste are mainly associated with six metals or metals groups, for example, Fe/steel, Al, Cu, Au, Ag, and platinum group metals (PGMs) [63].

In the e-waste, PCBs are the most important and valuable parts which contain 40% of metals value of e-waste. The majority of precious metals i.e. 80% of Au and PGMs and over 70% of Ag were locked in monitors, screens, and small IT equipment's [63]. It was summarized that 7500 ton of Ag and 320 ton of Au is consumed by electronic sector per year and recycling of which can generate $21 billion/ year [14]. Globally in 2016, 435 Kt of waste mobile phones were generated and recycling of which can generate 9.4 billion € [4]. According to Kaya [7], the quantities of Au, Ag, and Pd in the waste PCBs are higher than present in primary ores. Moreover, the global ore grade is abating and mines are forced to excavate to meet the total metal demand [64]. Kumar et al. [2] compared the concertation of metals in EEEs and average grade in ores. It was found that amount of Cu, Ag, Au, and Pd in PC boards was 20 (wt.%), 1000 (ppm), 250 (ppm), and 110 (ppm) whereas, in the mine/ore it was 0.6

(wt.%), 215 (ppm), 1.01 (ppm), 2.7 (ppm), respectively. The cost of processing and extraction of the same amount of metals from ore is 10-160 times higher than the waste mobile phones. Other than this, e-waste is a better resource to recover metals like gallium and indium; both the metals having an estimated life of 20 years before it completely runs out [50].

Nowadays, e-waste recycling has become a growing business and is providing jobs. According to Heacock et al. [65], informal e-waste recycling has provided jobs to almost 100,000 people in Guiyu, China. Whereas, in India, over 1 million people are involved in informal e-waste recycling [66]. Further, to deal with the total e-waste generated from 2020 to 30, developing nations need to develop new treatment facilities.

Table 2.3: Value of raw material in the WEEE [4]

Material	**Values (billion Euros)**	**Quantity (Kt)**
Au	18.84	0.5
Plastics	15.04	12,230
Cu	9.52	2,164
Al	3.58	2,472
Fe	3.58	16,283
Pd	3.36	0.2
Ag	0.88	1.6

2.7 E-waste recycling and its impacts

2.7.1 Informal recycling

In the developing countries like India, China, and Africa majority of e-waste is taken care by the informal sector. According to United Nation University report, globally, only 20% of e-waste is documented to be processed formally. The fate of 80% is not documented and is likely to be thrown in residual waste, dumped and recycled under substandard conditions. In India and China, only 5% of e-waste is documented to recycle formally, and the rest 95% is processed in informal sector [62]. To recover metals mainly Cu from e-waste, wires and other

plastic cables are burned in an open environment [67]. As a result, hazardous toxic compounds like polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are released into the atmosphere, hence, deteriorating the quality of air [68]. To recover metals like Au, Ag, and Pd; recyclers employ toxic chemicals like cyanide and strong acids like hydrochloric acid, nitric acid, and sulphuric acid. For example, numerous components such as gold plated pins, microchips, and condensers were extracted from waste PCBs through heating or burning and soaking with acids. The informal sector is able to extract only 25% of metals as against possible extraction efficiencies of over 95-99% in the formal sector.

2.7.2 Environmental and health impacts

Informal sector recovers metals Cr, Cd, Pb, Cu, Au, and Ag in an unscientific and unethical manner with minimum safety precautions [62]. The metals recovery under substandard conditions not only contaminate the surrounding resources like soil, water, air, and plants but also poses a risk of numerous health hazards [5]. The number of studies showed heavy metals contamination in soil, plants and drinking water of in and around e-waste recycling areas [6, 69, 70]. They reported the levels of heavy metal contamination beyond the limits set by the US Environmental Protection Agency (EPA) and WHO. For instance, surface soil of e-waste recycling area (Industrial area Mandoli, New Delhi, India) contained As (17.08 mg/kg), Cd (1.29 mg/kg), Cu (115.50 mg/kg), Pb (2,645.31 mg/kg), Se (12.67 mg/kg) and Zn (776.84 mg/kg) higher than those set by the US Environmental Protection Agency [6]. Luo et al. [71] reported higher concetrations of heavy metals in the wild plants (Cu - 94.3 mg/kg, Pb - 54.8 mg/kg, Cd - 2.62 mg/kg and Zn -143 mg/kg) and vegetables (Cd and Pb was 4.7 and 2.6 times higher than the food safety limit in China) from e-waste recycling area of south China. Similarly, groundwater near e-waste recycling was found to be contaminated with heavy metals; posing a threat to human health [72].

Pollutants such as PBDEs, PCBs, and toxic metals cause serious diseases and disorders through inhalation and direct skin exposure via dust, soil, and water [73]. For example, lead (Pb) has an irreversible effect on the developing nervous system, especially in children causing behavioral and learning disruptions [74, 75]. It also causes anemia, high blood pressure, kidney damage, miscarriages and brain damage. Similarly, exposure of Cd leads its accumulation in

the body causes kidney failure and bones damage [76, 77]. Cadmium is considered as a human carcinogen and also damages DNA. Inhalation or ingestion of Cd causes infertility, severe damage to lungs and even death also [75]. Zheng et al. [78] and Xu et al. [79] reported high Pb and Cd levels in children's blood at e-waste recycling area named Guiyu, China. Arsenic present in the e-waste is the source of chronic health effects including cancer of lungs, bladder, kidney, and liver [80]. The Hg and Cr present in relay, switches, batteries and printed wiring boards cause lung cancer, damage to the nose, throat, eyes, asthmatic bronchitis, ulcers, convulsions and skin [79]. Other than toxic metals, impact assessment studies conducted nearby e-waste recycling sites showed higher levels of PCB and PBDEs [81-84]. PCBs and PBDEs present in e-waste cause various neuropsychological disorders in children, including general cognition, memory, executive functions, attention, and motor function [85-88]. The studies reported that e-waste recycling in an unorganized sector under primitive conditions can cause health problems, cancer and neurological development disorders [19, 89-92].

2.7.3 Formal recycling

In the majority of countries, Government along with many stakeholders have taken a step to initiate well-organized e-waste recycling and management system. With the enforcement of strict legislation; many companies have adopted scientific, ethical and eco-friendly methods for e-waste recycling. Given the interest in this sector, more companies are slated to come up with state of art facilities to recycle the burgeoning e-waste. In developing countries (China and India) the large and entrepreneurial informal sector has been acting as the e-waste recycling hub. Realizing these lacunae, Government of every country is taking initiatives to cordon off informal e-waste recycling by applying strict regulatory instruments. Presently in India, central pollution control board (CPCBs) have authorized 178 recycling or dismantling units across different states of the country. The e-waste recycling is carried using automated/semi-automated/manual operations and have a capacity to recycle a total of 438085.62 MTA (Metric Ton per Annum) [93]. In China, only 109 formal e-waste recyclers are registered with a total recycling capacity of 133 million units of e-waste per year by these enterprises [62, 94].

However, the latest studies have shown surprising results of increased toxicity of metals in recycling workers from formal recycling plants of e-waste. Fujimori et al. [95] reported a higher level of Cu in the soil and 10-12 fold increase of life-threatening metals like Ni, Pb, Zn and Cd from formal recycling site of Philippines. Julander et al. [11] investigated heavy metals pollution in the air samples using exposure markers from formal e-waste recycling plants of Sweden. Their results showed eminent internal quantities of Cd, Cr, Hg, In, and Pb in the recycling personnel than the office personnel. Other than metals, brominated flame retardants are also reported in the blood and serum samples of workers from these recycling plants than other occupational group workers [96]. Therefore, it poses a great threat to workers engaged in formal recycling of e-waste and also to the environment but at a lower extent. The common route of exposure may be ingestion and inhalation of contaminated air and dust generated during formal e-waste recycling. To avoid the harmful impacts on workers regular health check-up; sanitized conditions; periodic environmental audits and impact assessment studies will help.

2.8 Existing recycling technologies

2.8.1 Pyrometallurgy

Pyrometallurgy is a conventional technology for recovery of metals from e-waste which requires high energy and has high-cost. The process involves incineration, smelting, drossing, melting, and roasting to recover metals [17]. Before the pyrometallurgical operation, a pretreatment step for e-waste is required which includes dismantling, shredding, and physical processing. This method has the advantage as it can accept any form of e-waste. Hence, metals like Cu, Au, Ag, Pt, and Pd can be recovered from e-waste as raw materials in the smelters. Globally, a limited number of pyrometallurgical processes are available for formal processing of metallic components of e-waste and these include Rönnskår smelter in Sweden, Aurubis in Germany, Noranda in Quebec, Canada, and Umicore in Belgium [10].

Though the method has limitations yet researchers attempted with a number of modifications in the process to improve the yield as well as to reduce pollution associated with the pyrometallurgical processes. In this context, Xie et al. [97] developed a novel cleaner

ultrasonic assisted pyrometallurgical technique with high recovery efficiency and minimized waste emission. They recovered 95.2–97.5 and 97.1–98.5% of Cu and Fe, respectively. According to Cui and Zhang [16], more than 70% of PCBs are treated in the smelter. Kaya [7] reviewed the industrial pyrometallurgical processes of metals recovery from e-waste, for example, Berlin Technical University in 1997 developed a recycling method and converted waste PCBs into a Cu-Ni-Si alloy, a mixed oxide (especially Pb and Zn) and environmentally friendly slag by a top blown reactor [98]. The Umicore's metal smelter and refinery in Belgium treated e-waste in IsaSmelt furnace to extract valuable metals along with Cu-bullion. In the subsequent process, Cu is extracted from the Cu-bullion by leaching and electrowinning followed by recovery of precious metals in the metal refinery [99]. In another smelter in Sweden (Boliden Ltd. Ronnskar), PCBs are added into Cu converter to extract metals like Ag, Au, Cu, Ni, Pd, Se, and, Zn; while the residual dust is treated separately to extract Pb, Sb, In, and, Cd [100]. The pyrometallurgical processes are known to emit a lot of toxic gases and compounds e.g., polybrominated dibenzodioxins (PBDD), polybrominated dibenzofurans (PBDF), dibenzo-p-dioxin, tribromobenzene, biphenyl, anthracene or phenanthrene, dibromobenzene, phenol, naphthalene, dibenzofuran, tetrabromobenzene and many more leading to serious pollution [101]. Further, the low final yield, loss of precious metals, generation of a large amount of slag and high capital investment are other major disadvantages associated with pyrometallurgical methods [7].

2.8.2 Hydrometallurgy

The hydrometallurgical process is widely used to recover metals from e-waste. These methods offer advantages of low capital cost and high metals recovery efficiencies compared to pyrometallurgical processes [16, 102]. The hydrometallurgical process involves the pretreatment step i.e. mechanical shredding of e-waste to liberate or expose the metals, leaching using a suitable lixiviant, purification of leachate and then recovery of metals [10]. The chemical lixiviant used for dissolution of metallic fraction of e-waste are thiosulfate, cyanide, sulfuric acid, aqua regia solution, nitric acid, and thiourea [15, 16, 26, 103, 104]. The precious metals leaching is commonly carried using cyanide, thiourea, thiosulfate, and halides as leaching reagents. However, it was reported that Au recovery is effective and economical

using cyanide as lixiviant but has environmental concerns associated with it [15]. The metals recovery from leached/pregnant solution is carried by solvent extraction, cementation, ion exchange and adsorption on activated carbon methods [105].

Silveria et al. [106] developed a process to extract In from LCD screens. They manually removed screens from mobile phones and subsequently pretreatment was done to remove polarizing films from the glass of LCD panels. The hydrometallurgical leaching recovered 96.4 wt.% of indium (In) from LCD powder using 1.0 M H_2SO_4 at 1:50 solid/liquid ratio, 90°C, 1 h, and stirring at 500 rpm. After leaching, 99.8 wt.% precipitation was attained with NH_4OH at pH 7.4. Kim et al. [107] achieved 71% Cu along with 98% Zn, 96% Sn and 96% Pb from waste PCBs using 2.0 M HCl solution at 50ºC and 400 rpm in 4 h. Jadhav and Hocheng [108] investigated the recovery of precious metals from waste PCBs. Prior to hydrometallurgical treatment, the waste PCBs were pretreated using sodium hydroxide (NaOH) to remove chemical coating present in PCBs. The complete recovery of metals was obtained by using 1 M HCl from 4 cm×4 cm PCBs at 150 rpm and room temperature in 22 h. According to Pant et al. [26], chemical leaching using a variety of chemical ligands such as diethylenetriaminepenta acetate (DTPA), ethylene diaminetetramaide (EDTA), and other chelators like oxalate and citric acid has also been reported for metal extraction. Calgaro et al. [109] used supercritical CO_2 (g) with H_2SO_4 + H_2O_2 as co-solvent to extract Cu from obsolete mobile phones. They recovered about 90% of Cu in presence of a mixture of 20% H_2O_2 in 2.0–2.5 M H_2SO_4, respectively, in 20 min. The technological feasibility of hydrometallurgical methods have been proven and metals recovery using chemical lixiviants are well-established and largely practiced in industry. However, e-waste treatment using these methods raises concerns about the formations of toxic, highly acidic or alkaline effluents, acid fumes, spent acidic medium and cleaning solvents which may pose risk of environmental contamination as well as acute to chronic exposure hazards to workers [7, 10, 16, 101, 105].

2.8.3 Biohydrometallurgy

Biohydrometallurgy/biometallurgy as a bioremediation strategy is an innovative approach developed to recover metals from e-waste and low-grade ores in an environmentally friendly, techno-economic and energy conservative way. Biohydrometallurgy is an

interdisciplinary field where aspects of microbiology (especially geomicrobiology), mineralogy, geochemistry, biotechnology, geology, chemical engineering, hydrometallurgy, and mining engineering are combined [32]. In the process, microorganisms play a role as biocatalysts in mining metals from metals bearing fractions and can be applied to exploit secondary sources e.g., e-waste. The process includes biooxidation and bioleaching.

Biooxidation process is microbial assisted oxidation of host minerals in which metals of interest are present. As a result, the undesired metal impurities were dissolved while the valuable metal of interest remains in the solid residue in a more concentrated form. Biooxidation is widely used to refine and remove impurities from the solid minerals during gold mining operations [32, 110].

Bioleaching refers to microbial assisted conversion/solubilization of the metals from solid metallic fractions, low-grade ores, and e-waste into their extractable which can be recovered from the solution [44, 45]. The work was focused to recover metals of interest i.e. Cu, Au, and Ag through bioleaching process, therefore discussed in the chapter rather than biooxidation.

The Rio Tinto mines in south-western Spain are typically credited to cradle biohydrometallurgy and have been exploited for their Cu, Au and Ag value since pre-Roman times. However, commercial bioleaching operation at industrial scale had been introduced to Tharsis mine in Spain 10 year ago [111]. Earlier, bioleaching process was used only for ores. With relatively low energy consumption and less requirement of reagents, the process of bioleaching has been proved as a promising economic alternative for metals extraction compared to traditional methods. The process allows metal recycling similar to the biogeochemical cycles, thus reducing the energy demand, and save the natural resources such as ores and landfill space [112]. Bioleaching has not been limited to ores but has opened up the possibility and paved the way for mining metals from solid metallic fractions, fly ash, industrial residues and complex material like e-waste. The process also offers the advantage of extracting metals which cannot be recovered by conventional methods [112]. Moreover, industrialists are considering clean and green technology such as bioleaching due to increasing pressure to adopt sustainable and eco-friendly processes [110]. Although the success of biometallurgy particularly in low-grade Cu minerals has been evident, yet 20 plants

commercialized in the past 30 to 40 years [113]. The application of bioleaching in case of e-waste is still in infancy.

2.8.3.1 Types of bioleaching

Two different types of bioleaching were reported in the literature i.e. one step and two step bioleaching. In one-step bioleaching, microorganisms and solid waste material are simultaneously added to the medium where microbial growth and metals dissolution take place together. The metals ions present in the solid waste induces toxicity and environmental stress which inhibits microbial growth thereby limits the lixiviant secretion and metal dissolution efficiency. Further, microorganisms were not able to tolerate higher pulp density, which limits its use for industrial-scale applications [24, 31, 35].

On the other hand, in the two-step bioleaching, microorganisms are first cultivated in the growth medium in absence of solid waste, till it reaches the optimum cell density (i.e. metabolically active state). After microbial growth, the waste is added aseptically in the medium flask. The two-step bioleaching has several advantages over one-step bioleaching: (1) e-waste can be recycled because biomass is not in direct contact with it, (2) in the absence of e-waste lixiviant production can be enhanced, and (3) higher pulp densities can be used compared to one-step bioleaching, resulting in enhanced metal mobilization [16, 18, 29, 32]. In addition, researchers have used spent medium leaching for metals extraction from e-waste. In this method, microbial cells are cultivated in the growth medium till the maximum production of metabolites occurs. After that cells are removed and cell-free medium containing biologically produced metabolites is used for leaching metals from solid waste [35, 114-116].

2.9 Bioleaching of e-waste

E-waste has been a major and fastest growing industrial waste in the 21st century. In the last decade, numerous studies have been published on metals bioleaching from e-waste. The number of microorganisms including mesophilic and moderately thermophilic acidophiles are receiving attention to extract metals from e-waste [20]. The major microorganisms known to play a vital role in metals bioleaching from e-waste belong to acidophilic group e.g., *A. thiooxidans*, *A. ferrooxidans*, *L. ferrooxidans*, and *Sulfolobus* sp. [117]. These bacteria drive

their energy from the oxidation of ferrous iron (Fe^{2+}) and elemental sulfur (S) or reduced sulfur compounds and in the process form ferric iron (Fe^{3+}) and sulfuric acid (H_2SO_4), oxidizing agents that promote metal solubilization [118]. In addition, heterotrophic bacteria (*C. violaceum*, *Pseudomonas* sp., *Bacillus* sp.), as well as heterotrophic fungi (e.g. *Aspergillus* sp., *Penicillium* sp.), have also been reported to mobilize metals from e-waste [117, 118]. The common mechanisms involved in bioleaching are acidolysis, complexolysis, redoxolysis, and bioaccumulation, respectively [111].

The earliest studies on e-waste bioleaching was accounted for Brandl et al. [24], who proved that microbial assisted processes can be utilized to extract metals from e-waste. Brandl et al. [24] used individual fungal strains *A. niger* and *P. simplicissimum* and a mixed culture of *A. thiooxidans* and *A. ferroxidans* to leach metals from e-waste and reported appreciable dissolution of Cu, Zn, Ni, and Al at 10 g/L pulp density. The microorganisms were termed as "computer munching microbes". The microbial generated inorganic and organic acids (lixiviant) are known to cause mobilization of metals. Further, their study found a poor microbial growth in presence of e-waste, hence proposed a two-step bioleaching process for efficient mobilization of metals. Ilyas et al. [119] performed bioleaching using a moderate thermophile *Sulfobacillus thermosulfidooxidans* and recovered more than 80% of Ni, Cu, Zn, and Al from e-waste. Liang et al. [120] established a process to extract metals from PCBs using *A. thiooxidans* and *A. ferrooxidans* (mixed culture) and extracted 94, 89, 90 and 86% of Cu, Ni, Zn, and Pb, respectively. Yang et al. [121] recovered 96.8%, 83.8%, and 75.4% of Cu, Zn and Al from waste PCBs using *A. ferrooxidans* at 15 g/L pulp density after 72 h. Chen et al. [122] examined column bioleaching of Cu from waste PCBs by *A. ferrooxidans*. After 28 days of column leaching, 94.8% of Cu was recovered from waste PCBs. Priya and Hait [123] using *Acidiphilium acidophilum* reported 79%, 39%, 29% and 10% of Cu, Ni, Zn, and Pb, respectively, from television PCBs at 10 g/L pulp density and <600 μm particle size after 60 days of leaching time. Though numerous studies have been reported to bioleach or recover metals such as Cu, Zn, Ni, and Pb using autotrophic microorganism, but the precious metals recovery is majorly carried using the heterotrophic group of microorganisms especially the cyanogenic microorganisms.

2.10 Cyanogenic bioleaching of e-waste

Cyanide is known to form well-defined metal-cyanide complexes with a variety of metals (such as Cu, Cr, Fe, Co, Ni, Zn, Ge, Tc, Ru, Rh, Pd, Ag, Pt, Au) that often exhibit high chemical stability and good water solubility [32]. In the e-waste, precious metals (Au, Ag, Pt, and Pd) extraction is gaining interest in the cyanidation process [118]. Cyanidation is extensively used to recover Au and Ag from ores concentrates. At present, approximately 90% of Au mining operations are using cyanide for gold recovery, in spite of its high toxicity [36]. Recently, an alternative approach is in consideration where chemical cyanide is replaced with the microorganism, especially the cyanogenic microorganism such as *C. violaceum*, *P. fluorescens*, *P. aeruginosa*, *P. putida*, and *B. megaterium* that produce cyanide to dissolve gold [35, 45]. The metals leaching using biogenic cyanide is termed as "biocyanidation"; and also expressed as 'alkaline bioleaching' or 'heterotrophic bioleaching' [16, 20, 117].

The cyanogenic microorganism produces hydrogen cyanide (HCN) as secondary metabolite and most of these bacteria belong to soil microflora [25]. The cyanide production occurs typically at the early stationary phase of bacterial growth [31]. According to Liang et al. [36], microbial mediated dissolution of gold produces little cyanide, when decoupled with subsequent detoxification are environmentally agreeable processes. The HCN is produced by bacteria such as *C. violaceum*, *P. fluorescens*, and *P. aeruginosa* and fungi such as *Marasmius oreades*, *Polysporus* sp., and *Clitocybe* sp., but only bacteria have been exploited for biocyanidation of gold from e-waste. The quantitative data on HCN production for many species is missing and is available only in the form of qualitative information [36, 118]. The HCN is produced from glycine using HCN synthase enzyme, however, the amount produced is limited e.g., maximum 20 mg/L of bacterial culture for *C. violaceum* [118]. In addition to cyanide production, cyanogenic microorganisms possess necessary enzymatic material to detoxify cyanide e.g., in case of *C. violaceum* it is β-cyanoalanine synthase. Thus, *C. violaceum* has been most extensively exploited for dissolution of gold followed by *Pseudomonas* sp.

The first evidence on formation of metals-cyanide complexes from metallic solid fraction such as PCB scrap by cyanogenic microorganisms is reported by Faramarzi et al. [33]. The authors reported mobilization of Ni as tetracyanonickelate from Ni powder and Au as

dicyanoaurate from electronic waste. The biocyandation of gold can be represented by Elsner's Eq. (2.1):

$$4Au + 8CN^- + O_2 + 2H_2O = 4Au(CN)_2^- + 4OH^- \qquad (2.1)$$

Brandl et al. [32] performed bioleaching of shredded PCBs using cyanogenic *C. violaceum* and recovered 68.5% of Au as dicyanoaurate. They also demonstrated the presence of cyanide-complexed Cu during bioleaching form e-waste possibly due to its higher content. Chi et al. [28] using *C. violaceum* increased Au mobilization to 11% from waste mobile phone PCBs by supplementation with H_2O_2. Pradhan and Kumar [31] investigated the bioleaching potential of individual bacterial strain and mixed culture from e-waste. Their results showed higher metals mobilization by mixed culture of *C. violaceum* and *P. aeruginosa* compared to individual bacteria. Tay et al. [124] genetically engineered *C. violaceum* and enhanced lixiviant production and thereby Au recovery from e-waste. As reported earlier, Cu presence interferes in gold cyanidation process. Therefore, Natarajan and Ting [8] applied chemical pretreatment using nitric acid to remove Cu from e-waste. The pretreated waste was subjected for bioleaching using mutated *C. violaceum* and enhanced Au recovery compared to wild *C. violaceum* [8]. In another study by the authors, Au recovery was enhanced to 30% with pH modification in spent medium leaching (cell-free metabolite) compared to Au (11%) recovered in two-step bioleaching at 0.5% pulp density [35]. Recently, a two-stage process has been proposed, in which oxidative leaching is carried using autotrophic bacterial like *A. thiooxidans* and *A. ferrooxidans* to remove base metals (particularly Cu) followed by bioleaching of precious metals using cyanogenic microorganism such as *B. megaterium* and *P. putida* [45, 125, 126].

2.11 Factors influencing bioleaching Process

Bioleaching like all other biological processes is influenced by various process parameters like pH, temperature, choice of microorganisms, precursor's molecules, and pulp density. Biotechnology will serve as promising technology in metallurgical processing, if the best combination of all these parameters is explored [10, 36]. Indeed, metals bioleaching from e-waste may find a potential industrial application only if the metals recovery efficiencies are improved, possibly through the optimization of influencing factors.

2.11.1 Choice of microorganism

Selection of microorganism is the most important parameter for the success of bioleaching. The precious metals like Au, Pd, and Ag are majorly recovered by cyanogenic microorganisms at alkaline conditions. Whereas, base metals extraction has been reported by autotrophic group of microorganisms in the acidic environment. Further, the process of bioleaching of metals from e-waste is influenced by the presence of toxic metals content as well as alkaline nature of e-waste. Therefore, selected microorganisms should be capable to tolerate higher concentrations of heavy metals and remain active under such circumstances. Prior to bioleaching, there is a need of proper acclimatization and selection of microbial cultures capable of tolerating higher toxicity of e-waste for efficient mobilization of metals from e-waste [20, 44].

2.11.2 Surface area

The bioleaching process faces the challenge of mass transfer. The particle size determines the contact surface and is a vital parameter in mass transfer. The overall contact surface increases as the particle size reduction and leads to better mass transfer. Further, reduction of particle size resulted in an increase in the number of particles. This arouses tension between solid particles and microorganisms results in cell damages or lysis. Therefore, the two aspects need to be balanced [127]. The researchers recommended particle size range from 40 to 200 µm for rapid metals dissolution [119, 128].

2.11.3 Pulp density

Increase in pulp density during bioleaching is known to hinder the microbial growth, and thereby the poor metals extraction rate. On the other hand bioleaching operation at lower pulp density is not economical from industrial point of view. Thus, optimum level of pulp density during bioleaching need to be investigated [125]. The researchers performed bioleaching experiments on different concentration of e-waste; for example, Natarajan et al. [30] reported maximum dissolution of Au at a pulp density of 5 g/L using genetically engineered *C. violaceum* strain pBAD. Pradhan and Kumar [31] performed bioleaching at 10, 50 and 100 g/L e-waste concertation to extract metals using cyanogenic microorganisms. They

reported maximum dissolution of metals at 10 g/L e-waste pulp density. Mara et al. [45] performed bioleaching of Au at 10 g/L pulp density using *P. putida*. The majority of bioleaching studies reported were carried on e-waste pulp density of 10 to 20 g/L. A decrease in metals extraction was reported beyond 20 g/L of e-waste pulp density [118].

2.11.4 Precursor molecule

It was reported that bioleaching using heterotrophic microorganisms can be enhanced by addition of precursor molecules such as glycine. Glycine is an immediate precursor of cyanide is formed by oxidative decarboxylation [129], and HCN is formed from glycine directly [33]. Faramarzi et al. [33] reported that glycine concentration greater than 10 g/L resulted in reduced bacterial growth and cyanide production whereas at lower glycine concentration the cyanide production is low. The optimum concentration of glycine can enhance the metal recovery, hence need to be optimized.

2.11.5 Temperature

The bacteria used in bioleaching are either mesophilic or thermophilic. It is necessary to optimize the temperature for bioleaching to proceed at a faster rate of metal extraction. The optimum temperature range for a given bacteria depends upon the type of bacteria used in the bioleaching e.g., majority of bioleaching studies using cyanogenic bacteria are carried at 30°C. The bioleaching rate is affected at suboptimal temperature [44]. Thus, in the bioleaching process, the temperature must be compatible with bacterial metabolism to support microbial growth to attain effective metal dissolution [10].

2.11.6 pH

The adjustment of right pH is essential for bacterial growth and is decisive in the dissolution of metals. The maximum bacterial growth as well as cyanide production takes place at initial pH range of 7-8 however, at this pH cyanide is present in the form of gaseous hydrogen cyanide (HCN) and may lost via volatilization. Since the pKa of cyanide is 9.3, conducting metals bioleaching experiments at alkaline condition will shift the equilibrium towards CN^- ions in Eq. (2.2). Thus, increases the availability of the aqueous cyanide ions and

hence increases the metal bioleaching efficiencies. However, microbial growth at alkaline pH is a challenge and cause of concern [42, 117].

$$HCN \leftrightarrow H^+ + CN^- \qquad (2.2)$$

2.12 Optimization Strategies

2.12.1 One-factor-at-a-time method

The OFAT approach examine only one parameter/variable at a time while keeping all other parameters/variables constant. The concentration of the parameters is changed over a desired range. The method has been a preferred choice among the researchers working in diverse fields, because of its ease and convenience. The numerous researchers have used OFAT to standardize parameters like pH, temperature, aeration, agitation, substrate concentration and carbon/nitrogen sources to enhance the end product yield [130, 131]. Several researchers have used OFAT method to standardize e-waste pulp density during bioleaching to extract maximum metals from e-waste [8, 30, 31, 35]. Chi et al. [28] optimized dissolved oxygen concentration and pH to enhance Au mobilization during bioleaching of e-waste using *C. violaceum*. Shin et al. [41] optimized glycine concentration and pH to extract Au from ore by *C. violaceum.* Jujun et al. [42] investigated the influence of pH, temperature, rotation speed and additive concentration on cyanide producing ability of *P. chlororaphis*. Then after, under optimized conditions *P. chlororaphis* was used to dissolve Cu, Au, Au and Ag from e-waste. However, the major limitation of OFAT method is that it does not provide estimates of interactions effect among the parameters studied [130].

2.12.2 Response Surface Methodology

To overcome the limitation of OFAT method, optimization has been carried using multivariate statistical method i.e. RSM. The RSM is a group of mathematical and statistical techniques, which is used to study a functional relationship/interaction between responses of interest and several variables which affect the response [40]. RSM was applied to find out/establish a relationship between the variables and the response, which under a given set of condition suggests a response value to determine the importance of the factors through

hypothesis testing [39]. Moreover, it provides the maximum or minimum response value by predicting the optimum condition of the variables. Before proceeding for RSM, it is important to select experimental design in order to find which experiments should be carried out in the experimental region being studied [130]. Several studies have claimed improvements in the end product yield using RSM over OFAT method [131]. However, optimization of factors affecting bioleaching of metals from e-waste using RSM is limited to Arshadi and Mousavi [125] and Arshadi et al. [40].

2.13 Process economy

The bioleaching process of recovering precious and critical metals from e-waste is an environmental friendly and economically feasible way of metals extraction. The biological agents/lixiviants responsible for leaching metals cut-off the external supply cost of these agents thereby reducing the cost of the process [22, 45, 132]. Jujun et al. [42] performed a cost economic analysis of recovering precious metals from crushed PCBs by *P. chloroaphis* and reported improvement in the cost by 0.26 dollars per 1 Kg of the mixed metallic particles. The operating cost of bioleaching may vary depending upon numerous factors such as plant location, cost of services at particular sites, cost of safe disposal, and recovery of metal ions from treated residue/leachate etc. In addition, the maintenance and management costs per annum need to be incurred [133]. Though the metals extraction through bioleaching from e-waste has been cited as economically viable in number of studies but lacks information on its economic aspects, hence limits the scope of a detailed cost-benefit analysis [109, 134]. Therefore, the commercial and economic viability of bioleaching process remains a knowledge gap which need to be addressed urgently.

2.14 Challenges in bioleaching of e-waste

Although significant research has been done on bioleaching of metals from e-waste yet the technology cannot receive a place for the industrial application. Despite of the presence of encouraging and economic driver i.e. precious metals in e-waste, the bioleaching setups have not progressed past the laboratory scale and is in its infancy. Bioleaching is a slow and time consuming process compared to chemical leaching. Since the high concentrations of metals is

toxic to bacterial cells therefore, bioleaching at higher e-waste pulp densities is another major limitation of bioleaching process.

The literature review has thus come up with the following research questions in bioleaching of metals i.e. Cu, Au, and Ag from e-waste using cyanogenic microorganisms:

- E-waste in terms of its composition is a complex and heterogeneous waste which arouses difficulty in extraction process design. Therefore, it is utmost of essence to characterize the heterogeneity of e-waste in order to minimize the discrepancies in the development a cost-effective and environmentally friendly process.
- The bioleaching of e-waste is majorly carried using autotrophic microorganism. Whereas, the heterotrophic microorganisms are limited to *C. violaceum*, *Pseudomonas* sp. and *Bacillus* sp. To improve the metals bioleaching yield from e-waste, novel microorganisms capable of sustaining higher metals concentration need to be identified.
- There is a pressing need for intensive research on bioleaching of e-waste concentrating on the optimization of process parameters to attain metals recovery at which process can be scaled-up to the industrial levels.

MATERIALS & METHODS

The research work entitled "*Bioleaching of metals (Cu, Au, and Ag) from waste computer printed circuit boards using cyanogenic microorganisms*" was carried out in the Environmental Biotechnology laboratory of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh during the year 2014-2018. The details of material used and methodology adopted are described in this chapter.

3.1 Media

3.1.1 Nutrient Broth (NB)

Peptone	:	0.5%
NaCl	:	0.5%
Meat Extract	:	0.15%
Yeast Extract	:	0.15%
pH	:	7.0 ± 0.2

3.1.2 Luria Broth (LB)

Casein enzyme hydrolysate	:	1.0%
Yeast extract	:	0.5%
NaCl	:	0.5%
pH	:	7.0 ± 0.2

In case of solid medium, NB/LB is supplemented with 1.5 ± 0.5% Agar

3.2 Chemicals, reagents, glassware and plastic ware

Analytical grade chemicals and reagents obtained from Hi-Media (India), Sigma-Aldrich (USA), and Merck (Germany) were used for the investigations. Molecular grade chemicals and products were procured from Promega (USA), and Thermo Scientific (USA) which include DNA extraction kit, PCR master mix, primers, Nuclease-free water, and DNA

ladder. The glassware used was purchased from Borosil (India), and the plasticware was procured from Eppendorf (Germany) and Tarsons (India).

3.3 Microbiological methods

3.3.1 Microorganisms and their maintenance

Microorganisms were isolated through enrichment culture technique from the soil samples and e-waste refuse of industry collected from Himachal Pradesh and Haryana, India. Other than this, *Chromobacterium violaceum* (MTCC-2656) was taken from Environmental Biotechnology Laboratory of Department of Biotechnology and Bioinformatics, JUIT, Solan; previously procured from Institute of Microbial Technology, Chandigarh, India. The bacterial cultures were preserved in glycerol stocks at –20ºC. For routine use; bacterial cultures were maintained on nutrient agar slants and stored at 4°C.

3.3.2 Sterilization

The glassware/plasticware used during experimentation were thoroughly washed in detergent water, running tap water followed by rinsing in distilled water. The sterilization of glassware was carried out in a hot air oven at 180ºC for one hour. All the media, distilled water solutions, and e-waste was sterilized by autoclaving at 121°C, 15 pounds per square inch pressure for 20 min (minutes) unless mentioned otherwise. To maintain aseptic conditions during microbiological experimentation like culturing, inoculation, sampling etc., laminar air flow (LAF) chamber was used.

3.4 To determine the heterogeneity in the metals content of waste CPCBs

3.4.1 Source of e-waste

Five different samples of waste CPCBs (P1-P5) were procured from the stockroom of Exigo Recycling, Panipat, Haryana, India (ExigoRecycling.com) and stored in zipper storage bags. The company collects the discarded electronic equipment from different parts/states/regions of the country (India). The collected waste undergoes various physico-

mechanical processes viz. segregation, dismantling, shredding and pulverization and then recycled to extract metals like Cu, Pd, Pt, Au, and Ag at the industry (Fig. 3.1). The leftover waste after recycling was further processed for its disposal through treatment, storage, and disposal facility (TSDF). The procured waste was used for bioleaching of Cu, Au, and Ag at the Jaypee University of Information Technology, Waknaghat, Solan, India. Prior to bioleaching experiments, the powdered CPCBs were sterilized in the autoclavable bags (HiMedia) at 121°C and 15 psi for 30 min and dried at room temperature (25±3°C) [43].

3.4.2 Particle Size distribution

The segregation of target metals in different particle sizes is helpful for process design during metal extraction [135]. To determine the particle size of waste CPCBs, standard test sieves as per IS 460: 1962 were used. The ground/crushed waste of sample size 50 g to 100 g were sieved and 1 g of the representative sample was used for compositional analysis. The particle sizes of five different morphological forms of CPCBs was: P1 (≥0.71 mm), P2 (0.35 to 0.71 mm), P3 (0.15 to 0.35 mm) and P4 (≤0.15 mm) and P5 (≤0.15 mm), respectively (Fig. 3.2). The waste CPCBs P4 and P5 have a similar particle size with variable metal composition.

3.4.3 Metal content analysis

The procured ground waste of CPCBs was subjected to its compositional analysis. The metal content analysis of waste CPCBs was done using aqua regia (HNO_3: HCl = 1:3) digestion method, a protocol used in several studies [15, 18, 31, 119]. In the method, 1 g of ground waste was added to 100 ml of aqua regia in a round bottom flask followed by refluxing at 100°C. The leachate was allowed to cool and final volume was made up to 100 ml by adding deionized water. The obtained leachate was then filtered through 0.45 µm glass fiber filter (PALL-GF-A/E-I) to ensure a particle free suspension and preserved for future analysis. The concentration of dissolved metal ions were determined through atomic absorption spectrometry [AAS, (PerkinElmer AAnalyst 400; PerkinElmer, Inc., Waltham, Massachusetts)] at following wavelengths (nm): Cu (324.8), Fe (248.3), Ni (232.0), Zn (213.9), Ag (328.1), Au (242.8), Co (240.7), and Cr (357.9) [31].

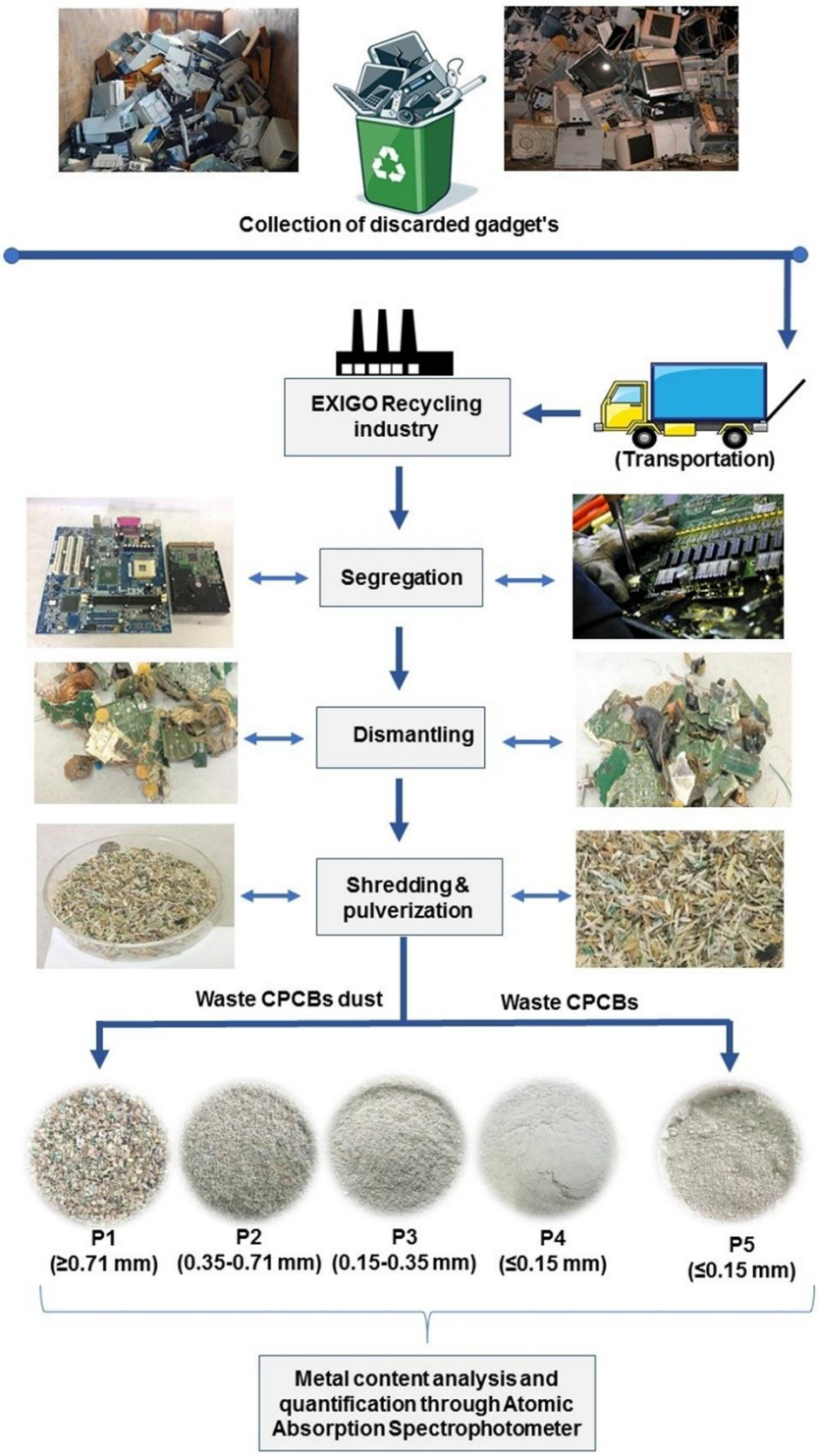


Figure 3.1: Diagram representing e-waste processing at Exigo Recycling Pvt. Ltd., Panipat, Haryana.

* The waste CPCBs P4 and P5 has a similar particle size with variable metal composition.

Figure 3.2: Pulverized CPCBs of different particle sizes: P1) ≥0.71 mm; P2) 0.35 to 0.71 mm; P3) 0.15 to 0.35 mm; P4) ≤0.15mm and P5) ≤0.15mm

3.5 Isolation, screening, and characterization of potent bacterial strains capable of bioleaching of metals (Cu, Au, and Ag) from waste CPCBs

3.5.1 Site characterization and sampling

Samples were collected from two different sites viz., Himachal Pradesh and Haryana of India. The soil samples (topsoil i.e. 0-15 cm) were collected in zipper storage bags from an abandoned gold mine (Fig. 3.3). The abandoned Goldmine is located at village Makdoa (30°50'56"N and 77°10'12"E) 15 Km from Solan town of Himachal Pradesh, along with the banks of Kewal River, and was closed down years ago due to the scanty quantity of Au. The soil samples within periphery (10-15 meters) of mine were also collected and pooled.

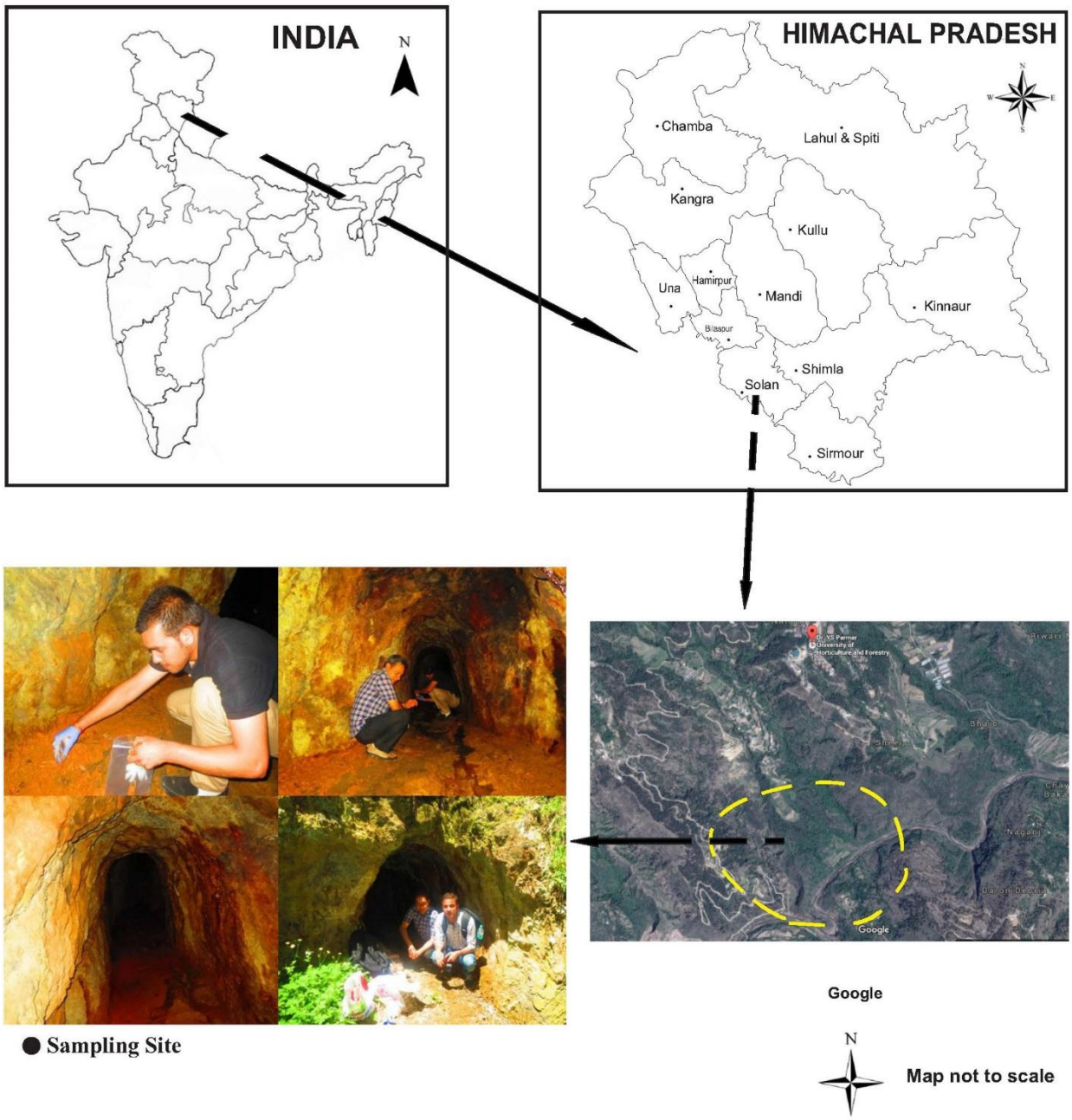


Figure 3.3: Location map and snapshot of abandoned gold mine (sampling site) of district Solan of Himachal Pradesh.

The second sampling site was the e-waste recycling facility i.e. Exigo Recycling Pvt. Ltd., Panipat, Haryana. This site was purposely selected to isolate the e-waste habituating bacterial strains. The e-waste refuse of CPCBs was collected and stored in zipper storage bags

from the e-waste recycling facility and stored in the laboratory at -20°C until analysis. Before proceeding for analysis, the soil samples procured from both the sites were homogenized aseptically, and the representative sample was then used for microbial isolation.

3.5.2 Physico-chemical analysis of gold mine soil

One gram of representative soil sample was ignited in a porcelain crucible at 400°C in a muffle furnace for 4-5 h. The ignited soil was treated with 10 ml of HCl 50% (*w/v*) for a time period of 1 h at 60-80°C. The leachate contained alkaline earth metals was transferred and retained. The leftover soil was treated with 10 ml of both hydrofluoric acid (HF) and HCl and evaporated till dryness. Then, after 5 ml of HF and HCl was added and evaporated to dryness, the leftover residues of soil were further dissolved in 10 ml of HCl. The final digested solution was mixed with the extract containing alkaline earth metals and then diluted with distilled water [6]. The diluted samples were filtered through 0.45 µm filters and analyzed through AAS.

3.5.3 Mining of cultivable bacterial population from the soil sample of the abandoned gold mine

The bacterial strains were isolated from the abandoned gold mine (Solan, HP, India) through enrichment culture technique. The soil sample was enriched in Luria broth (LB) medium (100 ml in 250 ml Erlenmeyer flask) containing sterilized e-waste at respective concentration. One gram of representative unsterile soil sample was added to LB medium supplemented with 10 g/L of e-waste pulp density of particle size ≤150 µm. The experimental flasks were incubated at 150 rpm and 30ºC in a shaker incubator (Thermo scientific MaxQ 8000; Thermo Fisher Scientific, Inc., Massachusetts) for a period of 6 days. After six days incubation, 5 ml of inoculum containing 2×10^8 CFU/ mL was transferred sequentially to the next set of flasks containing fresh medium supplemented with 25 g/L pulp density of e-waste. The flasks were incubated at a temperature of 30ºC, rpm of 150 for 6 days. The sequential enrichment was continued over a period of 30 days at e-waste pulp density of 50, 75 and 100 g/L, respectively (Fig. 3.4). After 30 days of incubation, samples were taken from the flask containing 100 g/L of pulp density and serially diluted followed by spread plating on nutrient

agar plates. The plates were incubated at 30ºC for 48-72 h and observed for the appearance of colonies. Colony forming units (CFU) were recorded on nutrient agar plates after 72 h, and was calculated as per Eq. (3.1):

$$\text{CFU} = \frac{\text{Bacterial plate count} \times \text{Dilution factor}}{\text{amount of sample plated}} \qquad (3.1)$$

Morphologically distinct colonies were selected and repeatedly sub-cultured to ensure the purity of the bacterial cultures [136, 137]. To avoid microbial contamination an un-inoculated control was incubated in parallel.

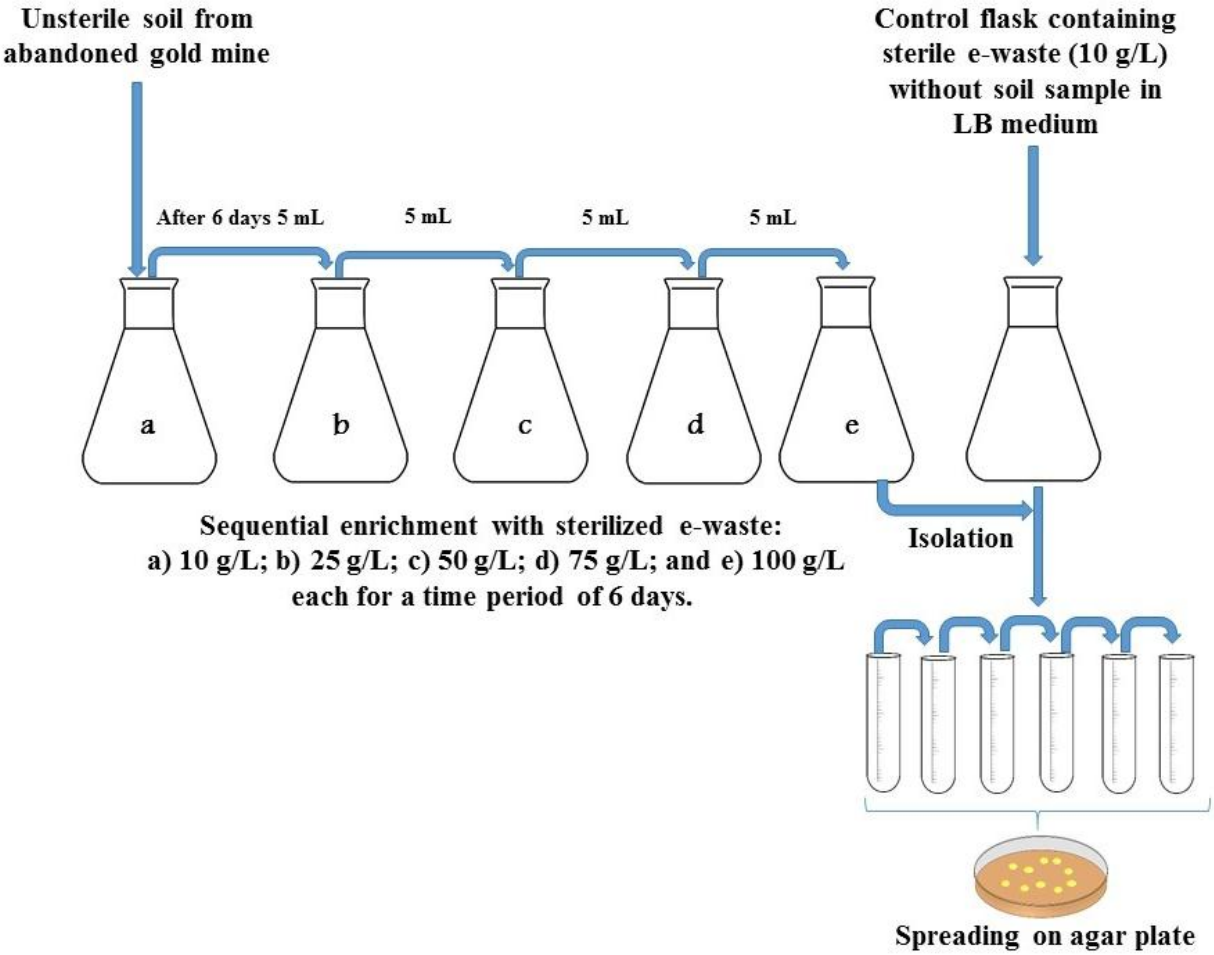


Figure 3.4: Diagrammatic representation of the methodology adopted for isolation of bacterial strains from abandoned gold mine of Solan, HP

3.5.4 Mining of cultivable bacterial population from the industrial e-waste refuse

The e-waste toxicity tolerant bacterial strain was isolated from industrial e-waste refuse (Panipat, Haryana) through enrichment culture technique. One gram of representative unsterile e-waste refuse sample was added to Luria broth (LB; 100 mL) medium flaks and incubated at 150 rpm and 30 ±2°C in a shaker incubator for 6 days. The experimental flasks containing medium and sterilized e-waste were kept as controls. The enrichment was performed by transferring 5 mL of the sample containing 2×10^8 CFU/ mL from the previous flask to next flasks having LB supplemented with 25 g/L of the sterile waste CPCBs. The flasks were incubated at 30 ±2°C and 150 rpm for 6 days. The sequential enrichments were continued with 50, 75, and 100 g/L of sterile waste CPCBs over a period of 30 days (Fig. 3.5). After 30 days of incubation, samples were taken from the end flask having 100 g/L CPCBs and serially diluted followed by spread plating on nutrient agar plates. The plates were incubated at 30ºC for 48-72 h and observed for the appearance of colonies. The CFU was recorded as per formula is given in Eq. (3.1). To obtain a pure culture, the isolated bacterial strains were repeatedly sub-cultured.

3.6 Identification and characterization of the isolated bacterial strains

The identification of bacterial strains isolated from two different sites was done based on their morphological, cultural and molecular characterization.

3.6.1 Morphological characterization

The isolated bacterial stains were accessed for their morphological features such as the appearance of the bacterial colonies, pigment production, Gram's reaction, and cell shape.

3.6.2 Characterization of isolates by 16S rRNA analysis

The identification of the isolated bacterial strains was carried using 16S rRNA gene sequencing. All the isolated bacterial strains were individually grown in LB medium for 24 h. For genomic DNA, 24 h fresh grown cells were harvested followed by isolation of DNA using Wizard genomic DNA purification kit (Promega; USA) as per protocol is given by the

manufacturer. The DNA was quantified spectrophotometrically by A260 and A280 measurements through a NanoDrop (Thermo Fisher Scientific, Inc., Massachusetts, USA). The isolated genomic DNA was subjected to 16S rRNA gene amplification using universal primers 27F and 1492R in the thermal cycler machine (Thermo Fischer Scientific). The PCR reaction of 20 µl volume contained 50-100 ng of template genomic DNA, 5 pmole of forward and reverse primers and PCR master mix (promega). The PCR reaction was started with initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 51.8°C for 1 min and 72 °C for 1 min and having a final extension at 72 °C for 7 min [21]. To eliminate any risk of contamination from extraneous DNA, the reaction mixture without the template was simultaneously run along with PCR reaction as a control.

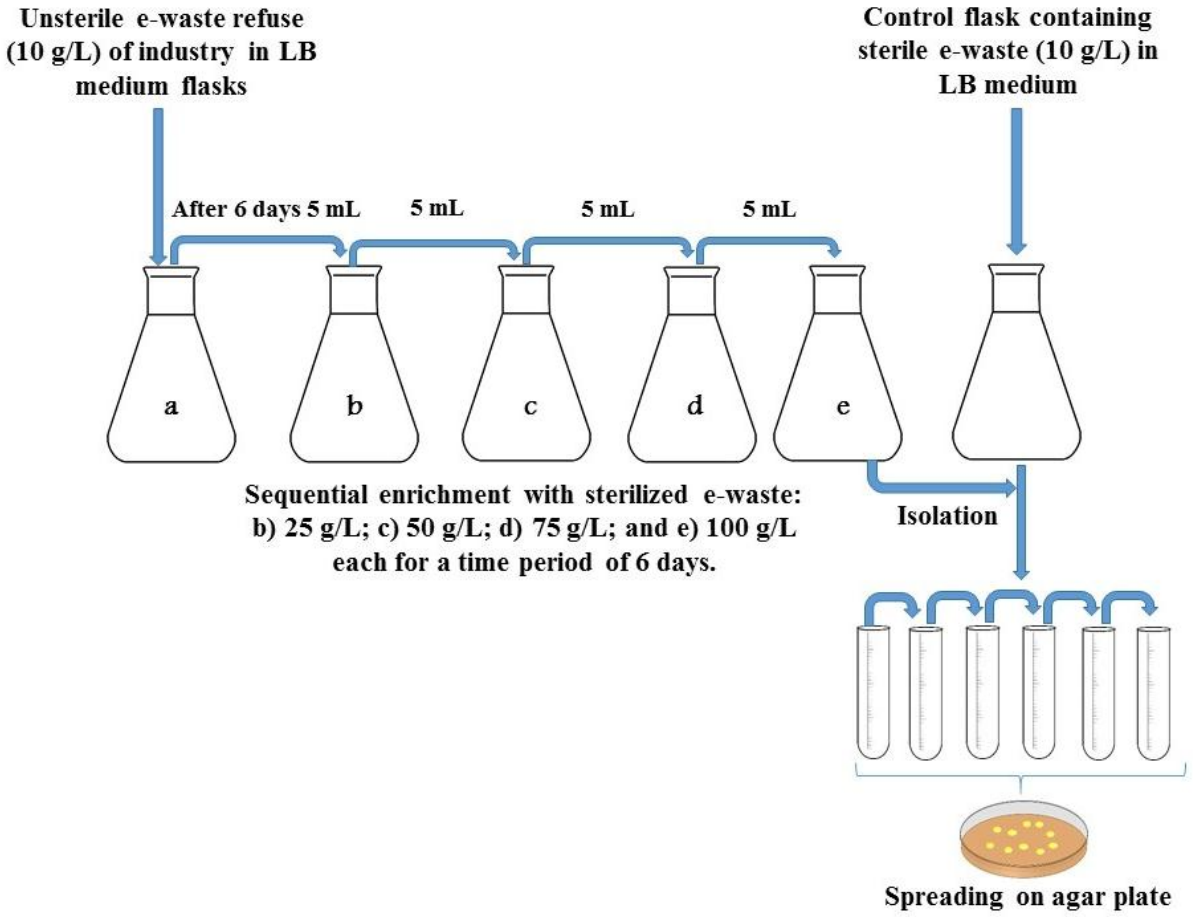


Figure 3.5: Diagrammatic representation of the methodology adopted for isolation of bacteria from e-waste refuse of industry.

3.6.3 Agarose gel electrophoresis of PCR product

The amplified PCR product of each test isolate was resolved by electrophoresis using 1.5% agarose gel in 1X Tris-acetate EDTA buffer containing ethidium bromide (0.5 µl/ml). The DNA ladder of 1 Kb was used as a marker. The gel was run at 120V for 2h using Bangalore Genei power system. The gels were viewed and the image was captured using gel documentation system (Bio-rad). The PCR amplicon/product of 1465 bp was seperated by running on the agarose gel (1.5%) electrophoresis followed by sequencing at Xcelris Labs Limited, Ahmedabad, Gujarat, India.

3.6.4 Sequence and phylogenetic analysis

The acquired 16S rRNA gene sequences were compared with the pre-existing 16S rRNA gene sequences available at GenBank database using BLAST(N) program of the National Center for Biotechnology Information, MD, USA. The alignment of sequences was carried by ClustalW program, and a phylogenetic tree was constructed using a neighbor-joining method in MEGA version 6.0 [138]. The Jukes and Cantor model was applied to compute the evolutionary distances. A bootstrap analysis of 1,000 replicates was used to evaluate the topology of the tree [139].

3.7 Screening of isolated bacterial strains for bioleaching of metals (Cu, Au, and Ag) from waste CPCBs-P4

Two-step bioleaching process was followed for solubilization of metals i.e. Cu, Au, and Ag from waste CPCBs because, one step is not recommended due to the toxicity waste CPCBs [24, 28]. In one-step bioleaching process, microorganisms were not able to tolerate higher pulp density, which limits its use for industrial-scale applications [40, 42, 126]. The two-step bioleaching has several advantages over one-step bioleaching: (1) e-waste can be recycled because biomass is not in direct contact with it, (2) in the absence of e-waste lixiviant production can be enhanced, and (3) higher pulp densities can be used compared to one-step bioleaching, resulting in enhanced metal mobilization [16]. The Fig. 3.6 depicts the diagrammatic representation of two step bioleaching. In the two-step bioleaching process, bacterial cells were cultured in the medium which lacks e-waste so that optimum biomass can

be attained. Subsequently, sterilized e-waste was added aseptically and allowed for metal mobilization for 7 days. Two-step bioleaching was performed in 250 mL Erlenmeyer flasks containing 100 ml LB medium [31]. The sterilization was performed in the autoclave at 121°C, a pressure of 15 psi for 15 min. The bacterial culture (5% *v/v*) containing 2×10^8 CFU/mL were inoculated to LB medium and incubated at 30°C and 150 rpm for 48 h so that bacteria attained optimum cell biomass. After 48 h of incubation, e-waste (1% *w/v*) sterilized by autoclaving was added to experimental flasks aseptically. The flask was then again incubated for an additional time period of seven days under the same set of conditions. An un-inoculated control was run in parallel. After 7 days of incubation, the experimental flasks were filtered to remove e-waste particles and centrifuged (Eppendorf Centrifuge 5804 R; Eppendorf India, Ambattur, Chennai, India) at 7000 rpm for 10 min to remove the cell biomass from the medium. The supernatant obtained was passed through 0.45 µm glass fiber filters and evaluated for the presence of metal ions using AAS. Other than metal ions, change in medium pH during bioleaching was also determined by a portable pH meter (Eutech pH Testr30; Thermo Fisher Scientific, Inc., Massachusetts).

3.8 Hydrogen Cyanide (HCN) Analysis

Cyanide production by bacterial isolates was estimated at their early stationary phase. The bacterial cells were grown in LB medium supplemented with 5 g/L glycine and incubated for 48 h. After 48 h, the cell biomass was separated from the medium by centrifugation at 7000 rpm for 10 min. The supernatant was collected and stored in a refrigerator at 4°C. The samples were then sent to Jeedimetla Effluent Treatment Ltd., (JETL) Hyderabad, India. Cyanide production was tested at JETL as per USEPA method 4500 CN^-/E.

3.9 E-waste toxicity assessment and dose-response analysis of potent bacterial strains

3.9.1 Toxicity assessment

To guarantee feasibility and success of bioleaching process; it is utmost important to examine e-waste toxicity upon the isolated bacterial strains [37].

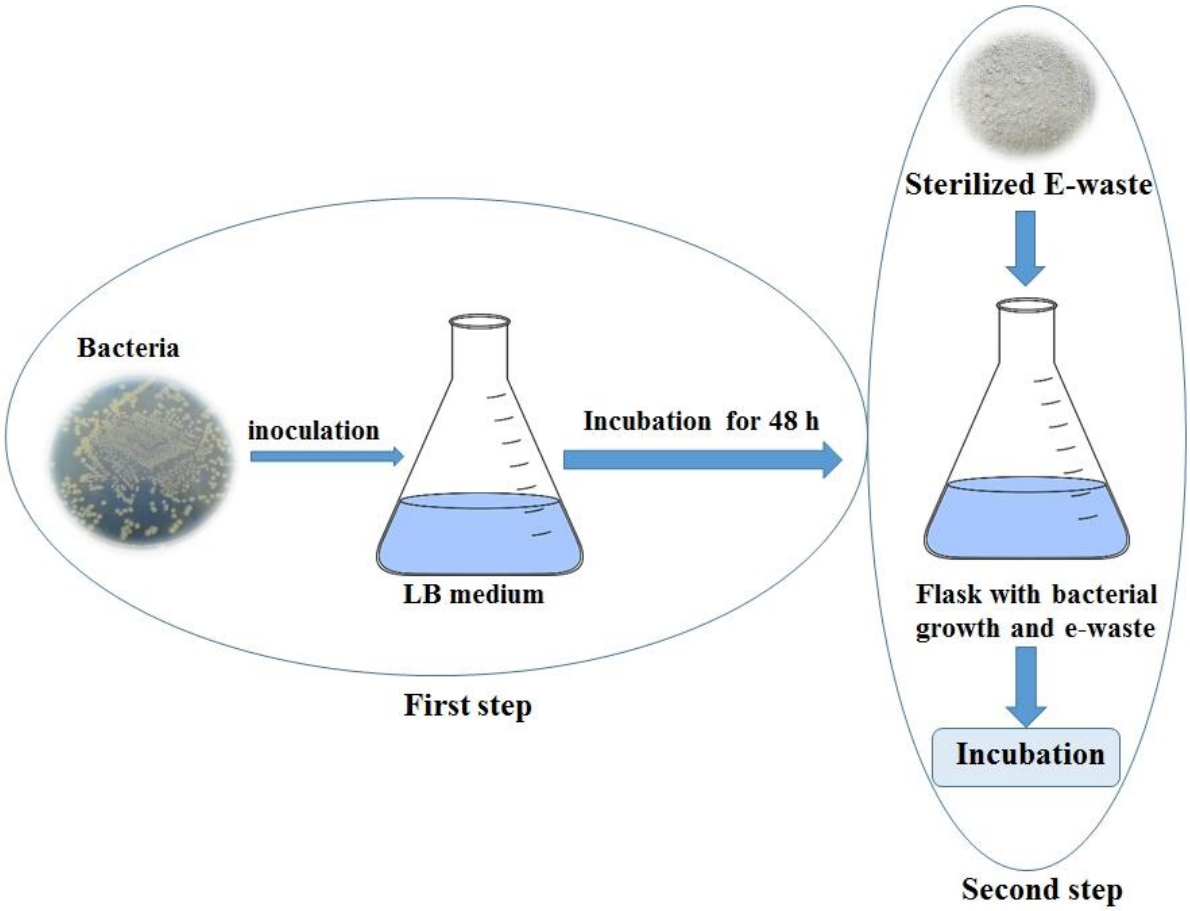


Figure 3.6: Diagrammatic representation of the two-step bioleaching process

The bacterial isolates selected during initial screening for bioleaching of Cu, Au, and Ag were investigated for their e-waste toxicity tolerance. Other than isolated bacterial strains, *C. violaceum* (MTCC-2656) was also examined for its tolerance levels. The bacterial cells of each strain were exposed to different e-waste pulp densities viz. 10, 50, 100, 150, 200, 300, 400 and 500 g/L, respectively. The plate count method was adopted to determine the toxicity tolerance. To determine e-waste toxicity, Clinical and Laboratory Standards Institute (CLSI) guidelines were followed. The experiments were conducted in the flasks (250 mL volume) having 100 mL LB medium. The medium flasks were inoculated with 1% (*v/v*) of inoculum containing 1.4×10^5 CFU/mL and sterile e-waste at respective pulp densities. A separate control which contains bacterial cells without e-waste was run in parallel. The experimental flasks (in duplicates sets) were incubated at 150 rpm and 30°C for 24 h. After 24 h of incubation, samples

were taken and serially diluted following enumeration of bacterial cells on nutrient agar medium by spread plate method. The bacterial growth was measured using CFU per gram of e-waste pulp density.

3.9.2 Dose-response Analysis

Dose-response is a quantitative analysis which provides estimates of the maximum treatment concentration and 50% effective concentration (EC_{50}) allowable for metals bioleaching [37]. Therefore, data generated from the toxicity assessment studies were plotted to generate dose-response curve using the GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, California). In this context, the percent inhibition response was computed as per Eq. (3.2):

$$\% \text{ IR} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100 \qquad (3.2)$$

Where *IR* is the inhibition response, *Control* is bacterial growth in the absence of e-waste, and *Test* is bacterial growth in the presence of e-waste.

Percent IR data obtained at respective e-waste pulp density was subjected to nonlinear regression. The equation followed to generate dose-response curves was *log (agonist) vs. normalized response-variable slope* [140]. On the basis of dose-response curves, the EC_{50} values for bacterial strains were determined. The EC_{50} is a statistically derived estimate for a chemical or pollutant at which 50% of microbial population reduced within a defined time period.

3.10 Selection of the factors influencing process parameters

Bioleaching due to its slow recovery rates and absence of conducive growth environment or optimized process parameters; has not been developed as a profitable process for metals extraction from the metallic repositories/e-waste/ores [10]. Hence, optimization of parameters is a topic of utmost importance in any of the industrial production processes because the little amendments can be critical for commercial/industrial success. The factor influencing the effectiveness of the metal bioleaching includes both biotic and abiotic

parameters. Biotic factors include the type of bacterial cultures and its inoculum size whereas, abiotic factors include factors like pH, temperature, aeration, and e-waste concentration etc. [31, 32, 38, 42]. The influence of biotic and abiotic factors during the process; help in achieving the maximum bioleaching of metals hence optimization is a critical component [141]. In order to speed up the rate of bioleaching process; following key parameters were selected with attention to previous work 1) pH [28], 2) Pulp density [142], 3) Temperature [42], 4) Precursor molecule [33], and 5) particle size [125]. During optimization; two-step bioleaching process was followed.

3.11 Optimization of the process for maximum recovery of metals (Cu, Au, and Ag) from waste CPCBs using one-factor-at-a-time (OFAT) approach

This is a traditional method of optimization in which all parameters were fixed, except the one under study. The detailed methodology is discussed as follow:

3.11.1 Effect of pulp density

Pulp density is a significant parameter in the bioleaching process. The concentration of pulp density influences metals leaching and growth of microorganisms [125]. Increased amount of pulp density surely will increase the metals concentration in the leaching environment, which may hinder the microbial growth resulting in reduced metals mobilization. On the other side lower quantities of pulp density may not be economically feasible for the process. Therefore it is essential to find out an optimum concentration of pulp density at which maximum bioleaching can be obtained [142]. In this context variable pulp densities (1, 5, 10%) were selected while taking other factors of pH (8), rpm (150), glycine (5 g/L) and incubation temperature (30°C) as constant.

3.11.2 Effect of temperature

Optimum activity of microorganisms (bacteria in our case) takes place in a specified narrow range of temperature. This indicates temperature dependent characters of bioleaching

[126]. To find out optimum range of temperature for metals mobilization all the bacterial strains were subjected for two-step bioleaching to variable temperature range (25, 30, 35, 40°C) while, keeping pH (8.0), rpm (150) and pulp density (1%) as constant.

3.11.3 Effect of glycine concentration

Glycine is a known immediate direct precursor molecule of cyanide, which forms HCN and CO_2 by oxidative decarboxylation [32, 41]. In the reaction, glycine loses four H to form HCN followed by cyanide formation by HCN synthase enzyme. It was reported that specific concentration of glycine enhanced end product formation i.e. cyanide production [33]. Since the mechanism of leaching was cyanide-based; medium was supplemented with different concentration of glycine (2.5, 5.0, 7.5 and 10.0g/L) to enhance cyanide production and metal mobilization. The other factors like pH (8.0), rpm (150), temperature (30°C) and pulp density (1%) were kept as constant.

3.11.4 Effect of pH

The concentration of cyanide is highly dependent on pH due to its pKa 9.3. Equilibrium of aqueous and gaseous cyanide can be written as shown in Eq. (3.3):

$$HCN \leftrightarrow H^+ + CN^- \quad (3.3)$$

The low pH below pKa value shifts the equilibrium toward right of the Eq. (3.3) and favors production of HCN rather than (CN^-). Hydrogen cyanide is a gas therefore, may lost by volatilization at low pH and not favorable for bioleaching point of view [30]. Gold recovery is reported to be effective above pH 10.0 and commercial bioleaching experiments are preferred at alkaline pH >10 to prevent the loss of HCN by volatilization [40]. However, most of cyanogenic bacteria solubilize gold at physiological pH of 7-8, because higher pH is not favorable for the growth of bacteria. Therefore, we examined bacterial strains for their bioleaching potential at pH 7, 8, and 9 keeping other factors like glycine, rpm, incubation temperature and pulp density as constant.

3.12 Optimization of process for maximum recovery of metals (Cu, Au and Ag) from waste CPCBs using response surface methodology (RSM)

The RSM was utilized to examine then interaction effect of parameters on bioleaching of Cu, Au and Ag from waste CPCBs. The RSM classify and enumerate the interaction between the process parameters [143]. In the RSM, the CCD is the most significant and widely exploited experimental design in the optimization studies. The CCD-RSM is used for modeling and developing the second-order response surface models [144]. Therefore, CCD-RSM was employed for *P. balearica* showing highest tolerance to e-waste toxicity. Four factors including pulp density, initial pH, temperature, and glycine concentration, were optimized to enhance the recovery of Cu, Au and Ag by CCD-RSM. The total number of experimental runs in CCD were equal to $2^k + 2K + n_0$ in which 2^k the factorial run, $2K$ is the axial run, n_0 is number of central point replications, and K is number of independent variables [145]. The system behavior was modeled through quadratic Eq. (3.4):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i \, x_j, \qquad (3.4)$$

where Y is predicted response, β_0 is the offset term, β_i is the linear effect, β_{ii} is the squared effect, and β_{ij} is the interaction effect [145].

The Design-Expert Software Version 10 (Stat-Ease, Inc., Minneapolis, Minnesota, USA) predicted 30 experimental runs and all were executed in duplicates sets. To determine the interactions among the variables, data obtained from the experimental runs was modeled. The significance of the factors and related responses was interpreted using ANOVA. The statistical significance and the quality fit of the models was determined through the *F*-test and coefficient of determination (R^2). Further, additional experiments were performed on the optimal conditions predicted by the RSM to validate the accuracy and adequacy of the model.

3.13 Morphological Characterization of waste CPCBs

The effect of bioleaching on morphology of the e-waste was studied through SEM (Scanning Electron Microscope) analysis using *C. violaceum* as it showed highest metals

mobilization among all the strains tested. Two-step bioleaching was performed under optimized conditions for 7 days. The residual e-waste left after bioleaching was used for SEM micrographic analysis. The LB medium containing e-waste without bacteria was kept as control. SEM micrograph analysis was done at central instrumentation facility of IIT Roorkee, Uttarakhand.

3.14 Bioleaching of metals from waste CPCBs-P5

3.14.1 Pretreatment of waste P5

To remove the interference of base metals especially copper during bioleaching of gold; nitric acid pretreatment was performed. The 10g of CPCBs waste was added to 30 ml of nitric acid (6 M) at room temperature under shaking conditions for 2 h. The waste CPCBs of particle size ≤0.15 mm were added to the nitric acid incrementally to avoid unnecessary frothing due to the production of nitric oxide gas. After 2 h of shaking, the mixture was centrifuged at 5000 rpm for 10 min and 5 ml of the sample was taken for metals content analysis. The residual waste was washed with deionized water and the cycle was repeated until traces of blue-green colour disappears. The pretreated waste CPCBs were dried and weighed to constant mass and subjected to metal content analysis using aqua regia (HCl: HNO_3 = 3:1) [8, 18].

3.14.2 Bioleaching study of pretreated and untreated waste

The bioleaching capabilities of bacterial strains against pretreated and untreated waste were tested under previously optimized condition using two-step bioleaching process as per procedure explained in section 3.7. Out of five bacterial strains, only three bacterial strains were used based on their metals mobilization potential.

3.15 Characterization and disposal of the residual waste CPCBs

Owing to its hazardous nature, e-waste cannot be discarded anywhere even after bioleaching. Therefore the residual of the bioleaching was further subjected to compositional analysis before disposal using aqua-regia based digestion as per protocol in section 3.4.3. The final disposal of the residual waste was done as per rules stated by MOEF &CC, 2016.

RESULTS & DISCUSSION

4.1 To determine the heterogeneity in the metals content of waste CPCBs

4.1.1 Metal content of different size fractions of waste CPCBs

For compositional analysis, received waste CPCBs were sieved into different size fractions. The metals content at different sizes i.e. P1 (≥0.71 mm); P2 (0.35 to 0.71 mm); P3 (0.15 to 0.35 mm) and P4 (≤0.15 mm) was determined using aqua regia digestion at 100°C for 1 h. The results revealed the presence of Cu in the majority of all the particle sizes. The highest concentration of Cu was found for the particle size ≥0.71 mm, while iron (Fe) was higher in the CPCBs of smaller size fraction ≤0.15 mm (0.63%) (Table 4.1). The Cu content in the CPCBs decreased with the decrease in particle size from ≥0.71 mm (29.29%) to ≤0.15 mm (1.38%) (Table 4.1). Similarly, Huang et al. [146] investigated the metallic composition of waste printed circuit boards (WPCBs) of different particle size fractions and reported a decrease in the Cu content with the decrease in the particle size from >0.5 mm to <0.075 mm. This may correspond to high ductility of Cu, which distributes it more in larger size fractions (≥0.71 mm) whereas Fe is highest in smaller size fraction (≤0.15 mm) due to brittle ferroalloys and magnets which tends to break easily during pulverization [135]. The precious metals such as Au (0.008%) and Ag (0.04%) also have a higher concentration in the smaller size fraction (≤0.15 mm). The results corroborate to the study by Sun et al. [135], reported the higher concentration of Cu in larger particle size fractions (1-8mm), whereas, precious metals were predominant in the samples smaller than 0.2 mm. The lower concentrations of precious metals (Au & Ag) in the particles size ≥0.71 mm may be because of low participation of precious metals and high amount of Cu which might have hindered the recovery of precious metals. Further, precious metals in waste CPCBs are already in a low quantity which may be entrapped within the metals present in bulk and less exposed to lixiviant in larger size fractions. However, size reduction (≤0.15 mm) may have liberated the entrapped precious metals which increased their participation and the contact surface for lixiviant, hence high concentration of Au and Ag obtained in the samples smaller than 0.15 mm. Other metals like Zn and Co also showed an increase in the metals content as the particle size decreased from ≥0.71 mm to ≤0.15 mm.

Whereas, Cr and Ni content was high in larger particle size fractions (Table 4.1). Overall, the metals content of waste CPCBs showed a large heterogeneity; for example, the amount of Cu varied from ~1.38% to ~29.29% in the waste CPCBs of different size fractions. Our results are in good agreement with those of Sun et al. [135] and Huang et al. [146], who reported that the Cu content in WPCBs can vary from 23% to 46% and 6.75% to 20.36%, respectively. The large variation/heterogeneity in the metal content of waste CPCBs within the same source arose difficulty in material characterization as well as the process design. This study revealed significant quantities of precious metals in the waste CPCBs smaller than 0.15 mm and therefore used in further experiments.

Table 4.1: Chemical composition of different particle size fractions of CPCBs

Metals	**Concentration (% *w/w*)**			
	P1	**P2**	**P3**	**P4**
Cu	29.29	4.72	4.62	1.38
Fe	0.01	0.11	0.21	0.63
Zn	0.006	0.001	0.01	0.07
Cr	0.12	0.02	0.11	0.04
Ni	0.12	0.017	0.09	0.06
Co	0.001	0.001	0.002	0.002
Au	0.002	0.001	0.002	0.008
Ag	0.002	0.006	0.03	0.04

P1 (≥0.71 mm); P2 (0.35 to 0.71 mm); P3 (0.15 to 0.35 mm); P4 (≤0.15 mm)

4.1.2 Effect of digestion time on the metallic content of waste CPCBs (≤0.15 mm)

The effect of aqua regia digestion time (1, 2, and 3 h) on metallic composition of waste CPCBs i.e. shredding dust (P4) and pulverized waste (P5) of particle size ≤0.15 mm was investigated and represented in Fig. 4.1. The results revealed significant differences in the leaching of Cu and Fe during 1, 2, and 3 h aqua regia digestion of waste CPCBs. In both the CPCBs i.e. P4 and P5, the maximum Cu and Fe was leached after 2 h aqua regia digestion (Fig. 4.1). Our results corroborate to the study by Duan et al. [147], who reported optimum Cu recovery after 2 h digestion at 70ºC using bronsted acidic ionic liquid. The maximum amount of Cu in waste CPCBs-P4 and P5 was 2.34±0.19% and 9.8±0.42%, respectively, whereas, the Fe content was 2.22±0.17% (P4) to 4.01±0.22% (P5). Overall, Cu was the dominant metals in

both CPCBs-P4 and P5. The high abundance of Cu can be attributed to lamination of Cu sheets onto the non-conductive substrate making up electric pathways to trace signals [148]. The chemical reaction involved in the dissolution of Cu is given in Eq. (4.1) [18].

$$3Cu + 6HCl + 3HNO_3 \rightarrow 3Cu^{2+} + 6Cl^{-} + 2NO \uparrow + 4H_2O \qquad (4.1)$$

The precious metals like Au and Ag in both P4 and P5 were lower compared to base metals. The precious metal Au and Ag in waste CPCBs was highest when digested for 1 h, thereafter increase in leaching time from 2 to 3 h resulted in decreased metal content. The maximum Au content in P4 and P5 was 0.008±0.001% and 0.03±0.002%, while the Ag content was 0.04±0.004% and 0.14±0.01%, respectively (Table 4.2). The concertation of Au and Ag in the present study is in good agreement with the range reported in the available literature [8, 42, 45, 148-150]. However, among the precious metals, the content of Ag in both CPCBs was higher than Au. This is because Au is mainly used for plating and in ICs whereas, Ag is present in plated pins, ICs, solder junctions with Sn, thin layer on CPCBs and fragments of alloys [135]. The chemical reaction involved in the dissolution of Au using aqua regia can be represented as per Eq. (4.2) – (4.4) [151].

$$2HNO_3 + 6HCl \rightarrow 2NO + 4H_2O + 3Cl_2 \qquad (4.2)$$

$$2Au + 9HCl + 3HNO_3 \rightarrow 2AuCl_3 + 3NOCl + 6H_2O \qquad (4.3)$$

$$3HCl + HNO_3 \rightarrow Cl_2 + NOCl + 2H_2O \qquad (4.4)$$

The abundance of Au and Ag in PCBs can be attributed to their extensive use in order to attain chemical stability and resistivity to oxidation and corrosion. These metals are known to maintain the longevity of electrical contacts when used in the form of a thin film over the PCBs to prevent oxidation [7, 148]. Other metals like Zn, Ni, Co, and Cr also showed variation in their leaching pattern during aqua regia digestion at different time intervals, however, the differences were statistically not significant. The maximum amount of base metals such as Ni, Co, Cr, and Zn in the waste P4 was 0.2±0.042, 0.11±0.02, 0.09±0.01, and 0.07±0.002%, respectively. Whereas, the concentration of base metals in waste P5 was 0.11±0.01 (Ni), 0.008±0.001 (Co), 0.15 ± 0.1 (Cr), and 0.04± 0.02 (Zn), respectively (Table 4.2).

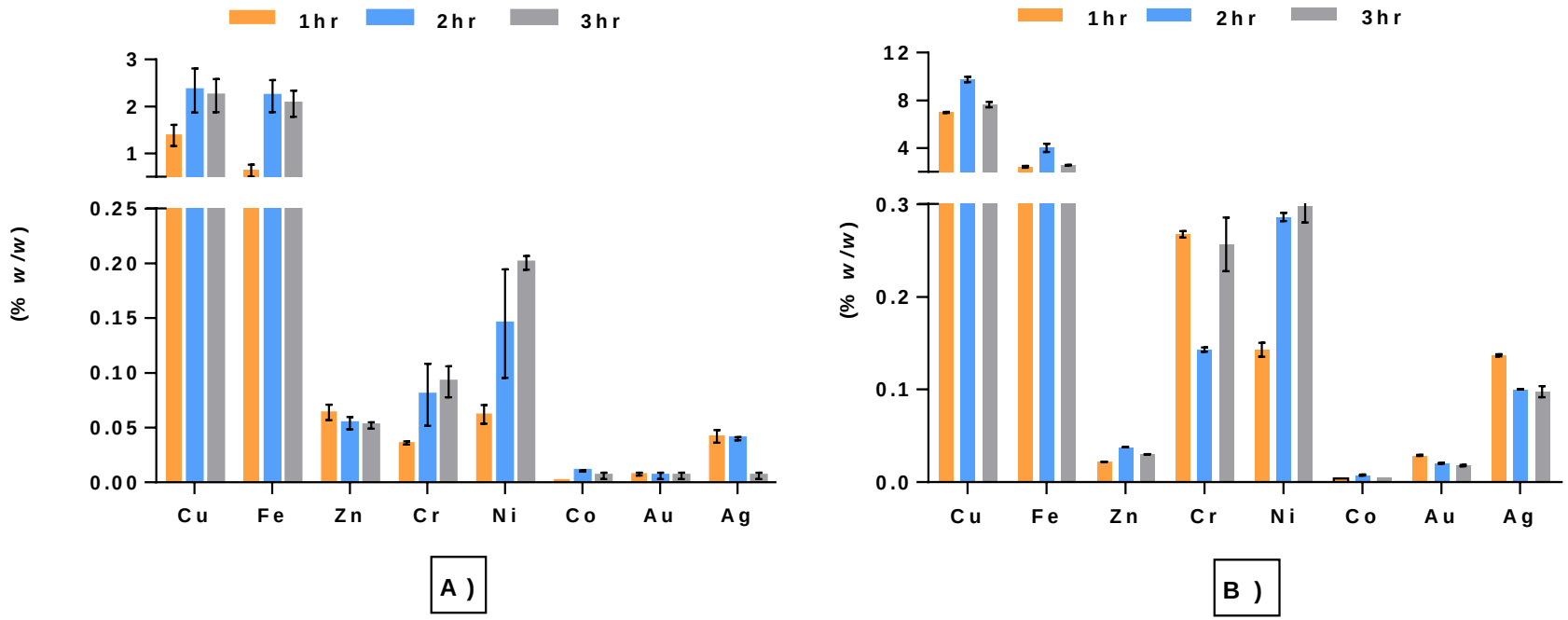


Figure 4.1: Effect of digestion time on metals content of waste CPCBs: (A) metal content of P4 (particle size ≤ 0.15mm); and (B) metal content of P5

The heterogeneity in the overall metallic content of CPCBs was observed when compared to the previous studies and is attributed to the source, type of e-waste used and industrial pre-treatment processes like segregation, manual dismantling, shredding and pulverization [10, 18, 31, 120, 135]. Moreover, it was observed that metallic content of waste P5 was higher than P4. The reason behind the low concentration of P4 is that it was the dust generated during recycling through physio-mechanical processes such as shredding at the industry, whereas the P5 used in present study was a virgin waste. The waste P4 and P5 were further used in the bioleaching experiments and metal content given in Table 4.2 was used to calculate the percentage (%) bioleaching in this study. The high abundance of precious and base metals along with their high weight percentage in the waste CPCBs and high obsolescence rate makes these a prime target to exploit for prospective profitable beneficiation.

Table 4.2: The metal content of waste CPCBs P4 and P5 using aqua regia

Metals	**Concentration (%)**	
	P4	**P5**
Cu	2.34 ± 0.19	9.8 ± 0.42
Fe	2.22 ± 0.17	4.01 ± 0.22
Ni	0.20± 0.04	0.3 ± 0.01
Co	0.11 ± 0.02	0.008 ± 0.001
Cr	0.09 ± 0.01	0.3 ± 0.09
Zn	0.07 ± 0.002	0.04± 0.02
Ag	0.04 ± 0.004	0.14 ± 0.01
Au	0.008 ± 0.001	0.03 ± 0.002

4.2 Characterization of site sampled in the study

4.2.1 Physico-chemical properties of experimental soil

A composite soil sample from four different locations of an abandoned gold mine was tested for physco-chemical properties. A composite sample was obtained by pooling the soil samples from all four locations. The results of the physical and chemical characterization of abandoned gold mine soil were presented in Table 4.3 and 4.4. It was observed that Au content in the mine was low which emphasized the reason for abandoning the mine. The amount of Au

present in the mine sample was 0.02 mg/g whereas, Au in the control soil taken from outside the mine was 0.01 mg/g. The presence of Au in the control samples may be due to the mixing of mine soil with that of control soil through human or other activities. In the mine soil, Fe (38 mg/g) was found in abundance. Our results were in good agreement with those reported from the Homestake gold mine at South Dakota, USA; i.e. gold deposits are typically associated with Fe formation [152, 153]. Other metals like Zn (0.4 mg/g), Cr (0.1 mg/g), Co (0.03 mg/g) and Cu (0.1 mg/g) were also present in significant quantities as shown in Table 4.3. The water samples collected from inside and outside the mine were also tested for the presence of metal ions. The pH, electrical conductivity and organic carbon content of the gold mine soil were 7.2, 0.02 $dscm^{-1}$ and 0.43%, respectively. The soil was high in available potassium (K-390.8 Kg/Ha) and nitrogen (N-163 Kg/Ha), while low to medium in phosphorus (P-44.8 Kg/Ha) availability (Table 4.4).

Table 4.3: Elemental composition of the soil procured from abandoned gold mine

Metals	**Goldmine soil (mg/g)**	
	Inside*	**Outside****
Au	0.02 ±0.06	0.01 ±0.01
Cu	0.1 ±0.06	0.03 ±0.01
Fe	38 ±0.6	3.6 ±0.2
Zn	0.4 ±0.01	0.08 ±0.06
Co	0.03 ±0.003	0.02 ±0.01
Cr	0.1 ±0.002	0.1 ±0.01
Ni	0.04 ±0.001	0.03 ±0.01
Ag	0.03 ±0.01	0.01 ±0.02
Al	20 ±1	31 ±1

*10-15 meters inside the mine; **10-15 meters outside the mine

Table 4.4: Physical characterization of the soil procured from the abandoned goldmine

pH	**EC ($dS\ m^{-1}$)**	**Organic Carbon (%)**	**Available Macro Nutrients (Kg/Ha)**		
			N	**P**	**K**
7.2	0.02	0.43	163.0	44.8	390.8

4.3 Isolation of bacterial strains for bioleaching of metals (Cu, Au, and Ag) from waste CPCBs

4.3.1 Mining of cultivable bacterial population from the soil sample of the abandoned gold mine

Bacterial isolation was achieved using enrichment culture technique for a period of 30 days. The results revealed the presence of eight different colony morphotypes on nutrient agar plates (Fig. 4. 2). All the eight morphological different colonies were subcultured to obtain a pure culture. All the eight bacterial isolates were characterized by their Grams nature and cell type. The colony morphology, cell type and Grams nature of the bacterial isolates are presented in Table 4.5. The un-inoculated control containing medium didn't show any microbial growth.

4.3.2 Mining of cultivable bacterial population from the industrial e-waste refuse

The industrial e-waste refuse was purposely selected in order to isolate e-waste habituating bacteria which can tolerate higher pulp density. Only a single bacterial strain was isolated after sequential enrichment from the flask containing 100 g/L pulp density of e-waste. The bacterial colonies were entire, flat, mucoid and circular with yellowish in colour (Table 4.5). The un-inoculated control containing medium along with sterilized e-waste did not show any microbial growth.

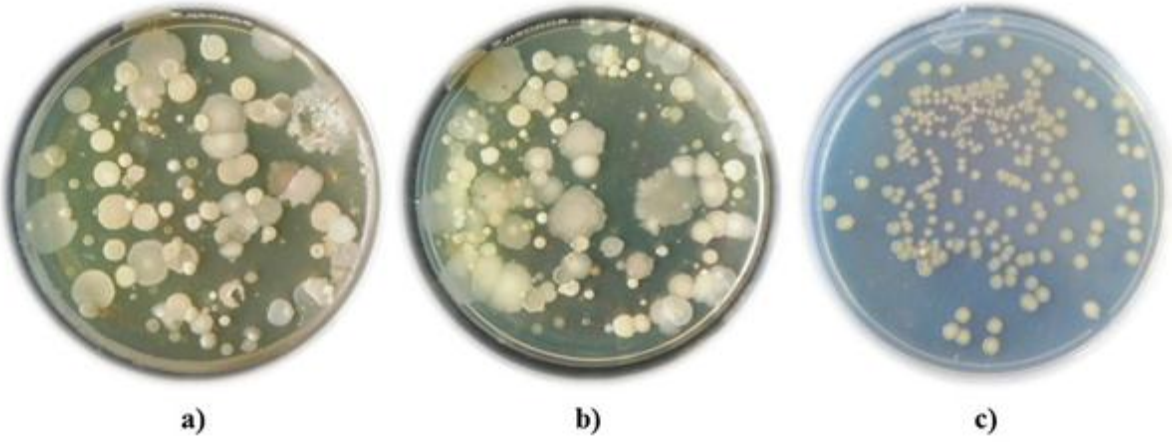


Figure 4.2: NA plates showing isolated bacterial strains from **a & b)** the abandoned goldmine soil and **c)** e-waste refuse of industry on nutrient agar plates

Table 4.5: Morphological characterization of the isolated bacterial strains

Isolate	Colony morphology	Gram's reaction	Cell shape
SAG1	Circular, entire, flat and white	+ve	Rods
SAG2	Irregular, undulate, flat, cream	+ve	Rods
SAG3	Circular, entire, flat, cream	+ve	Rods
SAG4	Circular, entire, umbonate, cream	+ve	Rods
SAG5	Circular, entire, raised, white	+ve	Rods
SAG6	Circular, entire, raised, cream	+ve	Rods
SAG7	Circular, entire, flat, yellow	+ve	Rods
SAG8	Irregular, lobate, flat, cream	+ve	Rods
SAE1	Circular, entire, flat, pale yellow	-ve	Rods

4.4 Identification and characterization of the isolated bacterial strains

4.4.1 Molecular identification using 16S rRNA gene amplification

To identify the isolated bacterial strains, good quality DNA of each bacterial strain was isolated. The concentration of DNA varied from 50-100 ng; estimated spectrophotometrically (Nanodrop, Thermo Fisher Scientific, Inc., Massachusetts, USA) by A260 and A280 measurements. After DNA extraction, primers 27 F and 1492 R were successfully used to amplify 16S rRNA gene from each of bacterial strain. As a result of amplification, an amplicon/ amplified product of expected size ~1500 bp (approximately) was obtained (Fig. 4.3). The amplified product was run on 1% agarose gel viewed under UV transilluminator and then sent for sequencing to Xcelris Labs Limited, Ahmedabad, India. The obtained sequences were checked for their quality.

4.4.2 Phylogenetic analysis of the bacterial isolates

The 16S rRNA sequences of each bacterial strain were analyzed using BLASTn search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of NCBI. The results revealed that the goldmine bacterial strains were the members of three different genera *Bacillus, Lysinibacillus*, and *Chryseomicrobium*. Whereas, the bacterial strain isolated from the e-waste recycling sites belongs to genus *Pseudomonas*. Based on the highest homology with those of available 16S rRNA sequences, bacterial strains isolated from goldmine were identified as *Bacillus*

megaterium SAG1; *Lysinibacillus sphaericus* SAG2; *Bacillus* sp. SAG3; *Bacillus amyloliquefaciens* SAG4; *Bacillus* sp. SAG5; *Brevibacterium frigoritolerans* SAG6; *Chryseomicrobium amylolyticum* SAG7 and *Bacillus safensis* SAG8, respectively. The bacterial strain SAE1 isolated from industrial e-waste refuse was identified as *Pseudomonas balearica* SAE1. The 16S rRNA gene sequences have been submitted to the GenBank database and are available under the accession no: KU163234-KU163241 for SAG1 to SAG8 and KU053282 for SAE1 as mentioned in Table 4.6. To trace out the evolutionary relationship of bacterial strains isolated from the goldmine and the e-waste recycling site, phylogenetic analysis was done using the neighbor-joining method with 1,000 bootstrap sampling. The phylogenetic tree of goldmine bacterial strains was shown in Fig. 4.4. The results showed the evolution of goldmine bacterial isolates from one phylogenetic group the Firmicutes. The prevalent occurrence of *Bacillus* genera from gold mine as well as other soil samples has been supported by many researchers. However, among the genus *Bacillus*, bioleaching of metals from e-waste has only been reported for *B. megaterium* [40, 125].

The bacterial isolate SAE1 of e-waste recycling site belongs to phylogenetic group Proteobacteria. The phylogenetic tree was shown in Fig. 4.5. Different *Pseudomonas* species, for example, *P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. chlororaphis* have been reported to solubilize metals from e-waste [31, 35, 42, 45]. In addition, species of *Pseudomonas* have been reported to solubilize RREs and inorganic phosphate [136, 154].

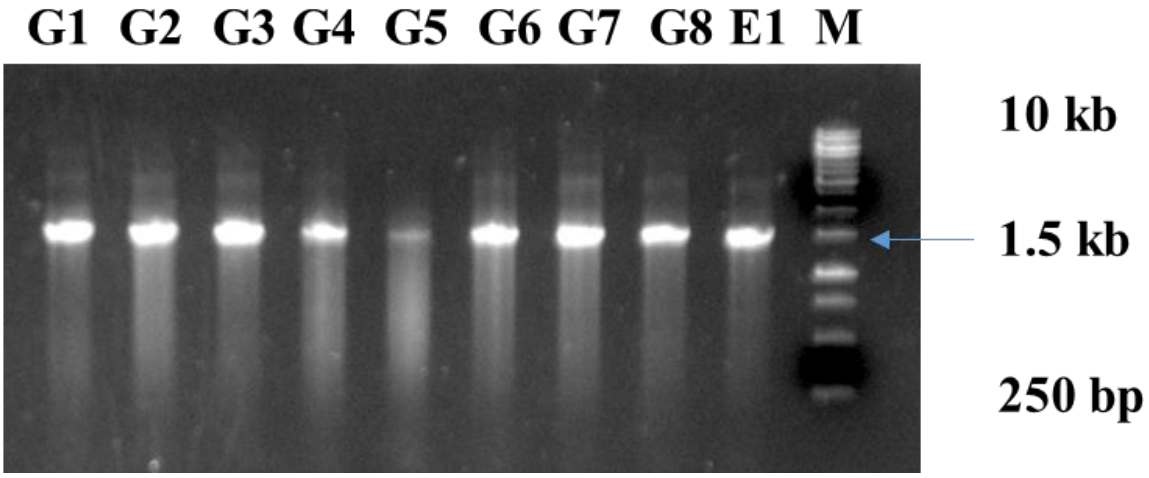


Figure 4.3: Gel image showing the 16S rRNA gene amplification of the bacterial strains

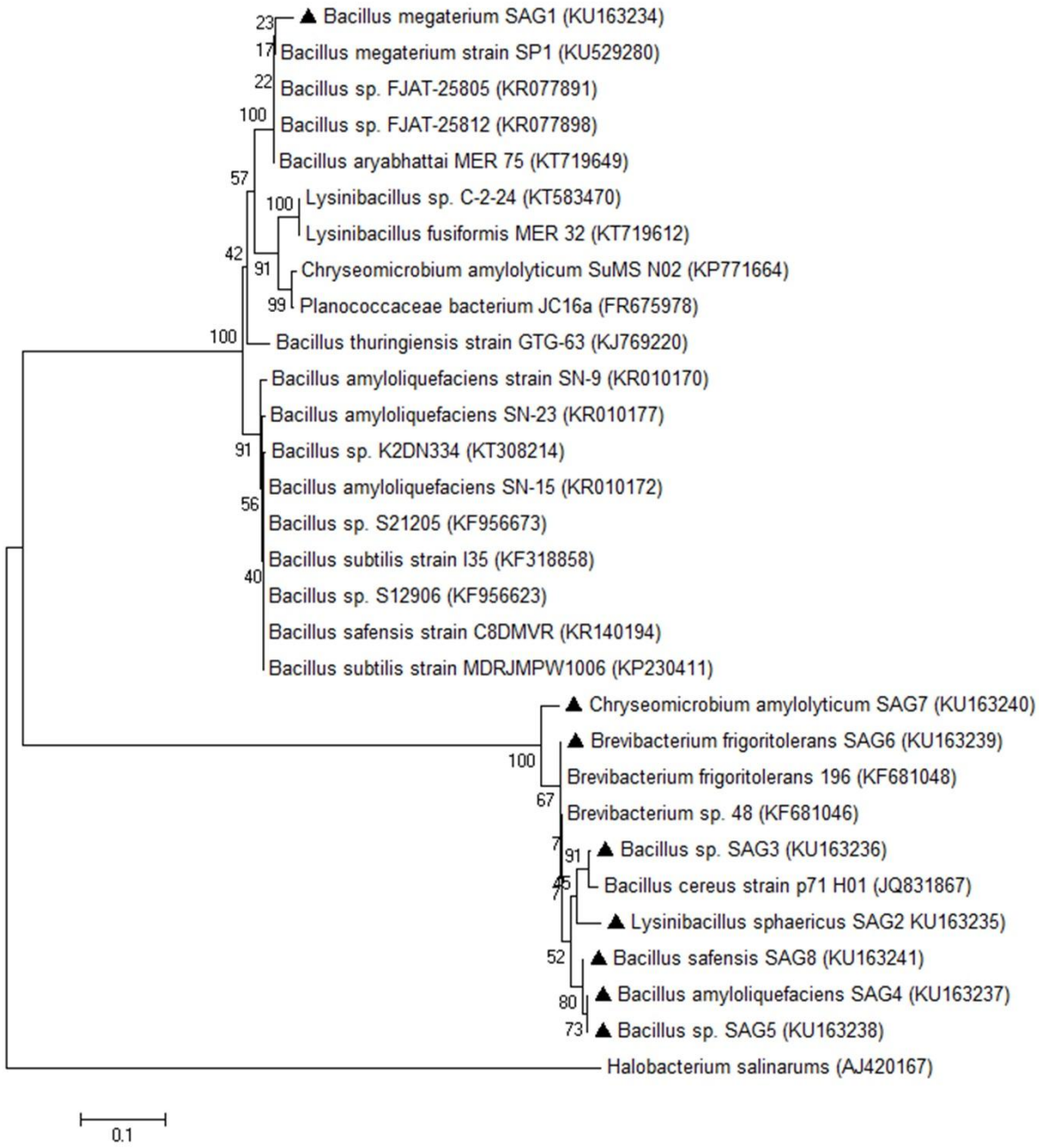


Figure 4.4: The phylogenetic tree of the goldmine bacterial strains. The Neighbour-joining tree was constructed base on the partial 16S rRNA gene sequences retrieved in the study and from the GenBank data base using Mega v. 6. The *scale bar* represents 0.1 substitutions per nucleotide position. Numbers at the node are the bootstrap values (%). *Halobacterium salinarums* (AJ420167) was selected as outgroup. Triangle represents goldmine isolates. Figure in the parenthesis ( ) represents the accession number of the bacterial isolates.

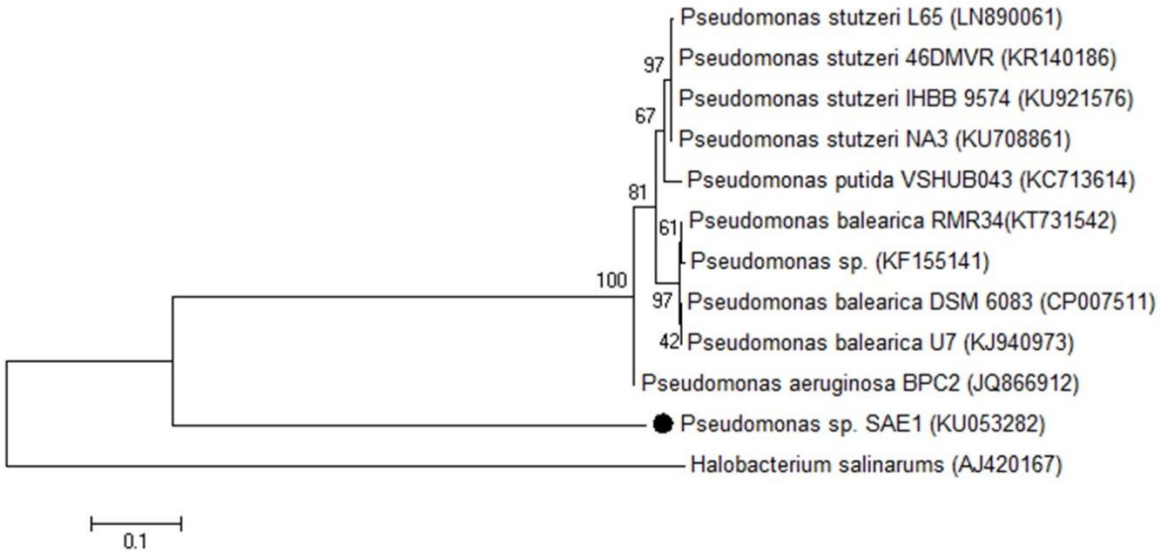


Figure 4.5: The phylogenetic tree of the bacterial isolate SAE1 isolated from e-waste recycling site. The Neighbour-joining tree was constructed using partial 16S rRNA sequences retrieved in this study and sequences of representative *Pseudomonads* isolates using Mega v. 6. The isolated bacterial strain SAE1 was represented by a black circle. Bootstrap values (1,000 replicates) were represented at the branching nodes. The scale bar represents, 0.1 substitutions per nucleotide position. *Halobacterium salinarums* was selected as out-group. Figure in the parenthesis ( ) represents the accession number of the bacterial isolates.

4.5 Screening of isolated bacterial strains for bioleaching of metals (Cu, Au, and Ag) from waste CPCBs-P4

The use of bacterial isolates native to mine or contaminated sites has been the most practical approach for establishing a bioleaching process because of their natural ability to tolerate higher concentrations of toxicants or pollutants [110]. Romo et al. [155] showed 70% of copper (Cu) removal from low-grade ore using a native consortium of *A. ferrooxidans* and *A. thiooxidans*. Cheng et al. [156] reported the presence of *Bacillus* sp., *Sporosarcina* sp. and *Pseudomonas* sp. in a bioleaching system and were capable to recover metals effectively from the Pb/Zn smelting slag. Therefore, *C. violaceum* along with nine bacteria (*B. megaterium*, *L. sphaericus*, *Bacillus* sp. SAG3, *B. amyloliquefaciens*, *Bacillus* sp. SAG5, *B. frigoritolerans*,

C. amylolyticum, B. safensis and *P. balearica*) were screened for their metal bioleaching potential. It was observed that all the bacterial isolates had the capability to mobilize metals from ground CPCBs (Fig. 4.6). *C. violaceum* exhibited maximum bioleach abilities of Au (58.4%) from the CPCBs followed by *P. balearica* (56.4%), *B. megaterium* SAE1 (55.26%), *L. sphaericus* (46.9%) and *Bacillus* sp. SAG3 (45.3%), respectively (Fig. 4.6). The amount of mobilized Cu was higher in case of *C. violaceum* (71.6%) followed by *P. balearica* (64.8) and *B. megaterium* (62.6%). The Ag leaching was higher in case of *P. balearica* (26.4%). The metal leaching efficiency of *C. violaceum* and *B. megaterium* is well known and documented [28, 31, 33, 35, 129]. However, the use of *P. balearica, Bacillus* sp. SAG3 and *L. sphaericus* in Au leaching is first reported in this study. It was observed that all the bacterial strains also exhibited Ag mobilization within a range of 2.89% to 26.4% (Fig. 4.6). Control flasks showed 18.22% mobilization of Cu under non-growth conditions after 7 days of incubation period. No leaching was observed in case of gold and silver under non-growth conditions. Based on Cu, Au and Ag mobilization efficiency, four bacterial strains viz. *P. balearica, B. megaterium*, *Bacillus* sp. SAG3 and *L. sphaericus* along with reference strain *C. violaceum* were employed for further optimization to enhance metals recovery from CPCBs.

4.6 Analysis of the cyanide producing potential of the potent bacterial strains

Since history, cyanide has been used to leach Au from ores and forms water-soluble complex [Au $(CN)^-_2$] with Au. Cyanogenic microorganisms like *C. violaceum*, *Pseudomonas* sp. and *Bacillus* sp. are known to produce cyanide as secondary metabolite [117], and solubilize metals from e-waste by forming water-soluble cyanide complexes [33]. These microorganisms produce biogenic cyanide at the stationary phase of their growth [31]. The use of biogenic cyanide has an advantage over chemical cyanide i.e. the bacteria possess necessary enzymatic machinery to detoxify the excess amount of free cyanide. Thus, the efficient bacterial strains were tested for their cyanide leaching potential. All the five bacterial strains showed a positive cyanide test which revealed that the biogenic cyanide is responsible for metal bioleaching from the e-waste. As reported by Natarajan and Ting [35], the cyanide production by *C. violaceum* was approximately 20 mg/L.

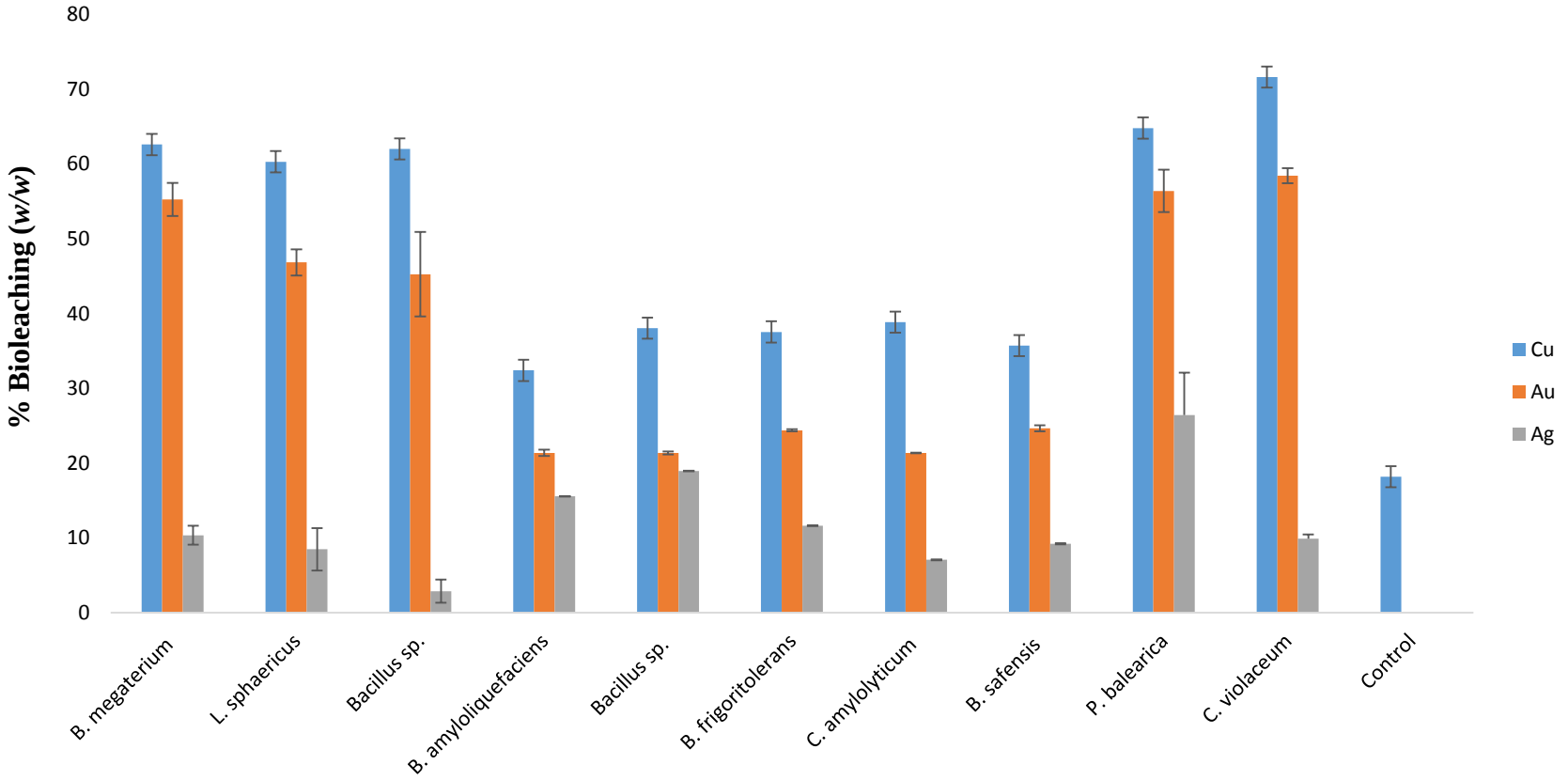


Figure 4.6: Percent bioleaching of Cu, Au and Ag, from waste CPCBs by isolated bacterial strains. The presented data is the average of those obtained from duplicate experiments. The results were significant at a $p < 0.05$ based on two-way ANOVA analysis. Control flask contained medium only.

Table 4.6: Identification and phylogenetic characterization of isolated bacterial strains from abandoned gold mine

Isolate (accession number)	**Phylogenetic Characterization based on 16S rRNA analysis**		
	Phylogenetic group (Division; Class)	**The blast hit in GenBank database (accession number)**	**Similarity (%)**
SAG1 (KU163234)	Firmicutes; Bacilli	*Bacillus megaterium* DSM319(NC_014103)	97
SAG2 (KU163235)	Firmicutes; Bacilli	*Lysinibacillus sphaericus strain GBPI_CDB145* (KR259214)	99
SAG3 (KU163236)	Firmicutes; Bacilli	*Bacillus* sp. D17-2 (KF479652)	97
SAG4 (KU163237)	Firmicutes; Bacilli	*Bacillus amyloliquefaciens* strain SN-23 (KR010177)	99
SAG5 (KU163238)	Firmicutes; Bacilli	*Bacillus* sp. S21205 (KF956673)	100
SAG6 (KU163239)	Firmicutes; Bacilli	*Brevibacterium frigoritolerans* strain 196 (KF681048)	100
SAG7 (KU163240)	Firmicutes; Bacilli	*Chryseomicrobium amylolyticum* strain SuMS_N02 (KP771664)	99
SAG8 (KU163241)	Firmicutes; Bacilli	*Bacillus safensis* strain C8DMVR (KR140194)	99
SAE1 (KU053282)	Proteobacteria; Gammaproteobacteria	*Pseudomonas balearica* DSM 6083 (U26418)	100

4.7 E-waste toxicity assessment and dose-response analysis of potent bacterial strains

4.7.1 Toxicity Assessment

E-waste contains a variety of toxic components including toxic metals and other brominated and chlorinated compounds; which inhibit the growth of microorganisms. The toxicity assessment studies are often used in toxicology to determine the quantitative estimates of maximum treatment concentration (EC_{100}) and 50% effective concentration (EC_{50}) of a chemical/pollutant allowable for metal bioleaching. Therefore, bacterial cells of *B. megaterium*, *L. sphaericus*, *Bacillus* sp., and *P. balearica* along with *C. violaceum* were

exposed to 10, 50, 100, 150, 200, 300, 400, and 500 g/L of e-waste pulp density. The growth in terms of CFU/mL was measured for each pulp density along with control. From the results, it was observed that *P. balearica* showed higher tolerance to e-waste toxicity i.e. 72% inhibition at 500 g/L of e-waste pulp density, among all the bacterial strains tested (Fig.4.7). This may be attributed to its habitat "e-waste recycling site", which might have developed metabolic machinery to resist the effect of toxic e-waste. However other bacterial strains such as *B. megaterium*, *L. sphaericus*, and *Bacillus* sp. and *C. violaceum* showed complete inhibition (100%) at a range between 200 to 300 g/L of e-waste pulp density (Fig.4.7). Among the mine isolates *Bacillus* sp. SAG3 showed higher tolerance to e-waste toxicity followed by *B. megaterium* SAG1 and *L. sphaericus* SAG2, respectively. The toxicity of e-waste upon bacterial strains is due to its toxic nature i.e. presence of toxic heavy metals and pollutants such as PBDEs [6, 29].

4.7.2 Dose-response analysis

To find out e-waste toxicity on bacterial strains, a quantitative dose-response curve was adopted for comparison (Fig. 4.8). The dose-response curve contains: (1) no-effect range (i.e. $C < EC_0$), (2) the range of increasing effect with increasing dose (i.e. $EC_0 < C < EC_{100}$), and (3) maximum effect range (i.e. $C \geq EC_0$) [37]. The EC_{50} and EC_{20} (Table 4.7) are of great significance since this directly determines the feasibility of bioleaching. The EC_{50} values for *P. balearica* SAE1, *Bacillus* sp. SAG3, *B. megaterium* SAG1, and *L. sphaericus* SAG2 were Log 2.5 (325.7 g/L), 2.1 (128.9 g/L), 1.9 (98.7 g/L), and 1.9 (90.8 g/L), respectively. Whereas, EC_{50} for *C. violaceum* was Log 1.9 (83.70 g/L). The R^2 values for dose-response curves of *P. balearica* SAE1, *Bacillus* sp. SAG3, *B. megaterium* SAG1, *L. sphaericus* SAG2 and *C. violaceum* were 0.97, 0.95, 0.92, 0.88, and 0.91, respectively. The 95% confidential intervals for these bacterial strains were given in Table 4.7. The EC_{50} values may vary with the type of e-waste and bacterial strain used. Therefore, EC_{20} values were calculated using GraphPad QuickCalcs online tool (http://graphpad.com/quickcalcs/ECanything1/). The EC_{20} for *P. balearica* SAE1, *Bacillus* sp. SAG3, *B. megaterium* SAG1, *L. sphaericus* SAG2, and *C. violaceum* were 149.6, 74.6, 49.5, 46.1, and 45.7 g/L CPCBs of the culture medium. This clearly indicates the toxicity of e-waste, the technological feasibility and viable operation range for bioleaching of metals from e-waste using native isolates.

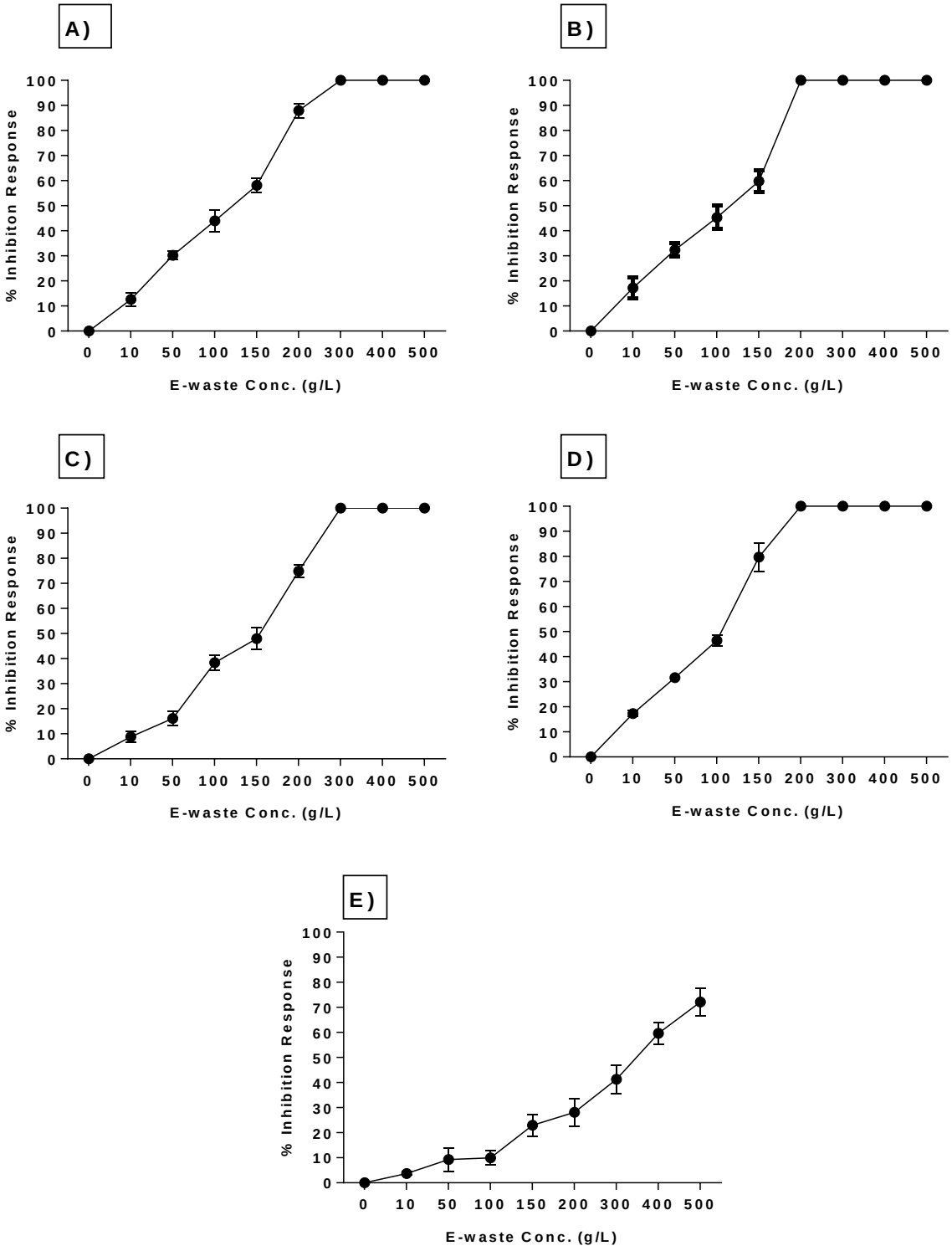


Figure 4.7: Percent inhibition response of different bacterial strains against e-waste toxicity A) *B. megaterium*, B) *L. sphaericus*, C) *Bacillus* sp., D) *C. violaceum*, and E) *P. balearica*

Table 4.7: Effective concentrations of e-waste toxicity for bacterial strains predicted from GraphPad Prism 6

Bacterial Strain	**EC_{50}**	**EC_{20}**	**95% confidential intervals**
	(g/L PCBs of culture medium)		
B. megaterium SAG1	98.7	49.5	81.9-118.9
L. sphaericus SAG2	90.8	46.1	71.3-115.8
Bacillus sp. SAG3	128.9	74.6	113.9-145.9
P. balearica SAE1	325.7	149.6	315.1-346.7
C. violaceum	83.7	45.7	68.7-102.0

4.8 Optimization of the process for maximum recovery of metals (Cu, Au, and Ag) from waste CPCBs using one-factor-at-a-time (OFAT) approach

4.8.1 Effect of Pulp density

The variation in bioleaching pattern of all five bacterial strains was observed when applied in presence of 10, 50, and 100 g/L e-waste pulp density (Fig. 4.9). The results revealed higher metal mobilization at 10 g/L e-waste pulp density which may correspond to the higher bacterial growth (Fig. 4.9d), thereby higher cyanide production. The percent bioleaching of Cu by different bacteria at 10 g/L pulp density was higher for *C. violaceum* (71.63%) followed by *P. balearica* (64.8%), *B. megaterium* (62.6%), *L. sphaericus* (60.3%), and *Bacillus* sp. (62.01%), respectively (Fig. 4.9a). The percent bioleaching of Au by different bacteria at 10 g/L pulp density was of the order: *C. violaceum* (58.4%)> *B. megaterium* (57.7%) >*P. balearica* (56.4%)> *L. sphaericus* (55.3%) > *Bacillus sp.* (53.8%) (Fig. 4.9b). However, the percent Ag mobilization was higher for *P. balearica* (26.5%) followed by *L. sphaericus* (10.5%), *C. violaceum* (9.91%), *B. megaterium* (6.5%) and *Bacillus* sp. (3.9%) (Fig 4.9c). Though the metal bioleaching was maximum for bacterial strain *C. violaceum* however, bacterial growth was higher in case of *P. balearica*. The higher metal bioleaching by *C. violaceum* (20 mg/L) may be attributed to its high cyanide producing ability which is low in case of *Pseudomonas* sp. (10-13 mg/L) [35].

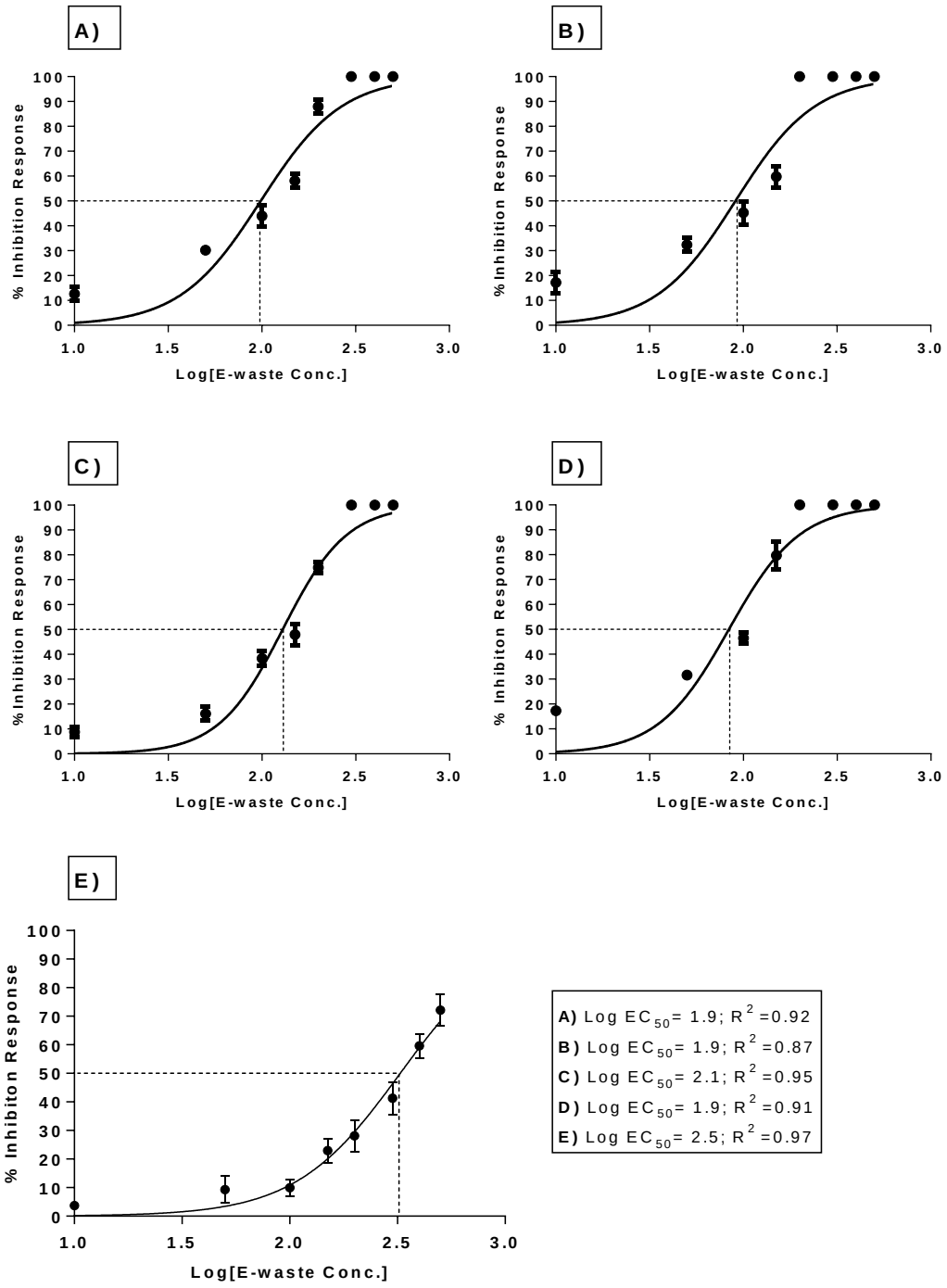


Figure 4.8: Dose-response curves of e-waste toxicity upon bacterial strains A) *B. megaterium*, B) *L. sphaericus*, C) *Bacillus* sp., D) *C. violaceum*, and E) *P. balearica*. The curve were generated following the equation "*log (agonist) versus normalized response--variable slope*" of GraphPad Prism 6

Further, it was observed that increase in e-waste pulp density from 10 to 50 and 100 g/L led to a significant decrease in percent Cu, Au, and Ag bioleaching. This is because, higher e-waste pulp densities increase the toxicity stress of both metallic and non-metallic components thus reduces bacterial growth (Fig. 4.9d) which, results in poor cyanide production [18], and ultimately results in low metal bioleaching. The percentage bioleaching of Cu, Au and Ag at 50 g/L pulp density was 37.2%, 28% and 2.2% for *C. violaceum*; 29.4%, 17.8% and 12.5% for *P. balearica*; 18.1%, 9.1% and 1.96% for *B. megaterium*; 8.7%, 6.96%, and 2.6 for *L. sphaericus* and 8.5%, 7.5% and 1.7%, for *Bacillus* sp., respectively. At pulp density 100 g/L, the percentage of Cu, Au and Ag recovery was 12.6, 6.6 and 1.3% for *C. violaceum*; 8.3, 5.1 and 1.8% for *P. balearica*; 5.1, 4.5 and 1.7% for *B. megaterium*; 3.2, 3.7 and 1% for *Bacillus* sp. and 2.8, 3.1 and 1.3% for *L. sphaericus*, respectively.

Among all the bacterial strains analyzed, the highest percentage of Cu and Au was bioleached by *C. violaceum* whereas, the percentage of Ag bioleached was highest by *P. balearica*. During bioleaching, the percentage of Ag bioleached was significantly low compared to Cu and Au. Our results are in well agreement with Pradhan and Kumar [31] and Mara et al. [45], they reported optimum metals mobilization at 10 g/L pulp density. Natarajan and Ting [8] investigated the effect of varying concentrations of e-waste on Au and Cu mobilization by *C. violaceum*. They achieved optimum mobilization of Cu and Au at pulp density 5 g/L. Though the metals bioleaching efficiency by cyanogenic bacteria is high at low pulp density 2-5 g/L [8, 125], the process may not be economically viable. Therefore, higher pulp density (10 g/L) may be considered for metals extraction as reported in other studies using PCBs as a source [31, 119].

4.8.2 Effect of Temperature

The effect of different temperatures i.e. 25, 30, 35, 40°C on metal bioleaching by different bacterial strains was investigated. The results revealed a significant difference in bioleaching (%) pattern of Cu, Au, and Ag by different bacterial strains. From the Fig. 4.10, it was observed that as the temperature increased from 25-30°C metal bioleaching also increased; a further increase in temperature from 30-40°C has led to decrease in metal bioleaching by all the bacterial isolates.

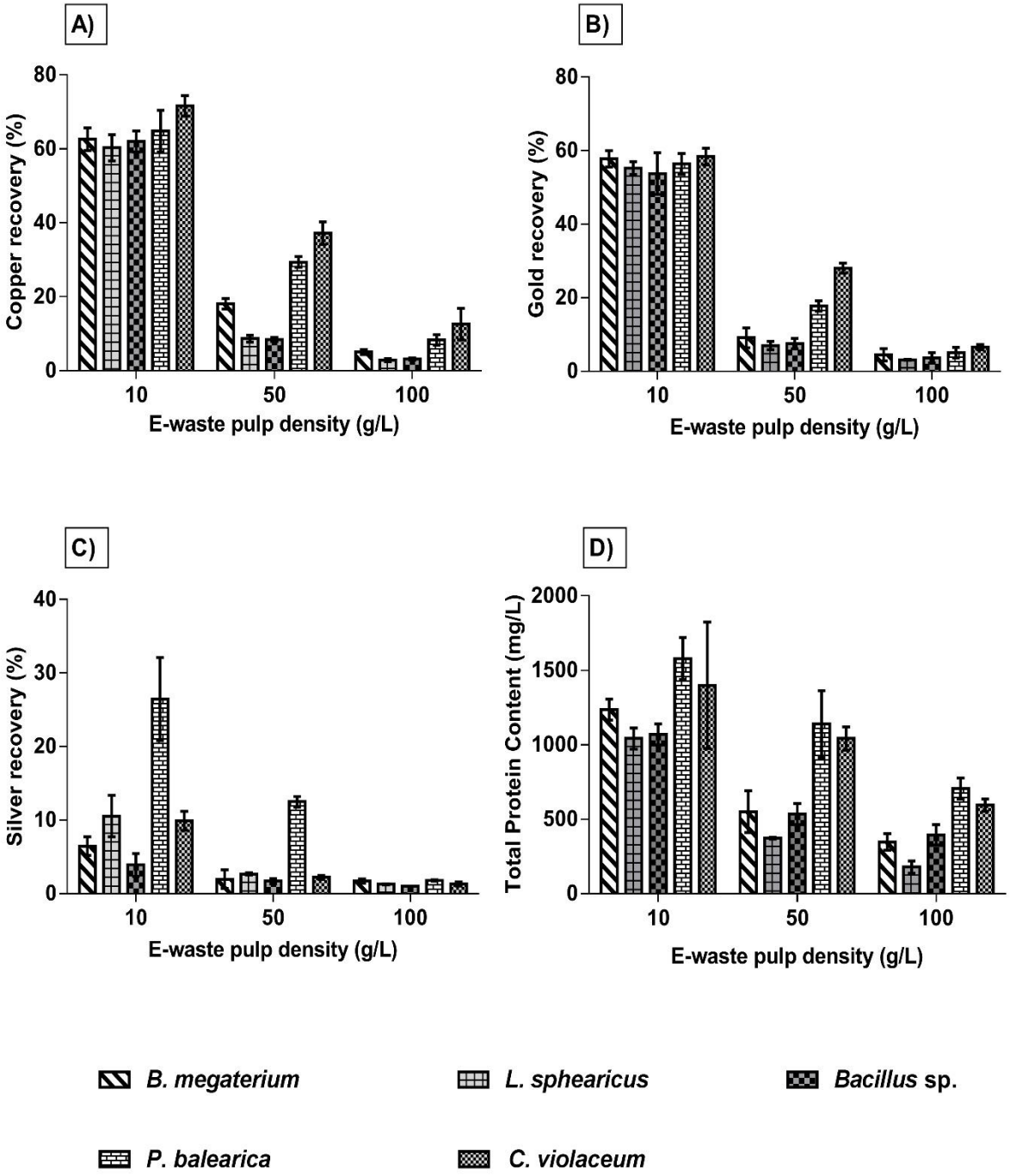


Figure 4.9: Effect of pulp density on percent (%) bioleaching of metals (Cu, Au, and Ag) from waste CPCBs by different bacterial strains: a) % Cu bioleaching; b) % Au bioleaching; c) % Ag bioleaching; Total Protein Content (mg/L). The presented data in this figure is the average of those obtained from duplicate experiments.

However, the differences in metal bioleaching at 30 and 35°C was statistically not significant. This may be because the maximum cyanide production also takes place at a temperature range of 30-35°C [157]. The maximum metals (Cu, Au, and Ag) were bioleached at 30°C whereas, the minimum was at 25 and 40°C, respectively. Among all the bacterial isolates, the amount of Cu bioleached was in order: *C. violaceum* (71.8%) > *P. balearica* (67.2%) >*B. megaterium* (66.1%) > *L. sphaericus* (64.9%) > *Bacillus sp.* (61.8%) (Fig. 4.10a). The Au bioleaching at temperature 30°C was 58.9%, 57.3%, 56.2%, 56%, and 51% for *C. violaceum*, *B. megaterium*, *P. balearica*, *L. sphaericus* and *Bacillus sp.* (Fig. 4.10b); however, percent bioleaching of Ag was higher for *P. balearica* (26.6%) followed by *L. sphaericus* (10.53%), *C. violaceum* (7.31%), *B. megaterium* (6.7%), and *Bacillus sp.* (3.8%), respectively (Fig. 4.10c). Among all the bacterial isolates, *C. violaceum* showed higher bioleaching of Cu and Au at 30°C whereas, Ag bioleaching was higher in case of *P. balearica*. Our results are in accordance with other bioleaching studies that reported optimum bioleaching of metals from e-waste at 30°C using cyanogenic microorganisms [8, 125, 129].

At temperature 35°C, the percent bioleaching of Cu, Au and Ag was 64.2%, 42.6% and 3.7% for *C. violaceum*; 65.8%, 42.9% and 22.5% for *P. balearica*; 64.9%, 44.5% and 3.3% for *B. megaterium*; 58.04%, 48.3%, and 6.1% for *L. sphaericus* and 58.04%, 50.3% and 2.3%, for *Bacillus* sp., respectively. At temperature 40°C, the percent bioleaching of Cu, Au and Ag was 48%, 19.9% and 1.6% for *C. violaceum*; 44.5%, 19.8% and 15.2% for *P. balearica*; 44.8%, 20.4% and 1% for *B. megaterium*; 47.6%, 19.4% and 2.8% for *L. sphaericus* and 41.7%, 16.4%, and 1% for *Bacillus* sp., respectively. At temperature 25°C, the percent bioleaching of Cu, Au and Ag was 60.7%, 42% and 2.6% for *C. violaceum*; 53.2%, 41.2% and 12.5% for *P. balearica*; 49.9%, 39.9% and 3% for *B. megaterium*; 51.4%, 40.1%, and 2.9% for *L. sphaericus* and 50.5%, 36.7% and 2.4%, for *Bacillus* sp., respectively.

The percent metal mobilization values dominates by Cu> Au> Ag. This may be due to the fact that in the e-waste material value dominates by Cu which might have consumed all the lixiviant. The presence of high amount of Cu interferes in Au and Ag cyanidation process and hence, low Au and Ag recovery [8, 24]. Other than metals ions, bacterial growth was estimated in term of total protein content. The results of the study revealed that the metals bioleaching

was significantly affected by temperature variations. However, the difference in the growth pattern of bacterial strains at varying temperature range (25-40°C) was statistically not significant. This corresponds to the fact that metals bioleaching strongly depends upon the amount of lixiviant production and is produced by bacterial strains at a specific temperature range (30-35°C). This is the reason that at a temperature range from 30-35°C bacterial strains has attained maximum bioleaching metals from e-waste. The survival of bacterial strains at a wide temperature range makes them a suitable candidate for industrial scale applications.

4.8.3 Effect of glycine concentration

Since hydrocyanic acid is produced directly from glycine [32], the optimum concentration was determined for different bacterial species in the presence of pulp density 10 g/L. The microbial growth and metal bioleaching ability of different bacterial isolates at varying glycine concentration are presented in Fig. 4.11. It was observed that increase in glycine concentration from 2.5 to 5.0 g/L have increased the metals bioleaching except *for C. violaceum* and *P. balearica* where metal bioleaching increased up to 7.5 g/L of glycine concentration. However, in both the cases i.e. *C. violaceum* and *P. balearica,* the metal bioleaching efficiency was statistically not significant at a glycine concentration of 5.0 to 7.5 g/L, therefore 5.0 g/L is considered as optimum. *B. megaterium* showed highest metal bioleaching at concentration 5.0 g/L (Cu-65.7%, Au-56.9%, Ag-6.7%) followed by 7.5 g/L (Cu-64.6%, Au-38.2%, Ag-4.7%), 10.0 g/L (Cu-55.9%, Au-36.8%, Ag-1.5%) and 2.5 g/L (Cu-52.5%, Au-40%, Ag-4.01%), respectively.

In case of *L. sphaericus* maximum bioleaching was recorded at 5.0 g/L (Cu-64.3%, Au-55.8%, Ag-10.5%) followed by 7.5 g/L (Cu-58.5%, Au-38.15%, Ag-7.4%), 2.5 g/L (Cu-49.3%, Au-43.1%, Ag-5.9%) and 10.0 g/L (Cu-46.2%, Au-33.8%, Ag-1.3%), respectively. Similar trend was observed with *Bacillus* sp. having maximum metals bioleaching at 5.0 g/L (Cu-63.5%, Au-52.5%, Ag-4.2%), respectively. *C. violaceum* was able to bioleach maximum metals at 7.5g/L (Cu-76.9%, Au-62.2%, Ag-7.5%) followed by 5g/L (Cu-74.1%, Au-60.9%, Ag-6.8%), 2.5g/L (Cu-66.2%, Au-47.2%, Ag-5.1%) and 10g/L (Cu-71.5%, Au-33.8%, Ag-2.5%).

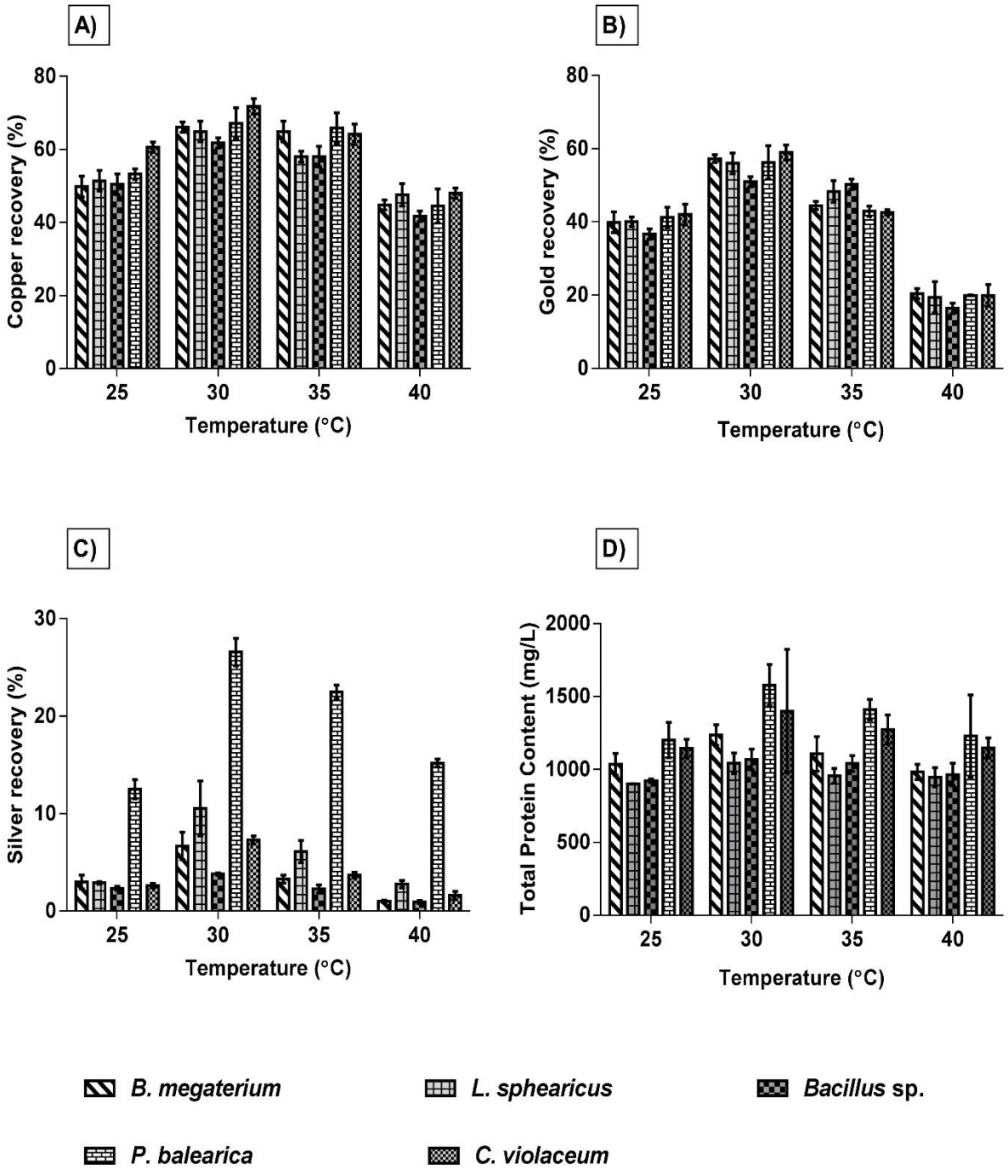


Figure 4.10: Effect of temperature on percent (%) bioleaching of metals (Cu, Au, and Ag) from waste CPCBs by different bacterial strains: a) % Cu bioleaching; b) % Au bioleaching; c) % Ag bioleaching. The presented data in this figure is the average of those obtained from duplicate experiments.

A similar trend was observed for *P. balearica* i.e. 7.5g/L (Cu-69.7%, Au-62.1%, Ag-27.9%) > 5g/L (Cu-64.2%, Au-59.7%, Ag-25.6%) > 10g/L (Cu-62.1%, Au-40.7%, Ag-21.3%) > 2.5g/L (Cu-53.6%, Au-46.6%, Ag-19.4%), respectively (Fig. 4.11). Similar findings were observed by Shin et al [41], reported 5 g/L glycine concentration as optimum during bioleaching of gold from ore using *C. violaceum*. Faramarzi et al. [33] determined a range of 8-10 g/L glycine concentration as optimum for highest cyanide production as well as recovery of tetracyanonickelate using *C. violaceum.* Other than metals ions, bacterial strains showed a decrease in growth from 2.5 to 10 g/L glycine concentration (Fig. 4.11d). This is due to enhanced concertation of amino acid i.e. glycine in the medium induces toxicity to the bacterial cells which led to reduced growth as well as low lixiviant production; and hence, a decreased metals recovery [41].

4.8.4 Effect of pH

The metal mobilization ability of bacterial strains was tested at different pH (7, 8, and 9) in LB medium. It was observed that maximum metals mobilization takes place at pH 9.0 followed by 8.0 and least at pH 7.0 (pH 9.0> 8.0> 7.0) except *L. sphaericus* which showed maximum metals mobilization at a pH of 8.0 (Fig. 4.12). Though the metals bioleaching was highest at alkaline pH 9.0 however, bacterial growth was maximum at physiological pH 7. This is because at pH 7 cyanide is mainly present in the form of HCN gas which is volatile and less soluble in water due to its pK_a value 9.3. The pH (9), equilibrium favors formation of more cyanide ions (CN^-), increases its availability for metals solubilization/ complexation [35], and is stable for the longer duration [34].

Among all the bacterial strains tested, average metals bioleaching was maximum with *C. violaceum* followed by *P. balearica, B. megaterium, Bacillus* sp. and *L. sphaericus* (Fig. 4.12). The percentage of Cu bioleached at pH 9.0 was higher for *C. violaceum* (87.5%) followed by *P. balearica* (77.4%), *B. megaterium* (72.8%) and *Bacillus* sp. (71%) (Fig 4.12a). The percentage of Au bioleached by different bacteria at pH 9.0 was higher for *C. violaceum* (73.7%) followed by *P. balearica* (68.5%), *B. megaterium* (66.6%) and *Bacillus* sp. (64.5%) (Fig. 4.12b).

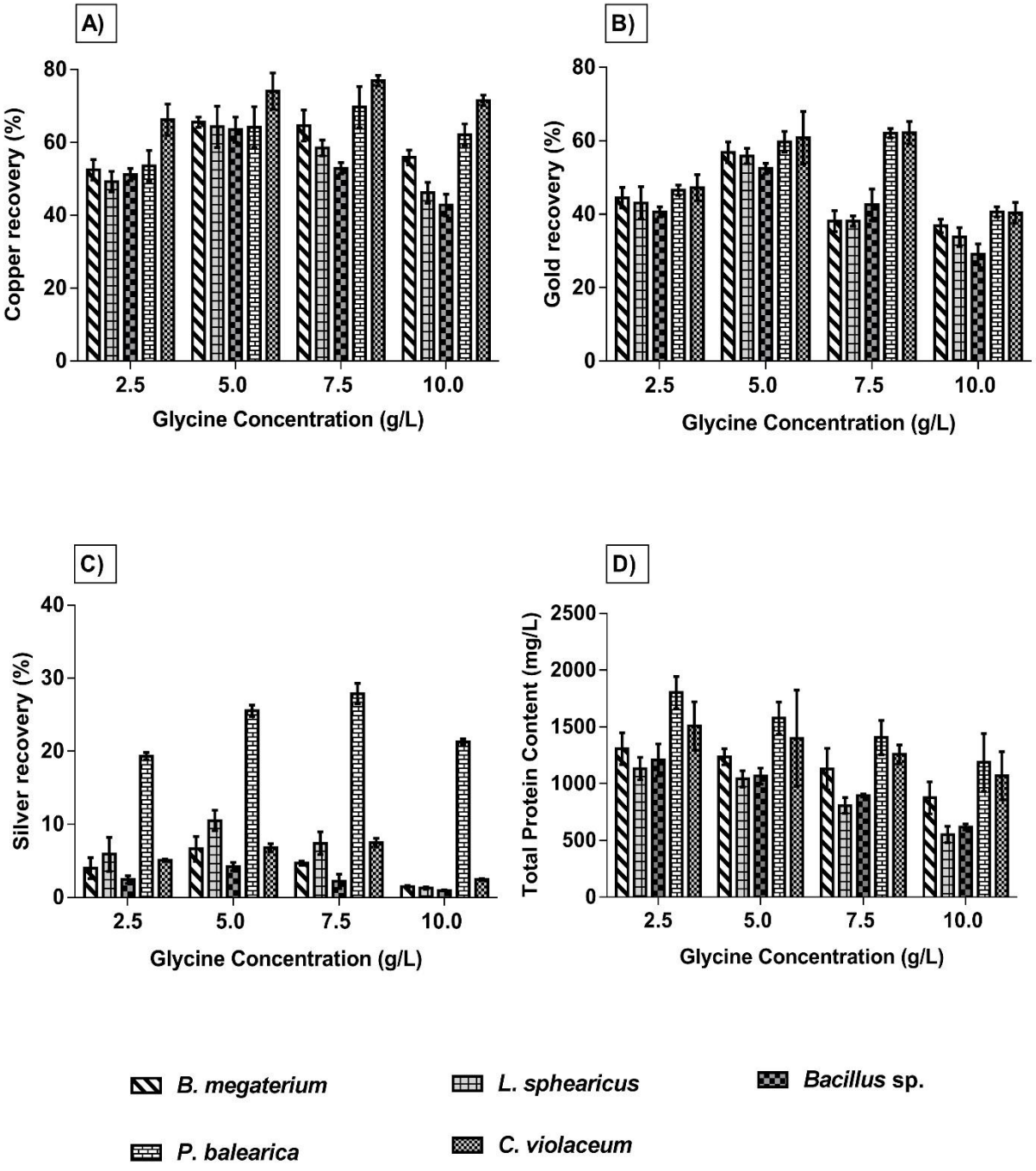


Figure 4.11: Effect of glycine concentration on percent (%) bioleaching of metals (Cu, Au, and Ag) from waste CPCBs by different bacterial strains: a) % Cu bioleaching; b) % Au bioleaching; c) % Ag bioleaching. The presented data in this figure is the average of those obtained from duplicate experiments.

In case of Ag, maximum bioleaching was recorded at pH 9.0 with *P. balearica* (33.8%) followed by *C. violaceum* (13.02%), *B. megaterium* (15.32%) and *Bacillus* sp. (8.7%) (Fig. 4.12c), respectively. *L. sphaericus* showed maximum mobilization of 64.3%, 58.4% and 10.53% for Cu, Au, and Ag, respectively at pH of 8.0. The lowest percentage mobilization of Cu, Au, and Ag at pH 7 was 61.7%, 51.2% and 4.1% by *C. violaceum*; 53.9%, 46.4% and 17.4% by *P. balearica*; 53.3%, 47.9% and 3.6 by *B. megaterium*; 49.2%, 45.3% and 3.1% by *L. sphaericus* and 54.8%, 40.6 and 5.02 by *Bacillus* sp., respectively.

The results of the study corroborate to other reports [8, 28], which showed higher metals recovery at alkaline pH of 9.0, 9.5, 10 and 11, respectively. Chi et al. [28] performed bioleaching of Cu and Au from waste mobile phone PCBs using *C. violaceum*. They reported that the dissolution of Cu and Au increased with increase in pH from 8-11, respectively. Natarajan and Ting [8] reported the highest recovery of Au at pH 9.5 using mutated *C. violaceum*. In another study, they reported 30% and 95.7% of Au and Cu recovery using spent medium leaching at pH 10 [35]. It was evident that pH diversely affects metal mobilization during bioleaching and alkaline pH was associated with increased metal bioleaching efficiency in our case. The alkalophilic cyanogenic microorganisms have a great application in Au and Ag leaching from mine tailings. There are few reports of silver leaching at alkaline pH using cyanogenic microorganisms from metals containing solid waste [32, 42].

4.9 Bioleaching assay under optimized conditions

To validate the optimized process parameters; bioleaching experiments were performed under all the optimized conditions. Two-step bioleaching of e-waste was carried for a time period of seven days at a pulp density of 10 g/L, glycine concentration of 5 g/L; a temperature of 30ºC; a particle size of ≤0.15 mm and a pH of 9.0 except for *L. sphaericus* where optimized pH was 8.0. Bioleaching profile of Cu, Au, and Ag by all bacterial strains was shown in Fig. 4.13. The maximum bioleaching of Cu, Au, and Ag occurred at the 7^{th} day of incubation; thereafter a saturation in metals bioleaching was observed. Among all the bacterial strains tested the reference strain *C. violaceum* showed maximum bioleaching of Cu (87.5±7%) and Au (73.6±4%) (Fig. 4.13). Whereas, the bioleaching of Ag (33.8±4%) was higher in case of *P. balearica.*

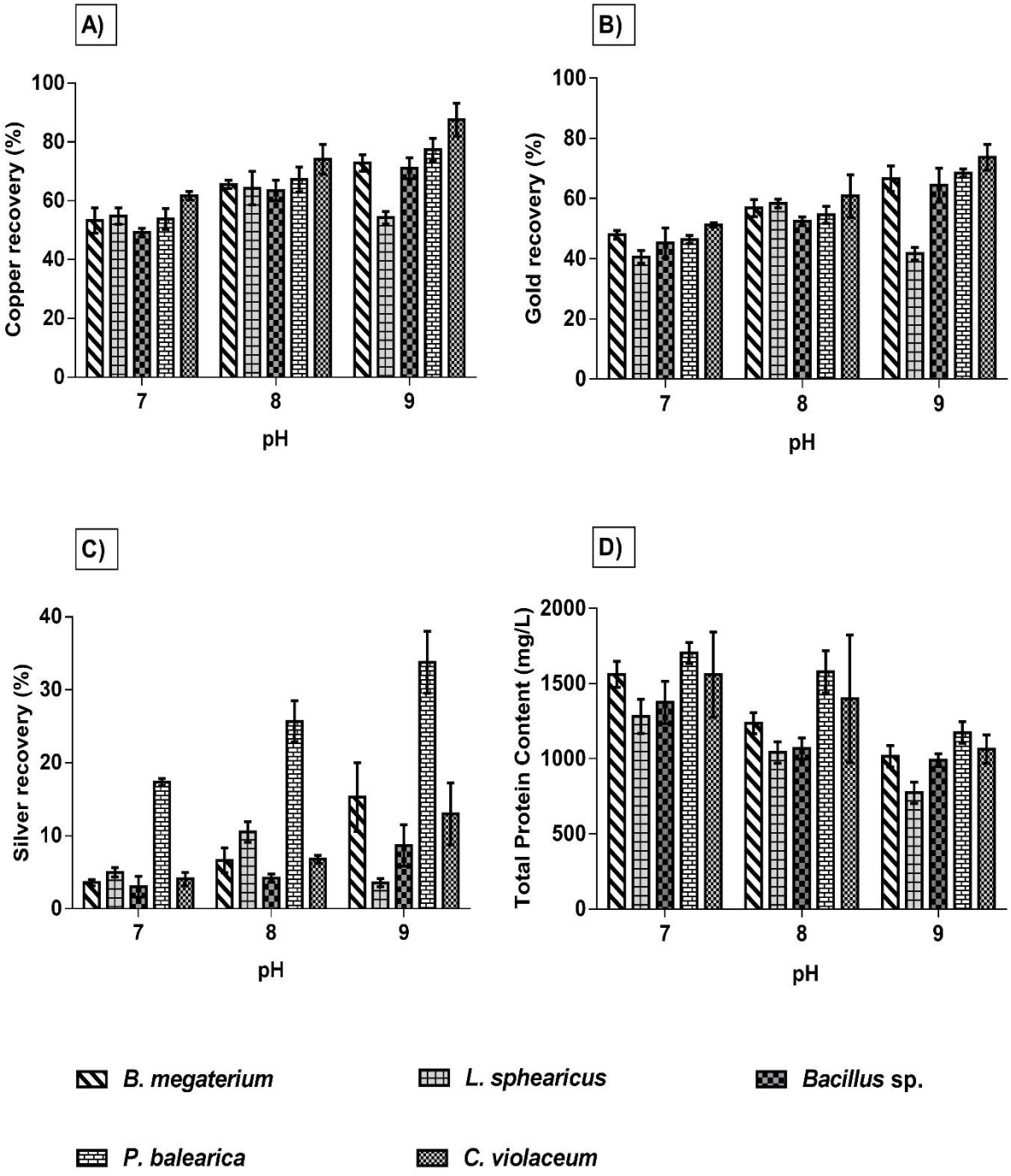


Figure 4.12: Effect of pH on percent (%) bioleaching of metals (Cu, Au, and Ag) from waste CPCBs by different bacterial strains: a) % Cu bioleaching; b) % Au bioleaching; c) % Ag bioleaching. The presented data in this figure is the average of those obtained from duplicate experiments.

Among the isolated bacterial strains, *P. balearica* showed highest metal mobilization (Cu-77.4±7%, Au-68.5±5%, Ag-33.8±4%) followed by *B. megaterium* (Cu-72.7±5%, Au-66.6±6%, and Ag-15.3±3%) (Fig. 4.13). The lowest mobilization of Cu (64.3%) and Au (57.4%) was attained by *L. sphaericus* whereas, Ag (8.7%) was least mobilized by *Bacillus* sp. The results of this study revealed that the Cu bioleaching was the highest among all the metals tested. Preferred Cu leaching compared to Au and Ag may be due to two reasons: 1) higher Cu content in the WPCBs which might have consumed all the cyanide ions and gold (E^0 $Au^{3+/}$Au: 1:52 V) is nobler than copper (E^0 Cu^{2+}/Cu: 0.34 V) [8, 28].

The results of the study are in good agreement to those previously reported in the literature using cyanogenic microorganisms. For example, Faramarzi et al. [33] obtained a maximum dissolution of 14.9% of Au (dicyanoaurate) from shredded PCBs using *C. violaceum* in a one-step bioleaching. Brandl et al. [32] attained 68.5% and 5% of Au and Ag dissolution from shredded PCBs and jewelry waste using cyanogenic microorganisms. Pradhan and Kumar [31] reported 83, 73, and 8% recovery of Cu, Au, and Ag, respectively, from e-waste using a mixture of *C. violaceum* and *P. aeruginosa*. Another study reported that *Pseudomonas chlororaphis* leached 52.3, 12.1 and 8.2% of Cu, Au, and Ag, respectively, from WPCBs [42]. In addition, Natarajan and Ting [35] enhanced Au mobilization through spent medium bioleaching using bacterial cell-free metabolite. They recovered 30% of Au content of electronic scrap. The findings indicated the potential of cyanogenic bioleaching of metals from the electronic waste material, and represent a novel type of microbial metal mobilization termed as "biocyanidation" [32, 158], that might find an industrial application.

Other than metal ions, final pH of the leachate was also analyzed (Fig. 4.14). The pH dropped due to acidification during microbial growth by secretion of organic acids [18]. However, after e-waste addition during two-step bioleaching, final pH of the leachate increased gradually until 7 days of the incubation period for all bacterial strains (Fig. 4.14). Using *C. violaceum*, the pH increased from 9 to 9.48 during 7 days of bioleaching period. In case of *P. balearica*, pH increased from initial 9 to 9.3 after 7 days of bioleaching. Among mine isolates, using *B. megaterium* with CPCBs pH increased gradually up to 9.45 until the seventh day of the incubation period.

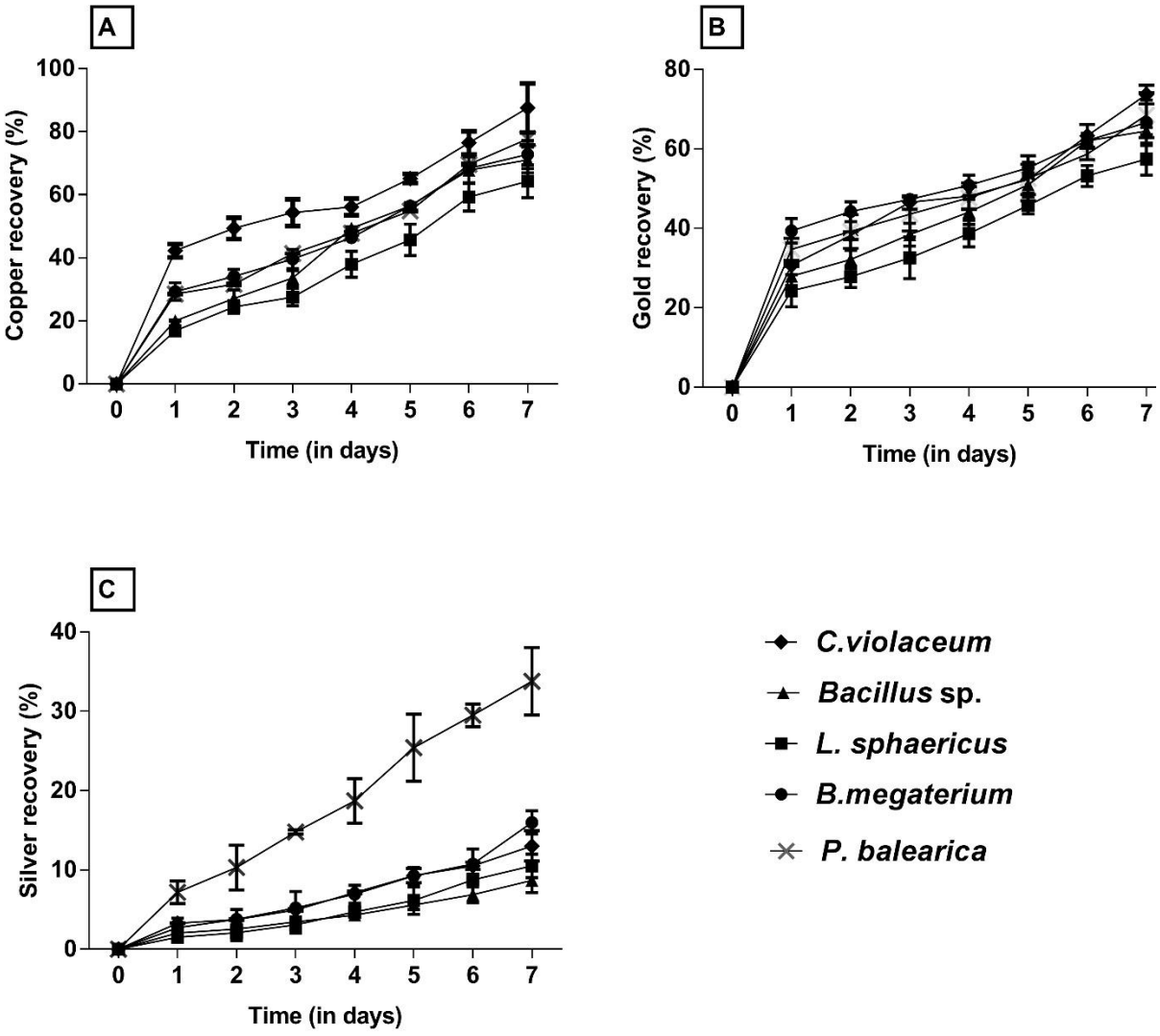


Figure 4.13: Effect of optimized parameters on percent (%) bioleaching of metals (Cu, Au, and Ag) from waste CPCBs by different bacterial strains: a) % Cu bioleaching; b) % Au bioleaching; c) % Ag bioleaching. The data presented in this figure presents is the average of those obtained from duplicate experiments.

Motaghed et al. [143] reported an increase in pH from 7 to 9 during bioleaching of spent catalysis using *B. megaterium*. Pradhan and Kumar [31] studied bioleaching of metals from e-waste using *P. fluorescence*. They reported an increase in pH from 7.2 to 9.2 after 7 days of bioleaching describing that cyanide was the reason behind increased pH. This is because, at physiological pH (7.0), cyanide is mainly present in the form of HCN due to its

pKa value (9.3). This value decreases to approximately 8.3 in the presence of salts, reducing its volatility. Hence, pH increased by the formation of CN^- and formulates complexes with metals [31]. Chi et al. [28] indicated that the growth of both *C. violaceum* and *P. fluorescens* improved the pH continuously from 7 to 9 for 4 days and then became constant. The similar results were attained by Brandl and Faramarzi [129], concluded that as a result of the growth of *C. violaceum*, the pH increased continuously from 7 to 9 within 4 days that remained constant afterward.

Our results are in good agreement with Sahni et al. [18], who studied bioleaching of metals from SIMW using *C. violaceum*. They reported that in the absence of subscriber identity module waste (SIMW) pH decreased from the initial pH, however, after addition of SIMW; pH (9.39 approx.) increased continuously up to 4 days. Arshadi et al. [40] and Arshadi and Mousavi [125] studied bioleaching of metals from CPCBs and MPPCBs using *B. megaterium*. In their study during first 2 days of bioleaching, the pH dropped from the startup point 10 and then pH increased up to 4 days. They mentioned that HCN production by bacteria caused pH to decrease whereas pH increased when HCN reacted with metals to form metal cyanide complex.

4.10 Elucidation of the kinetics involved in the process

A kinetic study for metals bioleaching from e-waste (PWBs) was performed using different cyanogenic microorganisms viz. *B. megaterium, L. sphaericus, Bacillus* sp., *P. balearica* and *C. violaceum*. The metals bioleaching increased till the seventh day and thereafter reached to saturation level. The data obtained after bioleaching was plotted against time for zero, first and second order reaction, respectively. The linear plot suggested that bioleaching process follows first-order reaction kinetics (Fig. 4.15) and showed high R^2 (correlation factor) value (Table 4.8). To plot first order graph Eq. (4.5) was followed by using boundary conditions (time) t = 0 to 7 days and (concentration) [A] = 0 to 7 days:

$$\ln[A]_t = -kt + \ln[A]_0 \qquad (4.5)$$

Where ln = natural log; $[A]_t$ & $[A]_0$ = concentration of metals leached at time 0-7 days

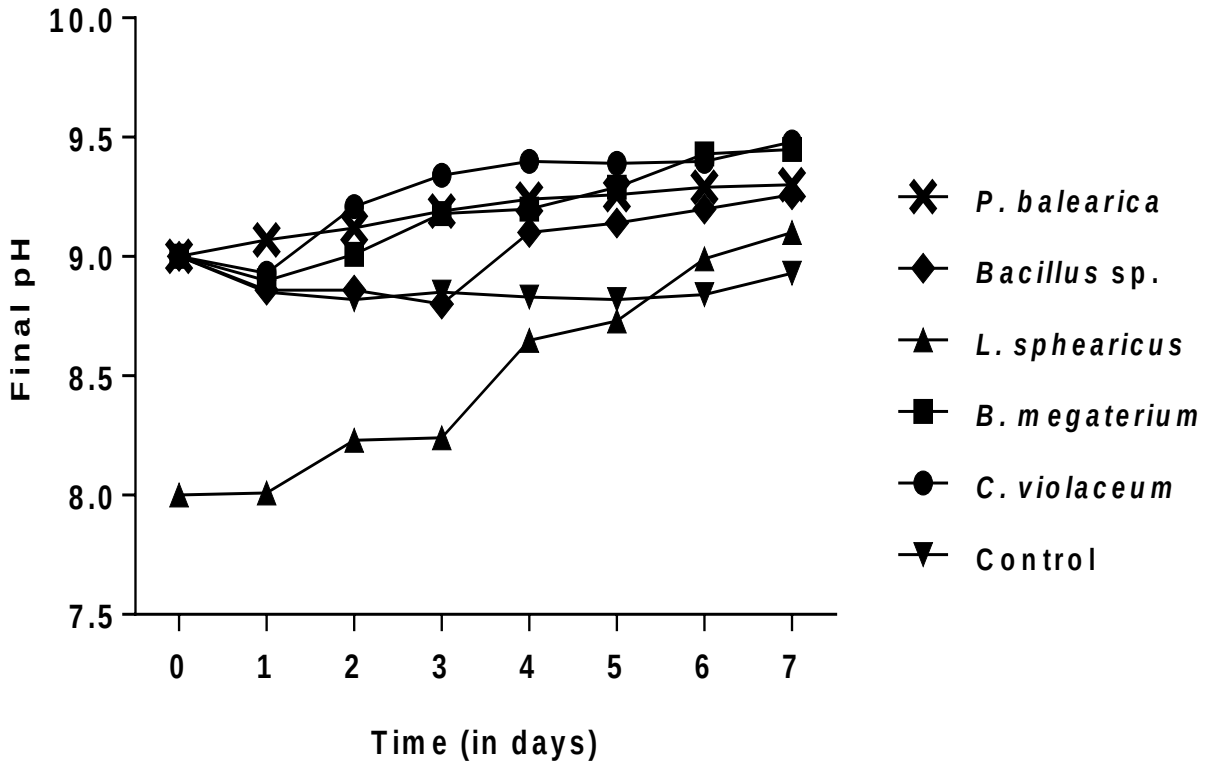


Figure 4.14: Trends in pH change during bioleaching with waste CPCBs.

Table 4.8: Bacterial strains representing the correlation coefficient (R^2) for first-order kinetics

Bacterial strain	The line of best fit and correlation coefficient		
	Au	Ag	Cu
B. megaterium	0.9945	0.9934	0.9976
L. sphaericus	0.9932	0.9915	0.9815
Bacillus sp.	0.9894	0.998	0.9835
P. balearica	0.9946	0.9733	0.9909
C. violaceum	0.969	0.9838	0.9785

The reaction rate for a solid-liquid reaction is usually controlled by (1) diffusion of reactant (HCN in case of cyanogenic bacteria) from liquid film to the solid product (CPCBs), (2) reactant diffusion through solid product layer, and (3) chemical reaction in the studied system at the surface of solid or product layer [159]. Bioleaching of Cu, Au, and Ag from waste CPCBs

depends upon biogenic cyanide production when treated with cyanogenic microorganisms. The chemical reactions for bioleaching of Cu, Au, and Ag are represented in Eq. (4.6) – (4.8):

$$4Cu + 8CN^- + O_2 + 2H_2O = 4Cu(CN)_2^- + 4OH^- \quad (4.6)$$

$$4Au + 8CN^- + O_2 + 2H_2O = 4Au(CN)_2^- + 4OH^- \quad (4.7)$$

$$4Ag + 8CN^- + O_2 + 2H_2O = 4Ag(CN)_2^- + 4OH^- \quad (4.8)$$

The Cu formed various complexes with cyanide like $[Cu(CN)_2^-]$, $[Cu(CN)_3^-]$ and $[Cu(CN)_4^-]$, depending upon the pH conditions [28]. In the same set of experimental conditions; bioleaching rate (mgL^{-1} day^{-1}) was calculated and was found to be dependent on metal (Cu Au and Ag) concentration present in the e-waste. The metals with higher concentration in the waste PCBs (Cu- 23.4 mg/g) are bioleached at faster rates as compared to Au and Ag, respectively (Table 4.9). Similar findings have been reported by Pradhan and Kumar [31].

Table 4.9: Evaluation of bioleaching parameters with different bacterial strains under the same set of experimental conditions.

Bacterial Cultures	**Metal leaching rate*(mgL^{-1} day^{-1})**		
	Cu	**Au**	**Ag**
B. megaterium	24.4	0.075	0.095
L. sphaericus	21.5	0.071	0.063
Bacillus sp.	23.8	0.074	0.051
P. balearica	25.9	0.077	0.202
C. violaceum	29.3	0.082	0.078

*Metals leaching rate (mgL^{-1} day^{-1}) $=\frac{A}{T}$; where A is maximum metal bioleaching concentration, T is time (seven days). Data represented as the average of those obtained from duplicate experiments

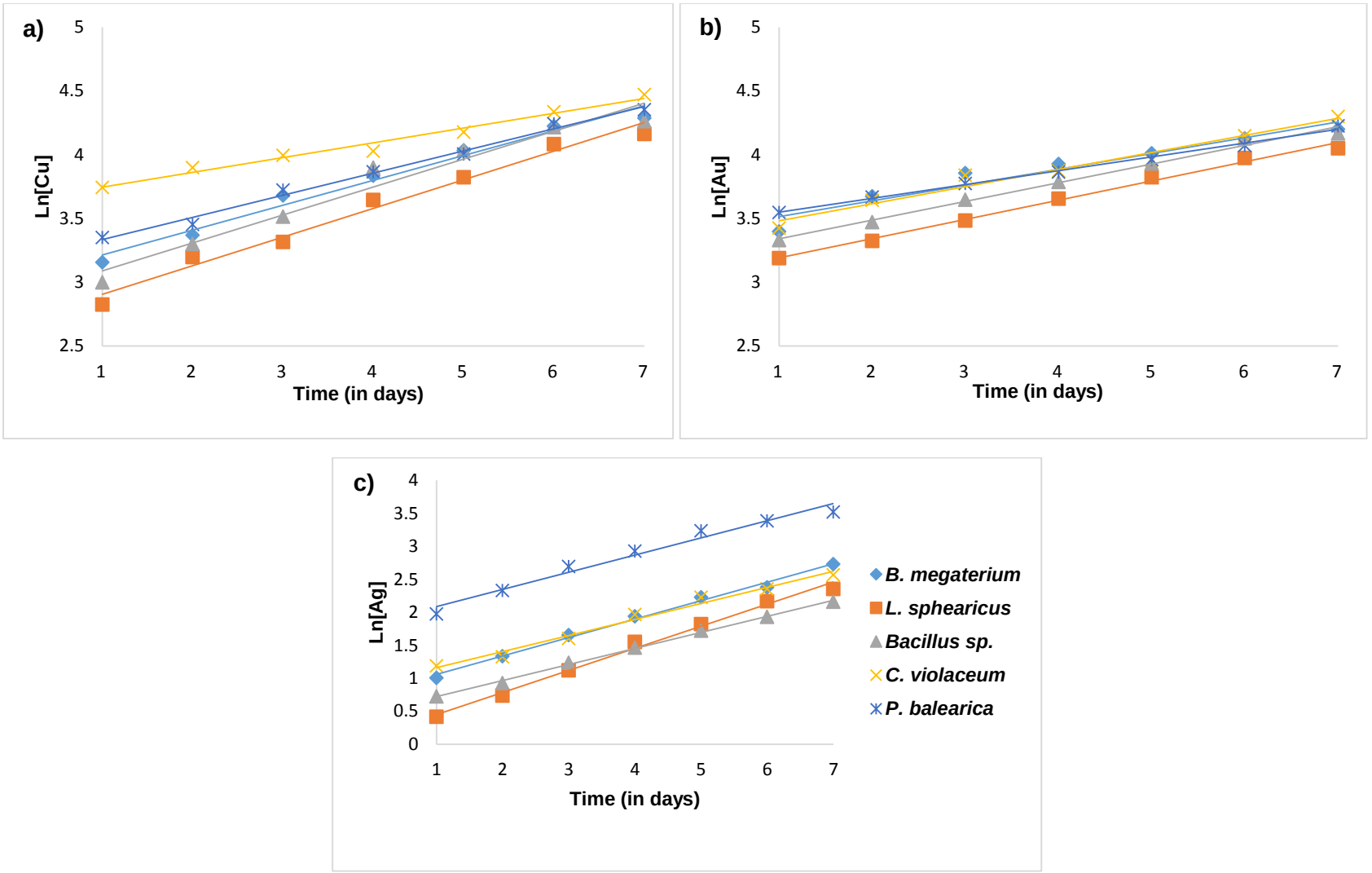


Figure 4.15: Kinetic modeling results showing first order reaction kinetics for metals bioleaching from waste CPCBs: a) Cu; b) Au; c) Ag.

4.11 Optimization of the process for maximum recovery of metals (Cu, Au, and Ag) from waste CPCBs using response surface methodology (RSM)

4.11.1 Statistical evaluation

In order to find interaction and cumulative effect of parameters on bioleaching of metals, we performed statistical optimization using RSM [142]. The process parameters such as pulp density, pH, glycine concentration, and temperature were optimized using CCD-RSM to enhance the dissolution of Cu, Au, and Ag during the bioleaching process. The 30 runs of experiment including 16 factorials, 8 axial, and 6 center points were conducted as suggested by RSM. The experimental design and their responses both predicted as well as the experimental were represented in Table 4.10. Three quadratic models were suggested by RSM based on the statistical results of the ANOVA for bioleaching of Cu, Au and Ag, respectively (Table 4.11). According to the statistical data of Table 4.11, the "model *F*-values" equal to 29.9 (Cu), 8.03 (Au) and 26.9 (Ag) depicting the significance of models. The *p* values depicts that the possibility of errors occurrence is <0.01% and thus validates the model. The factors having a *p* value < 0.05 affects the Cu, Au, and Ag recovery significantly. The "adequate precision" value greater-than (>) 4 is desirable; a measure of signal-to-noise ratio [160]. The adequate precision value for quadratic model Cu, Au, and Ag was 19.8, 10.4, and 19.3, respectively, indicates the adequate signal and can be used to navigate the design space established by CCD [39]. The coefficient of variance (CV) for quadratic model Cu, Au and Ag was 7.1, 15.3 and 15.6% which confirms the accuracy and reliability of the experiments conducted [160]. The higher coefficient of determination (R^2) is essential to confirm the validity and reproducibility of models [125, 142]. The R^2 values i.e. 0.96, 0.88, and 0.96, for the polynomial quadratic model Cu, Au, and Ag confirms that the models are proficient and reproducible under given experimental conditions. The Fig. 4.16a, b, c presents the actual vs. predicted responses for percent bioleaching of Cu, Au, and Ag from waste CPCBs. The actual responses were those values obtained experimentally, whereas the predicted responses were proposed by CCD-RSM. The adjacency of scattered points toward the 45° line confirmed the adequacy of the model in predicting the responses [142]. The Fig. 4.16d, e, f presents the

normal probability plot for bioleaching of Cu, Au and Ag. The data on a straight line in the probability plot depicts a normal distribution and thus supports the acceptability of the least-squares fit [39]. The influence of the process parameters on bioleaching of Cu, Au, and Ag was studied using three-dimensional (3D) response surface plots (Fig. 4.17, 4.18, 4.19).

4.11.2 Model Fitting

On the basis of 30 experimental responses, CCD-RSM proposed three polynomial quadratic model for percent bioleaching of Cu, Au, and Ag from waste CPCBs. The suggested polynomial equations for Cu, Au, and Ag are given in Eqs. (4.9) – (4.11):

$$Y_{Cu} = 61.28 + 1.80A - 9.64B - 3.72C + 1.52D - 0.48AB + 0.37AC - 0.17AD + 1.81BC - 2.24BD - 0.64CD - 4.5A^2 - 0.82B^2 - 4.86C^2 - 6.68D^2 \quad (4.9)$$

$$Y_{Au} = 50.08 + 0.72A - 9.25B - 2.57C + 2.09D - 0.50AB - 0.29AC + 0.09AD + 1.23BC - 0.32BD - 1.10CD - 3.92A^2 - 0.66B^2 - 4.37C^2 - 5.75D^2 \quad (4.10)$$

$$Y_{Ag} = 23.34 + 1.06A - 6.43B - 1.81C + 0.25D - 1.61AB - 0.67AC - 0.33AD + 2.24BC - 0.93BD - 0.42CD - 2.97A^2 + 0.10B^2 - 2.93C^2 - 4.47D^2 \quad (4.11)$$

According to Rastegar et al. [161], the smaller p values ($p < 0.05$) represent significance (statistically) of the corresponding coefficient with a high confidence level. The model's p-value for bioleaching of Cu (0.0001), Au (0.0001) and Ag (0.0001) was statistically significant at a high confidence level. It was observed that the individual variables *A, B, C* and *D* interaction variable *BD* and quadratic variables A^2, C^2 and D^2 have a significant ($p < 0.05$) effect on Cu recovery. In case of Au, individual variable *B* and *C* and the quadratic variables A^2, C^2, and D^2 showed a significant effect on Au recovery at a p value < 0.05. Whereas, for Ag recovery, individual variables *A, B,* and *C*; quadratic variables A^2, C^2, and D^2; and interaction variables *AB* and *BC*, were significant ($p < 0.05$) (Table 4.11). The terms individual variable, interaction variable, and quadratic variable play a key role in attaining the best conditions for bioleaching of Cu, Au, and Ag, respectively.

Table 4.10: The CCD-RSM experimental plan and their responses

Sr. No.	A	B	C	D	Experimental response			Predicted response		
					Cu (%)	Au (%)	Ag (%)	Cu (%)	Au (%)	Ag (%)
1	7.5	15	7.5	30	43.2	39.5	12.3	39.9	32.9	9.4
2	8	10	10	25	39.1	34.2	11.7	42.6	38.7	12.4
3	8	10	10	35	45.3	38.2	12.5	49.2	41.1	14.6
4	8.5	25	7.5	30	34.7	18.9	8.0	38.8	28.9	10.9
5	9	10	10	25	48.3	40.5	14.9	48.3	40.4	17.02
6	9	20	5	35	39.3	35.9	7.9	37.4	30.9	5.8
7	8	20	5	35	34.6	32.7	6.3	35.9	29.8	6.2
8	8.5	15	7.5	30	59.9	50.3	22.4	61.3	50.1	23.4
9	8.5	15	7.5	30	62.1	49.5	23.7	61.3	50.1	23.4
10	8	20	10	25	33.1	23.8	6.4	32.4	24.3	9.1
11	8	10	5	25	51.7	40.1	15.7	53.2	43.5	18.3
12	9	20	10	35	33.2	31.9	5.0	33.1	25.5	4.5
13	8.5	5	7.5	30	84.3	72.4	40.1	77.3	65.9	36.6
14	8.5	15	7.5	30	61.7	49.9	24.1	61.3	50.1	23.4
15	8	20	5	25	35.8	25.1	7.9	35.7	24.2	6.1
16	8	20	10	35	31.4	25.7	8.2	30.1	25.4	7.6
17	9	10	10	35	52.4	42.8	17.4	54.2	43.2	17.9
18	8	10	5	35	60.8	48.2	21.1	62.3	50.3	22.2
19	8.5	15	7.5	30	63.2	48.3	23.9	61.3	50.1	23.4
20	9	20	10	25	35.9	26.6	9.8	36.1	23.9	7.0
21	8.5	15	7.5	20	32.5	23.9	6.0	31.5	22.9	4.9
22	8.5	15	2.5	30	50.1	32.9	13.9	49.3	37.8	15.3
23	9	20	5	25	40.6	31.1	7.1	37.9	25	6.9
24	9.5	15	7.5	30	46.8	25.8	11.2	47.1	35.9	13.6
25	8.5	15	7.5	30	62.9	51.9	23.2	61.3	50.1	23.4
26	8.5	15	12.5	30	36.6	28.7	9.9	34.4	27.4	8.0
27	9	10	5	35	63.8	57.2	28.8	65.8	53.6	28.2
28	8.5	15	7.5	40	39.7	26.7	5.5	37.6	31.2	5.9
29	8.5	15	7.5	30	57.9	50.7	22.9	61.3	50.1	23.4
30	9	10	5	25	54.3	46.6	26.4	57.3	46.4	25.6

Where, *A*, *B*, *C*, and *D* represent the pH, e-waste pulp density (g/L), glycine concentration (g/L), temperature (°C), and the multiple of two variables represent interactions among each other.

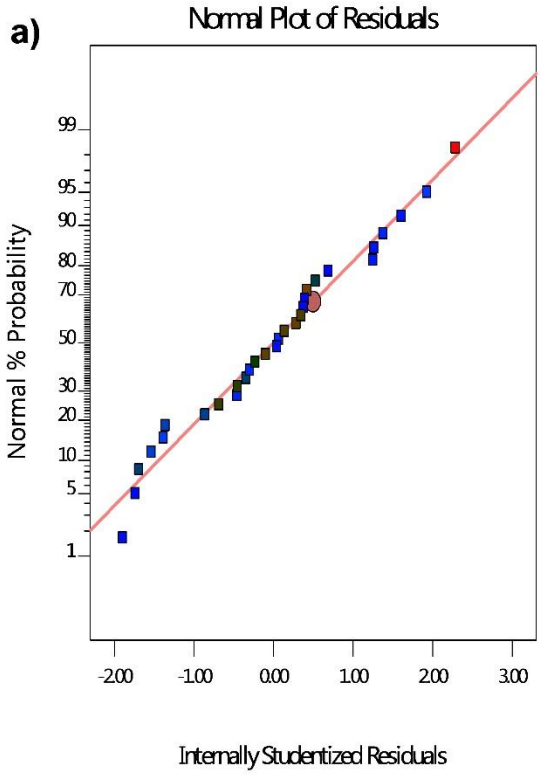
Table 4.11: ANOVA for percent bioleaching of metals (Cu, Au, and Ag)

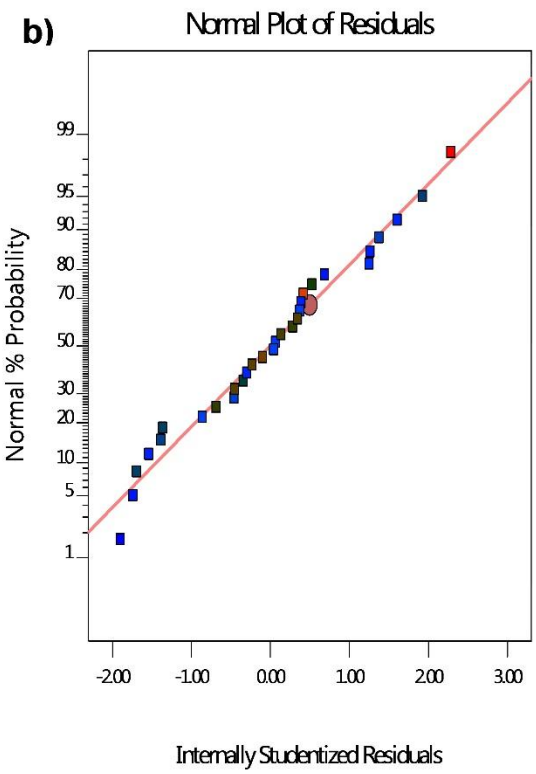
Response	Source	Sum of squares	df	Mean Square	F-value	*p*-value
Cu recovery (%)	Model	4775.6	14	341.1	29.9	0.0001
	A-pH	77.7	1	77.7	6.8	0.02
	B-Pulp Density	2228.1	1	2228.1	195.9	0.0001
	C-Glycine Concentration	332.1	1	332.1	29.2	0.0001
	D-Temperature	55.5	1	55.4	4.9	0.04
	AB	3.8	1	3.8	0.3	0.6
	AC	2.2	1	2.12	0.2	0.7
	AD	0.5	1	0.5	0.1	0.9
	BC	52.5	1	52.5	4.6	0.05
	BD	80.4	1	80.4	7.1	0.02
	CD	6.5	1	6.5	0.6	0.5
	A^2	545.9	1	545.9	47.9	0.0001
	B^2	18.4	1	18.4	1.6	0.2
	C^2	648.5	1	648.5	57	0.0001
	D^2	1223	1	1223	107.5	0.0001
	Residual	170.7	15	11.4		
	Pure Error	20.5	5	4.1		
	Cor Total	4946.2	29			
	$R^2 = 0.96$					
Au recovery (%)	Model	3865.6	14	276.1	8	0.0001
	A-pH	12.5	1	12.5	0.3	0.5
	B-Pulp Density	2053	1	2053	59.6	0.0001
	C-Glycine Concentration	158.9	1	158.9	4.6	0.04
	D-Temperature	105.1	1	105.0	3.1	0.1
	AB	4.2	1	4.1	0.1	0.7
	AC	1.3	1	1.3	0.1	0.8
	AD	0.2	1	0.1	0.01	0.9
	BC	24.3	1	24.3	0.7	0.4
	BD	1.6	1	1.6	0.1	0.8
	CD	19.4	1	19.4	0.5	0.4
	A^2	420.9	1	420.9	12.2	0.003

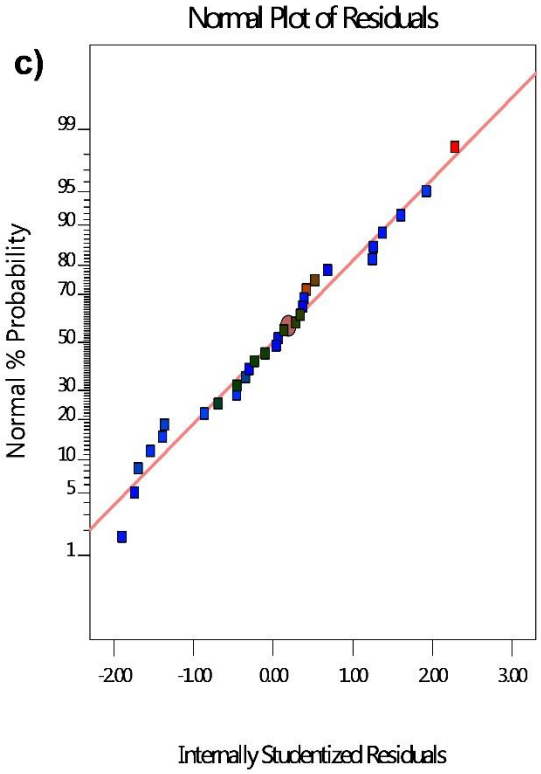
	B^2	11.9	1	11.9	0.3	0.5
	C^2	523.1	1	523.1	15.2	0.001
	D^2	909.9	1	909	26.4	0.0001
	Residual	515.9	15	34.4		
	Pure Error	6.9	5	1.3		
	Corr. Total	4381.5	29			
	$R^2 = 0.88$					
Ag recovery (%)	Model	2102.9	14	150.2	26.9	0.0001
	A-pH	26.9	1	26.9	4.82	0.04
	B-Pulp Density	990.5	1	990.5	177.3	0.0001
	C-Glycine Concentration	78.5	1	78.5	14.2	0.001
	D-Temperature	1.5	1	1.5	0.2	0.6
	AB	41.3	1	41.3	7.4	0.01
	AC	7.1	1	7.1	1.2	0.3
	AD	1.7	1	1.7	0.3	0.6
	BC	80.3	1	80.3	14.3	0.001
	BD	13.9	1	13.9	2.5	0.1
	CD	2.7	1	2.7	0.5	0.4
	A^2	242.2	1	242.2	43.3	0.0001
	B^2	0.2	1	0.2	0.1	0.8
	C^2	234.8	1	234.8	42.1	0.0001
	D^2	546.9	1	546.9	97.9	0.0001
	Residual	83.7	15	5.5		
	Pure Error	2.2	5	0.45		
	Corr. Total	2186.7	29			
	$R^2 = 0.96$					

4.11.3 Response Plots

a) **Copper recovery:** Fig. 4.17a represents the influence of pH and pulp density on Cu recovery. It was observed that both the factors have a significant independent effect on the mobilization of Cu. The mutual interaction between the factors did not show a significant effect on Cu mobilization. The bioleaching of Cu was maximum at low pulp density (10 g/L) and alkaline pH (8.8 approx.). Fig. 4.17b represents the effect of glycine concertation and pH on Cu recovery.


a)
Normal Plot of Residuals
Normal % Probability
99
95
90
80
70
50
30
20
10
5
1
-2.00
-1.00
0.00
1.00
2.00
3.00
Internally Studentized Residuals


b)
Normal Plot of Residuals
Normal % Probability
99
95
90
80
70
50
30
20
10
5
1
-2.00
-1.00
0.00
1.00
2.00
3.00
Internally Studentized Residuals


c)
Normal Plot of Residuals
Normal % Probability
99
95
90
80
70
50
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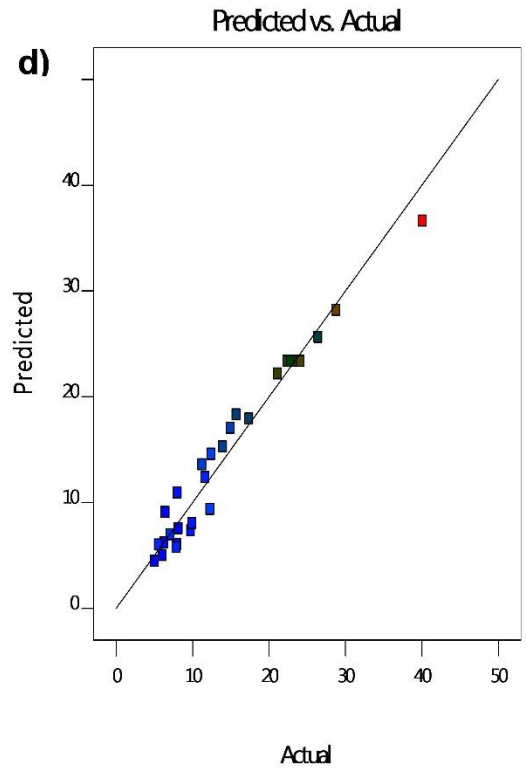
-1.00
0.00
1.00
2.00
3.00
Internally Studentized Residuals


d)
Predicted vs. Actual
Predicted
40
30
20
10
0
0
10
20
30
40
50
Actual

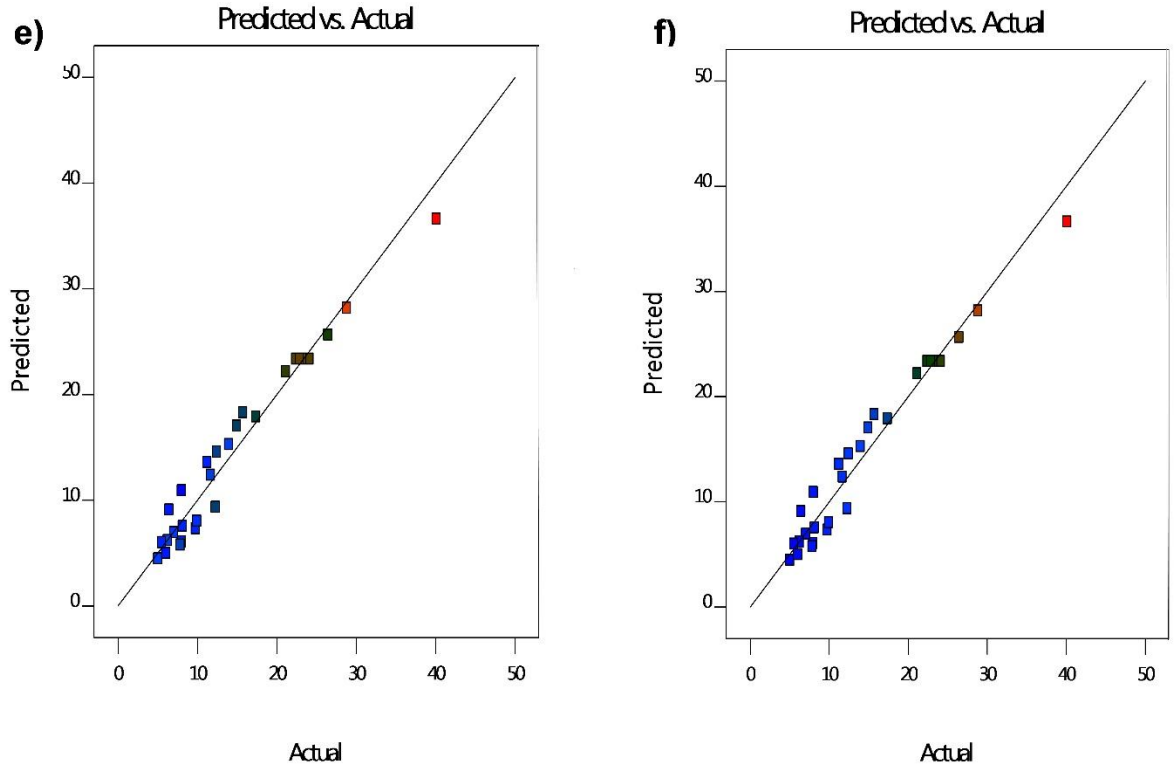


Figure 4.16: Actual vs. predicted plots **a)** Cu recovery (%), **b)** Au recovery (%), **c)** Ag recovery (%); and normal probability plots **d)** Cu recovery (%), **e)** Au recovery (%), **f)** Ag recovery (%)

The maximum Cu recovery was attained at glycine concentration of approximately 7.5 g/L thereafter, a decrease in Cu mobilization was observed. According to Arshadi and Mousavi [125], cyanide production can be enhanced using glycine until it does not lead to reduced bacterial growth. Fig. 4.17c represents the effect of temperature and pH on Cu recovery. It was observed that increase in temperature up to 31ºC (approx.) resulted in increased Cu recovery. A further increase (from 31 to 35 ºC) in temperature resulted in decreased Cu recovery from CPCBs. The other researchers also reported who reported 30ºC as optimum for metals recovery during bioleaching using cyanogenic microorganism [18, 42, 45, 126]. This is due to the fact that maximum biogenic cyanide production takes place at this temperature range 30-35ºC [42, 126]. The circular contour in the Fig. 4.17b, c means that the factors did not show any interaction and have an independent effect on the Cu recovery. The Fig. 4.17d represents the effect of glycine concentration and pulp density. The maximum recovery of Cu was attained at glycine concentration (7.5 g/L) and pulp density (10 g/L). The interaction between the

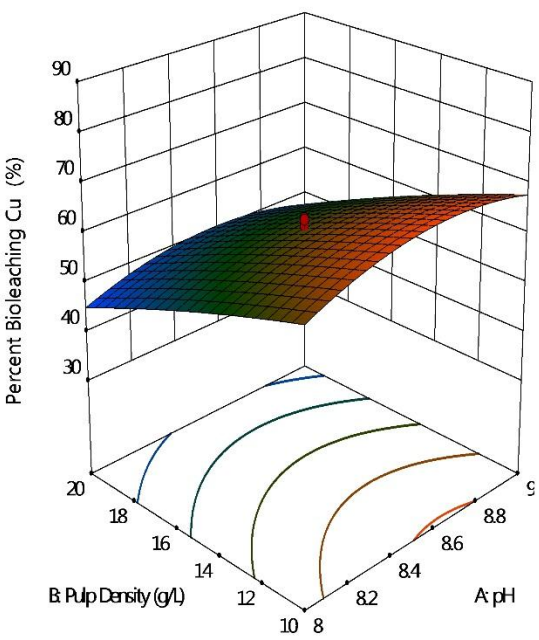
factors was statistically not significant. Fig. 4.17e represents the effect of temperature and pulp density on Cu recovery. It was observed that at temperature 31ºC (approx.) and pulp density 10 g/L a maximum recovery was attained. The elliptical line in the graph showed the interaction among the factors and was statistically significant. The circular contour in the Fig. 4.17f represents that temperature and glycine concertation have an independent effect on Cu recovery. *P. balearica* recovered 84% of Cu through CCD-RSM optimization from waste CPCBs.

b) **Gold recovery:** Two-step bioleaching was conducted for dissolution of Au from waste CPCBs. During bioleaching of Au from CPCBs, biogenic cyanide reacts with Au and form a water-soluble complex i.e. dicyanoaurate [33]. Brandl et al. [32] performed bioleaching of Au, Ag, and platinum (Pt) using cyanogenic bacteria from solid metallic waste. They recovered 68.5% of the total added Au as dicyanoaurate. Jujun et al. [42] reported 8.2% of Au dissolution from waste PCBs using *P. chlororaphis*. In another study, Mara et al. [45] performed pretreatment of e-waste by *A. thiooxidans*; subsequently, 48% of Au was recovered in 3 h using *P. putida*. Furthermore, Pradhan and Kumar [31] performed bioleaching of metals from e-waste and recovered 73% of the total Au present in the e-waste using a mixed culture of *C. violaceum* and *P. aeruginosa*. Other than this, Arshadi and Mousavi [125] used a pure culture of *B. megaterium* and extracted 13.26% of Au of the total present in the waste CPCBs under optimum conditions using RSM. Fig. 4.18a illustrates the interaction effect of pH and pulp density on Au recovery. The maximum Au recovery was observed at alkaline pH >8 to ≤9 and low pulp density (10 g/L). The higher Au recovery at alkaline pH (>8) is due to the pKa of cyanide. At pKa = 9.3 and pH value higher than 9.3, the dominant form of cyanide is CN^-. However, at pH 7, the dominant form of cyanide is HCN gas, which is volatile and may lose out from the solution [40]. The dissolution of Au involve an electrochemical process, as shown in Eqs. (4.12) – (4.14) [151].

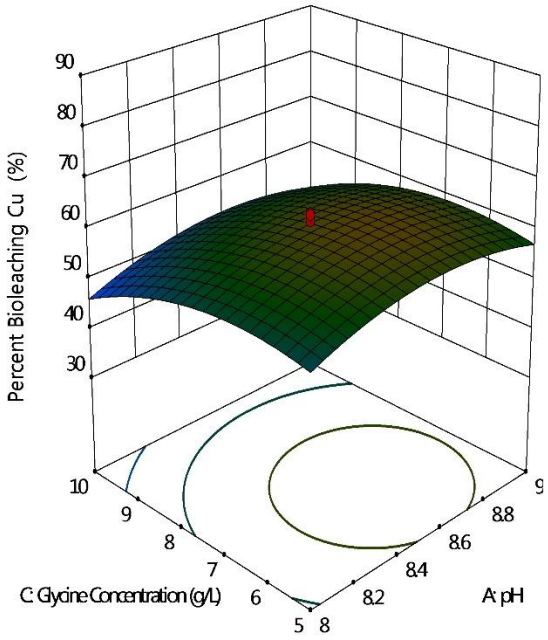
Cathode reaction: $$4Au + 8CN^- \rightarrow 4Au(CN)_2^- + 4e^- \quad (4.12)$$

Anode reaction: $$O_2 + 2H_2O + 4e \rightarrow 4OH^- \quad (4.13)$$

Overall: $$4Au + 8CN^- + O_2 + 2H_2O = 4Au(CN)_2^- + 4OH^- \quad (4.14)$$


Percent Bioleaching Cu (%)
90
80
70
60
50
40
30
B: Pulp Density (g/L)
20
18
16
14
12
10
A: pH
8
8.2
8.4
8.6
8.8
9

a)


Percent Bioleaching Cu (%)
90
80
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60
50
40
30
C: Glycine Concentration (g/L)
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9
8
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A: pH
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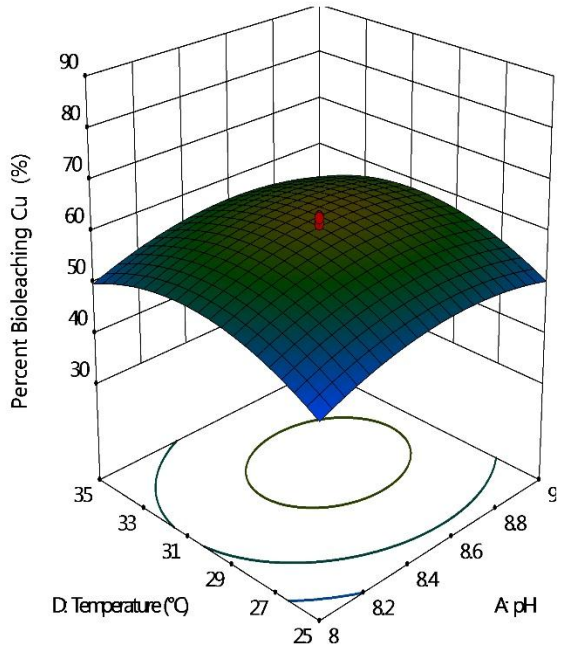
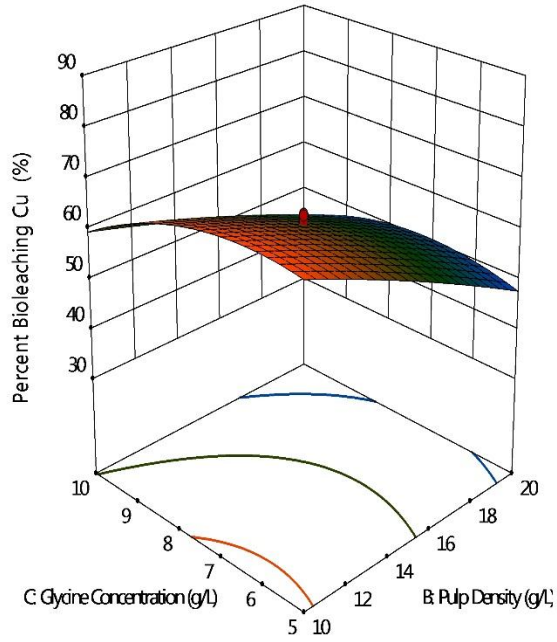
8.8
9

b)


Percent Bioleaching Cu (%)
90
80
70
60
50
40
30
D: Temperature (°C)
35
33
31
29
27
25
A: pH
8
8.2
8.4
8.6
8.8
9

c)


Percent Bioleaching Cu (%)
90
80
70
60
50
40
30
C: Glycine Concentration (g/L)
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8
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6
5
B: Pulp Density (g/L)
10
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d)

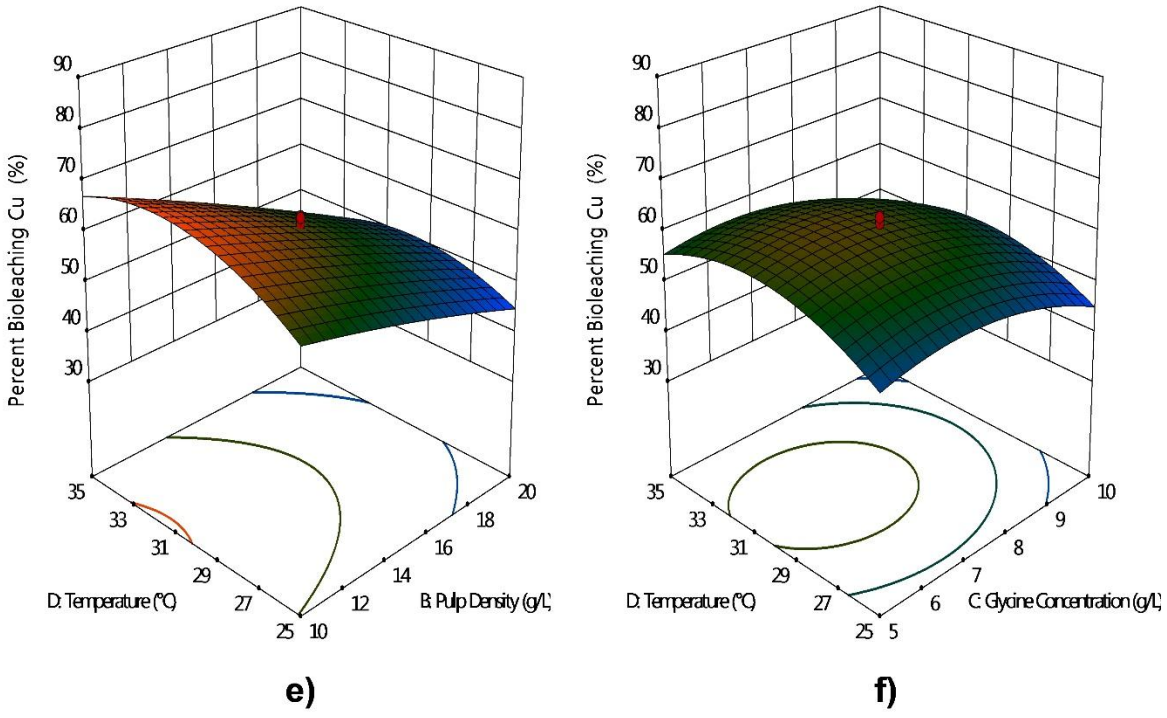
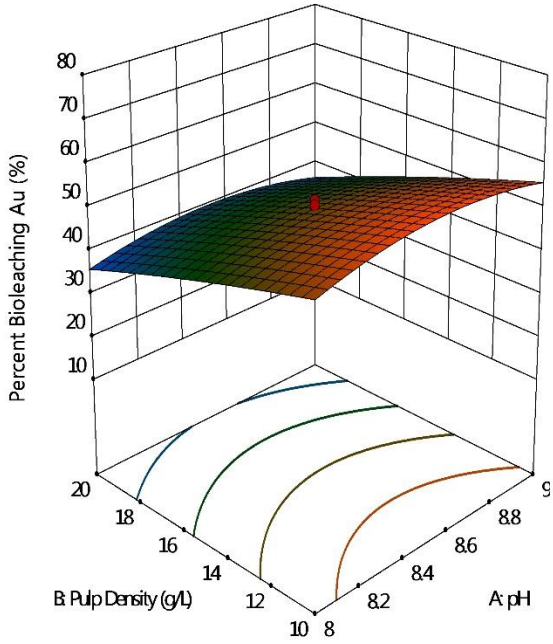


Figure 4.17: 3D surface plot for percent bioleaching of Cu by *P. balearica* **a)** pulp density & pH, **b)** glycine & pH, **c)** temperature & pH, **d)** glycine & pulp density, **e)** temperature & pulp density, **f)** temperature & glycine

The maximum Au recovery occurred at low pulp density ≤10 g/L and a further increase of which (from 10 g/L to 20 g/L) lead to decreased Au recovery. The elevated concentration of e-waste arises the environmental toxicity and stress to the bacterial cells, resulting in reduced bacterial growth [8]. Pradhan and Kumar [31] also reported that metals recovery decreased as the pulp density increased during bioleaching of e-waste using *C. violaceum*. The Fig. 4.18b represents the influence of glycine on bioleaching of Au from waste CPCBs. It was observed that the bioleaching of Au increased as the glycine concentration rises from 5 g/L to 7.5 g/L, however, greater-than (>) 7.5 g/L the Au mobilization decreased. This may be due to the toxic effects of glycine on bacterial cells which resulted in reduced bacterial growth, poor lixiviant production and hence, the low metals mobilization [41]. The Fig. 4.18c represents the influence of temperature and pH on bioleaching of Au. The Fig. 4.18b, c exhibit

circular contours and did not show significant interaction effect on the dissolution of Au. The Fig. 4.18d represents the effect of e-waste pulp density and the glycine concentration. The Au dissolution was maximum at 6 to 7 g/L (approximately) of glycine concentration and a low pulp density (10 g/L). The Fig. 4.18e represents the influence of pulp density and temperature on bioleaching of Au. The curves of contour showed the interaction among the influencing factors, but statistically not significant as shown in Table 4.11. The maximum dissolution of Au occurred at e-waste pulp density of 10 g/L and a temperature of 31°C (approximately). The Fig. 4.18f exhibits circular contours and the epicenter of the concentric circle represents the area where maximum mobilization of Au occurs. Overall, CCD-RSM optimization recovered 72.4% of Au.

c) **Silver recovery:** Fig. 4.19a illustrates the influence of pulp density and pH on bioleaching Ag form waste CPCBs by *P. balearica*. The bioleaching of Ag increased with increase in pH from 8-9, however, increased pulp density lead to decreased metal mobilization. The dissolution of Ag was maximum at low pulp density (10 g/L) at alkaline pH (approximately 8.8). The results are in accordance to Arshadi et al. [40], who extracted the maximum Au at alkaline low pulp density and alkaline pH from MPPCBs by *B. megaterium*. The Fig. 4.19b illustrates the interaction between the pH and glycine concentration and are statistically not significant ($p = 0.3$). The Fig. 4.19c represents the influence of temperature and pH on bioleaching of Ag. The p value 0.6 depicted that interaction effect between temperature and pH was statistically not significant (Table 4.11). The Fig. 4.19b, c exhibit circular contour during bioleaching of Ag which reveal that the factors have an independent influence on the response. The epicenter of circular contour is area where maximum dissolution of the Ag takes place. Fig. 4.19d illustrates the effect of glycine and pulp density on bioleaching of Ag. The Ag mobilization was maximum at low glycine concentration (approximately 6.5 g/L) and a pulp density of 10 g/L. Further, increase in both glycine concentration and pulp density lead to a decrease in Ag mobilization. This is because the higher amount of glycine and e-waste are toxic to bacterial cells and inhibit their metabolic activity thereby, ending in poor lixiviant production and low metals recovery [41].


Percent Bioleaching Au (%)
80
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20
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B: Pulp Density (g/L)
8
8.2
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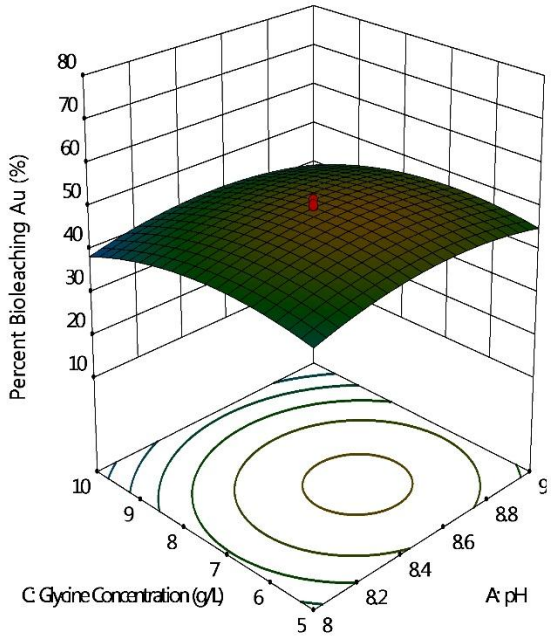
8.8
9
A: pH

a)


Percent Bioleaching Au (%)
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C: Glycine Concentration (g/L)
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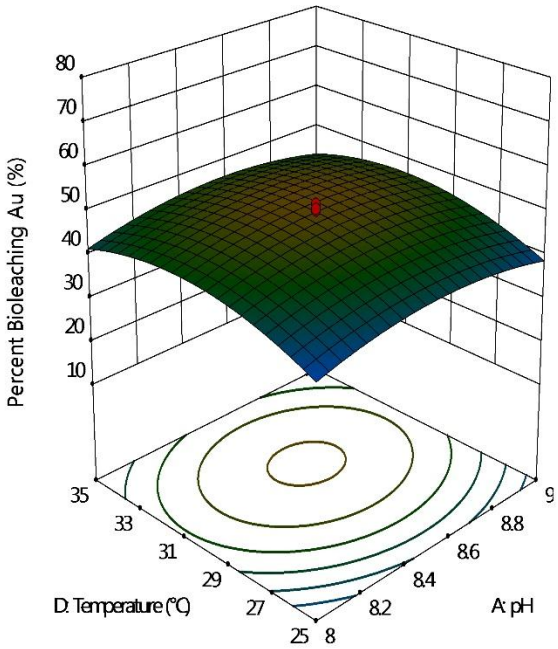
A: pH

b)


Percent Bioleaching Au (%)
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D: Temperature (°C)
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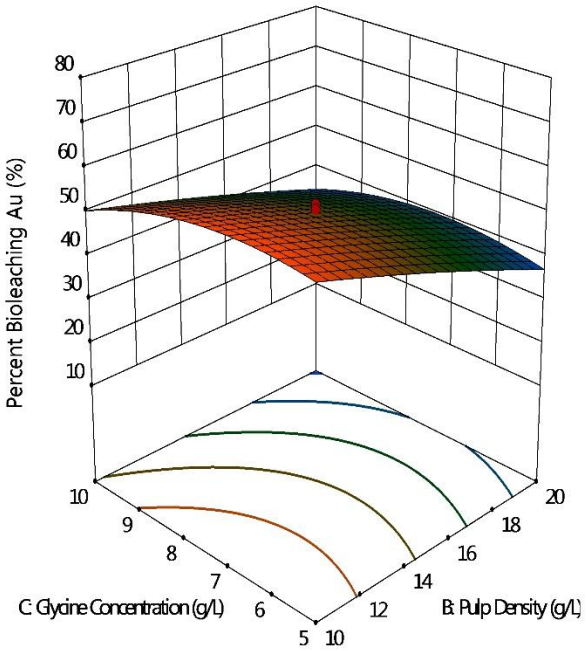
9
A: pH

c)


Percent Bioleaching Au (%)
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C: Glycine Concentration (g/L)
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B: Pulp Density (g/L)

d)

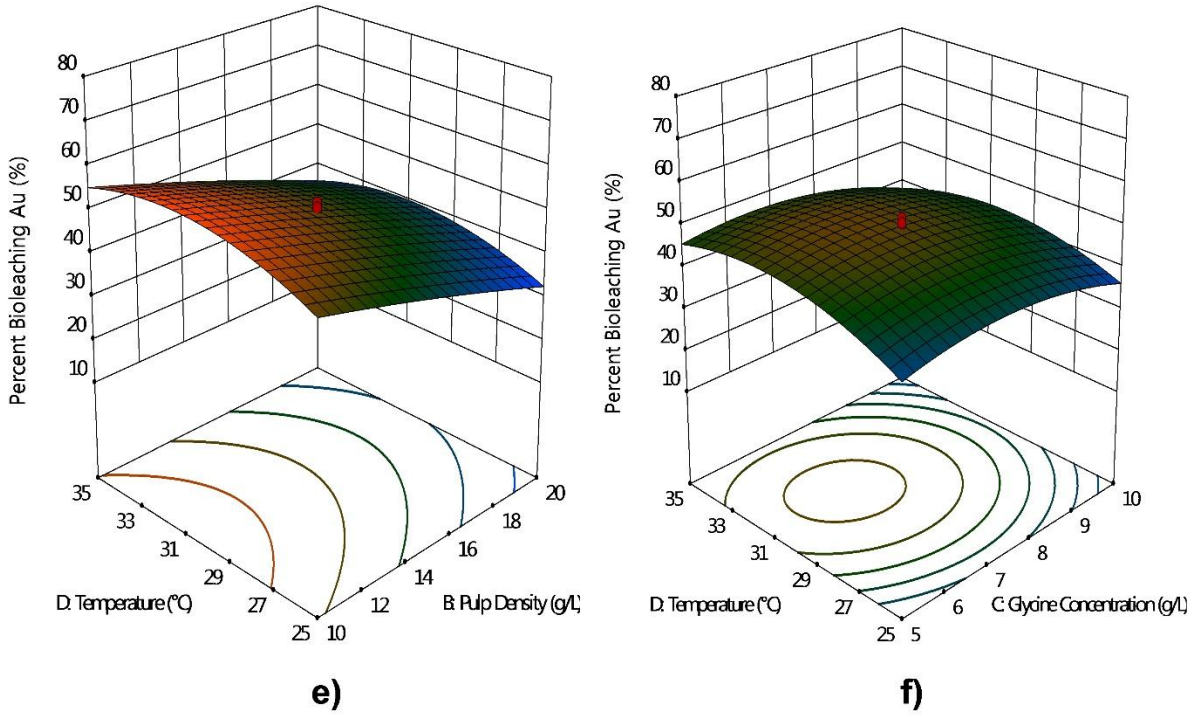
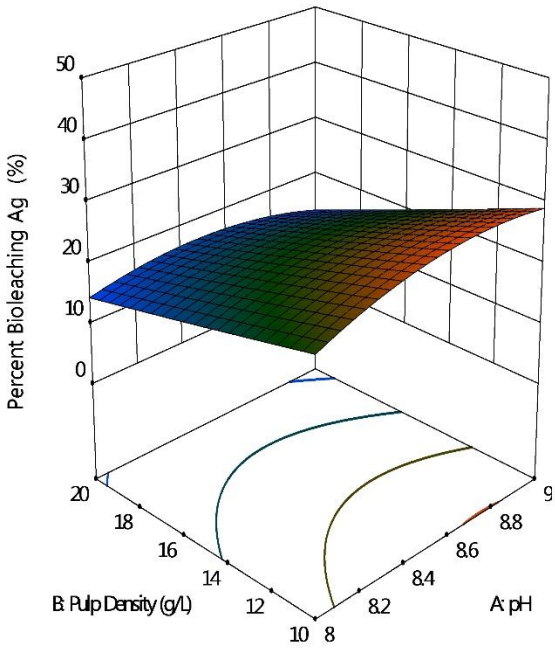


Figure 4.18: 3D surface plot for percent bioleaching of Au by *P. balearica* **a)** pulp density & pH, **b)** glycine & pH, **c)** temperature & pH, **d)** glycine & pulp density, **e)** temperature & pulp density, **f)** temperature & glycine

The interaction effect of glycine and pH on Ag recovery was significant ($p < 0.01$). The contours of Fig. 4.19d were elliptical curves that indicate the interaction among the parameters. The Fig. 4.19e illustrates the effect of temperature and pulp density on Ag mobilization. It was observed that Ag leaching increased with increase in temperature from 25°C to 32°C. This may correspond to the fact that maximum cyanide production takes place at a temperature range of 25°C to 35°C [42]. The Fig. 4.19e exhibit a circular contour that indicates no interaction of temperature and glycine concentrations on Ag recovery. The results revealed pulp density as the most influential parameter during metals mobilization from waste PCBs because the Ag mobilization decreased as pulp density increased. Overall, *P. balearica* recovered 40% of Ag using CCD-RSM from waste CPCBs.


Percent Bioleaching Ag (%)
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B: Pulp Density (g/L)
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A: pH
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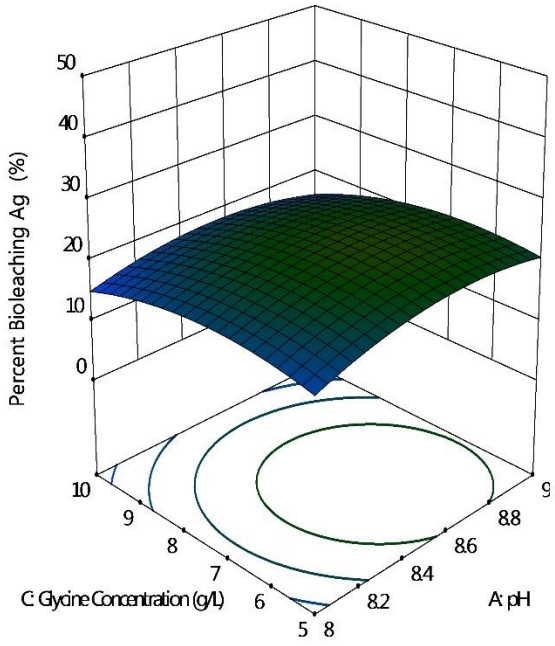
a)


Percent Bioleaching Ag (%)
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C: Glycine Concentration (g/L)
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A: pH
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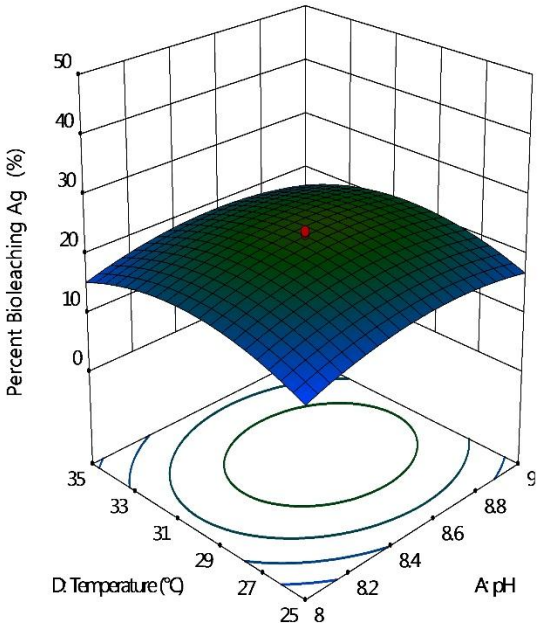
b)


Percent Bioleaching Ag (%)
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D: Temperature (°C)
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A: pH
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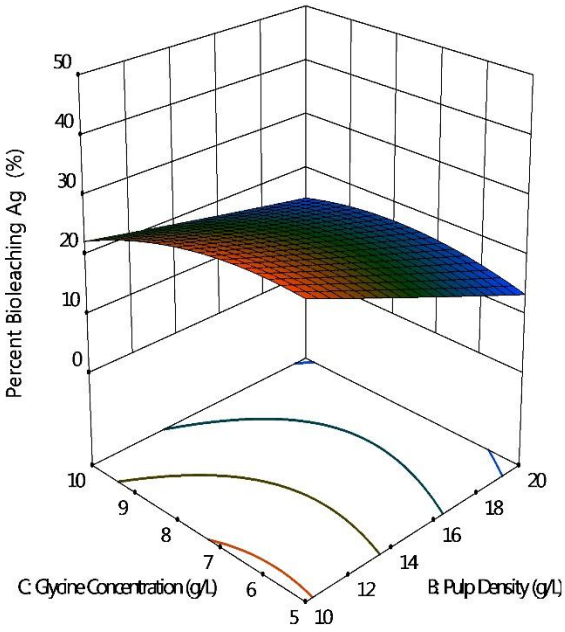
c)


Percent Bioleaching Ag (%)
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20
10
0
C: Glycine Concentration (g/L)
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6
5
B: Pulp Density (g/L)
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16
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20

d)

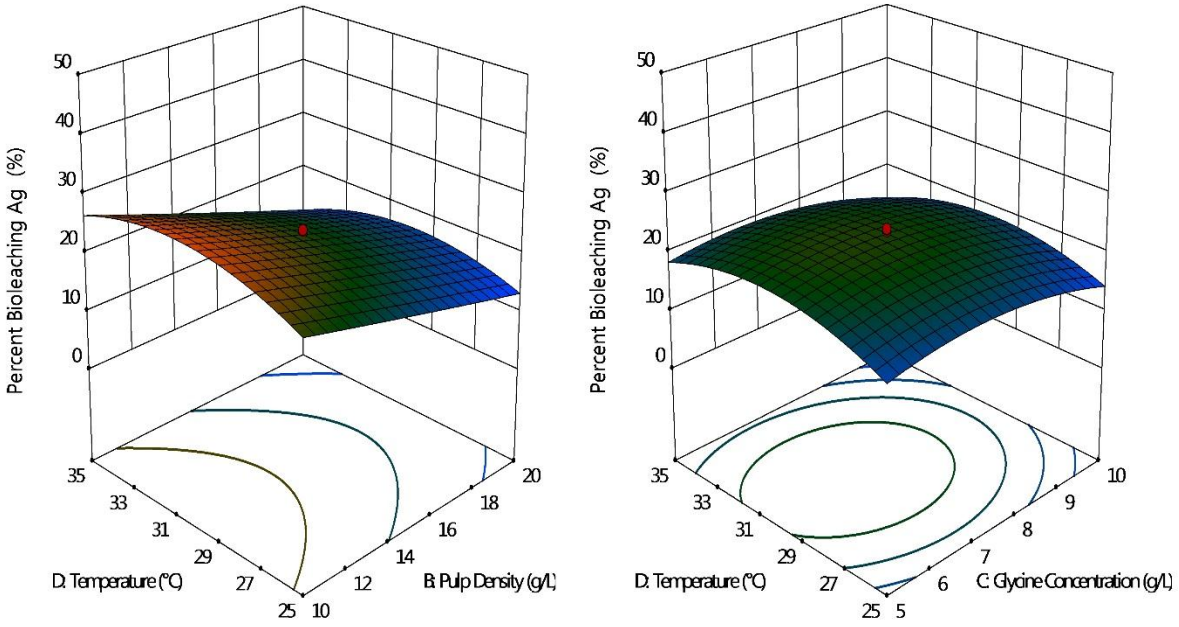


Figure 4.19 3D surface plot for percent bioleaching of Ag by *P. balearica* **a)** pulp density & pH, **b)** glycine & pH, **c)** temperature & pH, **d)** glycine & pulp density, **e)** temperature & pulp density, **f)** temperature & glycine

4.11.4 Model validation

The validity and accuracy of the predicted models were confirmed through bioleaching experiments (duplicates) in shake flaks under optimum conditions as proposed by the CCD-RSM. The CCD-RSM predicted a maximum dissolution of 80.4%, 67.8% and 40.1% of Cu, Au, and Ag, respectively from waste CPCBs, using *P. balearica* (Table 4.12). The optimum conditions predicted for maximum dissolution of Cu, Au, and Ag were as follows: 5 g/L pulp density, 8.6 pH, 6.8 g/L glycine concertation, and 31.2°C, respectively. The two-step bioleaching using *P. balearica* under above mentioned optimized conditions resulted in 81.7%, 73.9%, and 41.6% dissolution of Cu, Au, and Ag from waste CPCBs. The experimental responses are in accordance with the predicted responses, thus validating the accuracy of the models (Table 4.12). Control experiments in absence of bacteria showed mobilization of Cu (21.6%) only.

4.12 Bioleaching of metals (Cu, Au, and Ag) in Shake flasks of 2 L capacity

C. violaceum, *P. balearica*, and *B. megaterium* were subjected to two-step bioleaching in shake flasks of 2 L capacity containing 1 L LB medium. Bioleaching was performed under the conditions optimized through OFAT approach i.e. 5 g/L glycine concentration, 10 g/L pulp density, 9.0 pH and 30 °C, respectively. The three bacterial strains showed similar metals bioleaching as in case of 100 mL medium volume (Fig. 4.20). *C. violaceum* showed highest metals leaching of Cu (82.2±6%), Au (72.9±5%) and Ag (14.2±3%), respectively, after seven days of bioleaching. *P. balearica* was able to mobilize 73.8±5, 64.6±5 and 31.2±4% of Cu, Au, and Ag, respectively. *B. megaterium* mobilized 69.4±7, 65.2±6, 11.8±2% of Cu, Au, and Ag, respectively. Overall, *C. violaceum* showed maximum mobilization of Cu and Au whereas, Ag mobilization was maximum with *P. balearica*. However, to enhance the industrial suitability of the process; further, scale-up studies need to be carried out.

Table 4.12: Predicted vs. observed responses of Cu, Au & Ag under optimal conditions

Response (%)	**Predicted response**	**Observed response**	**95% PI low**	**95% PI high**
Cu recovery	80.4	81.7±3.4	71.2	89.6
Au recovery	67.6	73.9±5.9	51.7	83.6
Ag recovery	39.2	41.6±2.4	32.8	45.7

4.13 Morphological Characterization of waste CPCBs

The representative sample of homogenized waste CPCBs before and after bioleaching was observed for its morphology under the SEM (Fig. 4.21). The Fig. 4.21a represents the morphology of untreated e-waste samples demonstrated the presence of particles with varying sizes, texture and shapes. Majority of particles were rod-like with crystal and flakes on the surface. The similar morphology of e-waste sample was observed by [8, 148, 162]. According to Priya and Hait [148], the heterogeneity in the texture, size, and shape of particles was explicit of numerous tensile and shearing forcing in which pulverization of the PCBs was accomplished. The observed discrepancies in particle shapes and sizes may correspond to a

diversity of materials present in PCBs for example, silica, polymers, glass fibers and cellulose papers [163]. Following bioleaching, the surface of the CPCBs appeared eroded with exposed cervices and cracks (Fig. 4.21b). It was noted that prior to bioleaching; e-waste surface was rough however it became smooth after bioleaching.

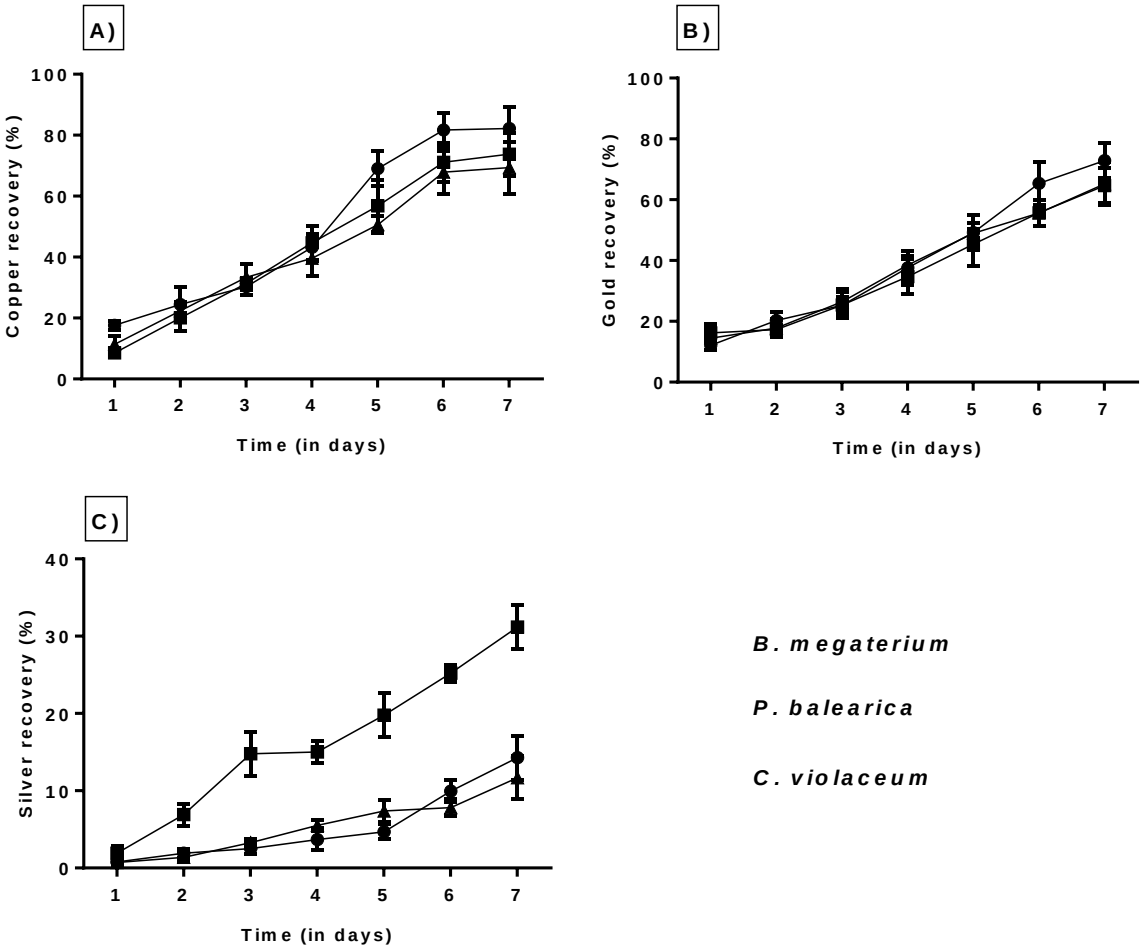


Figure 4.20: Bioleaching of metals from CPCBs P4 in shake flasks reactors of 2 L capacity under optimized conditions.

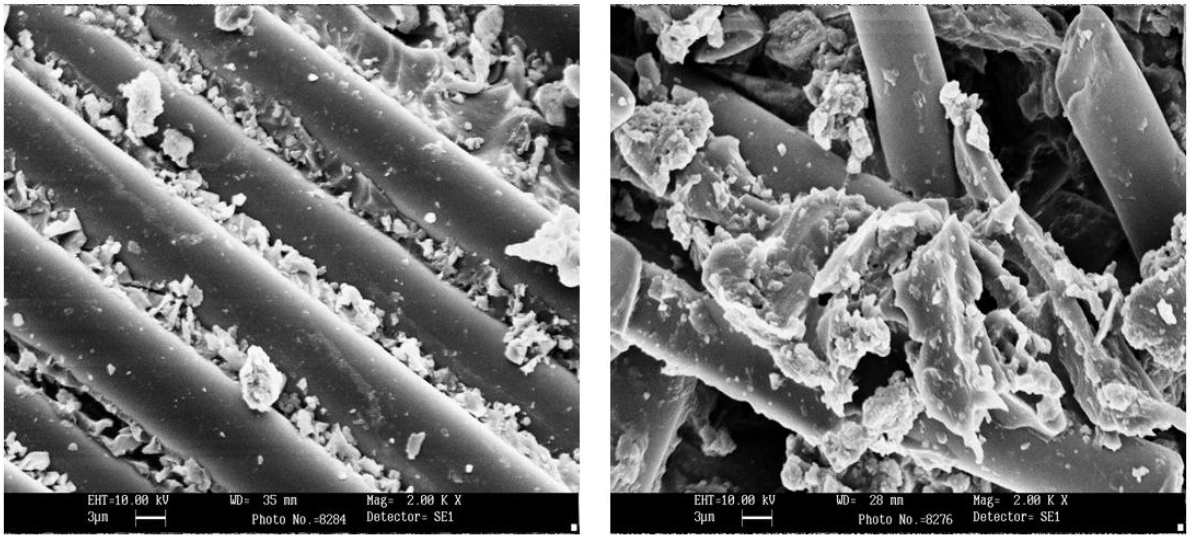
4.14 Bioleaching of metals from waste CPCBs-P5

4.14.1 Pretreatment of waste P5

The CPCBs waste P5 contains a high amount of Cu (9.8%) and base metals. The Cu and other base metals form complexes with cyanide and their presence at a higher

concentration in e-waste cause hindrance in the recovery of gold [164, 165]. Thus, CPCBs P5 was pretreated with nitric acid (HNO_3) to remove and recover Cu and other metals in order to enhance Au recovery. The pretreatment with acid leads to 82.1, 57.6, 70.4, 65.8, 47.4 and 90.2% of Cu, Ni, Fe, Cr, Ag and Zn recovery without significantly altering the concentration of Au (Table 4.13). Nitric acid-mediated dissolution of Cu is represented in Eq. (4.15). The results of the study are in accordance with those of Sahni et al. [18] and Natarajan and Ting [8], reported nearly 72% and 80% removal of copper.

$$3Cu + 8HNO_3 \rightarrow 3Cu^{2+} + 6NO_3^- + 2NO\uparrow + 4H_2O \qquad (4.15)$$



(A) **(B)**

Figure 4.21: SEM micrograph of (A) Untreated e-waste (B) E-waste after bioleaching

4.14.2 Bioleaching of untreated and pretreated waste P5

The literature reported various pretreatment methods in order to enhance gold recovery from e-waste. Natarajan and Ting [8] pretreated e-scrap with nitric acid and enhanced biorecovery of Au (22.5% of the total in pretreated e-scrap) using mutated *C. violaceum* grown at pH 9.5 and a pulp density of 0.5 % (*w/v*). In another study by Sahni et al. [18], SIMW was pretreated with nitric acid and subsequently 70.61% of Cu was recovered from the pretreated SIMW in a two-step bioleaching process using *C. violaceum*. The bioleaching of Au from

SIMW was significantly low because of the high concentration of Cu (20.68%) present in SIMW even after pretreatment compared to pretreated e-scrap (3.04%). In a recent study, Das and Ting [162] removed 85.5% of Cu from the total in the e-waste through a reaction mixture of H_2SO_4 and H_2O_2 (1:1, *v/v*). Following pretreatment, metabolically engineered *C. violaceum* pBAD strain recovered 36.4% and 24.7% of Au from the pretreated waste by two-step bioleaching and spent medium leaching, respectively. Other than chemical pretreatment, researchers have used microorganisms to remove Cu from e-waste so as to enhance Au recovery. Mara et al. [45] removed all the base metals from shredding dust of WEEE using *A. thiooxidans* in 8 days of bioleaching. In the second step, they used cyanogenic *P. putida* and extracted 48% of Au in 3 h. In another study, Isıldar et al. [126] used a mixed culture of *A. ferrivorans* and *A. thiooxidans* and removed 98.4% of Cu from e-waste. In the subsequent step, using *P. putida* they recovered 44% of Au from e-waste in 2 days of bioleaching.

Table 4.13: Chemical composition of pretreated and untreated waste P5 using acid digestion by aqua regia

Metals	Untreated (% w/w)	Pretreated (% w/w)	% Removal with pretreatment
Cu	9.8 ± 0.42	1.70±0.14	82.6
Fe	4.01 ± 0.22	1.6±0.08	60.1
Ni	0.11 ± 0.08	0.004±0.004	96.36
Cr	0.15 ± 0.001	0.1±0.008	33.33
Co	0.008 ± 0.09	0.004±0.008	50.00
Zn	0.04± 0.015	0.004±0.001	90.00
Ag	0.14 ± 0.009	0.07±0.001	47.36
Au	0.03 ± 0.002	0.025±0.004	15.44

To enhance the commercial viability of bioleaching process, it is necessary to investigate the bioleaching potential of bacterial strains on unprocessed or virgin waste. *C. violaceum*, *P. balearica* and *B. megaterium* were employed for bioleaching from waste CPCBs-P5. From the results of Fig. 4.22, it was observed that *C. violaceum* (48.4%) showed maximum leaching of Cu followed by *P. balearica* (44.3%) and *B. megaterium* (36.5%), respectively. Whereas, Au and Ag's leaching was significantly low. This may be due to high concentration (9.8%) of copper present in the waste P5, which might have consumed all the lixiviant produced by bacteria resulted in poor metal mobilization Au and Ag. The pretreated

waste showed higher bioleaching of Cu, Au, and Ag using cyanogenic microorganisms compared to untreated waste. Bioleaching of pretreated waste P5 resulted in 81.2, 79.9, and 69.8% recovery of Cu by *C. violaceum*, *P. balearica* and *B. megaterium*, respectively. The Au recovery from pretreated waste P5 was enhanced to 52.4%, 47%, and 42% compared to 7.1%, 4.6%, 4.2% bioleaching from waste P4 by *C. violaceum*, *P. balearica*, and *B. megaterium*, respectively. In case of Ag bioleaching from pretreated waste P5, *C. violaceum*, *P. balearica*, and *B. megaterium* showed 18.3, 25.9 and 16.3% mobilization of Ag. Un-inoculated control showed mobilization of Cu in both untreated (22.3±5%) and pretreated (31.9±5%) e-waste, but no Au and Ag were detected during the entire bioleaching period. The results showed that bioleaching using cyanogenic microorganisms from metals enriched waste CPCBs is not economical. However, a combination of chemical and biological methods may be successful in the recovery of metals from e-waste.

4.15 Comparative bioleaching assessment among waste CPCBs P4 and P5

It is of interest to comparatively assess the percentage bioleaching of metals by cyanogenic microorganisms among waste P4 and P5. It was observed that the percentage bioleaching of metals was higher for waste P4 than P5. The percentage of Cu bioleached form waste P4 by *C. violaceum*, *P. balearica* and *B. megaterium* was 87.5±7, 77.4±7, and 72.7±5%, respectively, whereas, in case of waste P5 the percentage of Cu bioleached was 48.3±1.4, 44.2±1.2, and 36.3±0.5%, respectively. The percentage bioleaching of precious metals i.e. Au and Ag were significantly low in the waste P5 compared to waste P4 (Table 4.14). The amount of Au bioleached by *C. violaceum* from waste P4 was 73.6±4% whereas, from waste P5 only 7.1±0.1% Au was extracted. This is because the metal content in the waste P4 was significantly lower than in P5 (Table 4.2). In addition to overall metal content, the amount of Cu in P4 (2.34%) is lower than in P5 (9.8%) because of which lixiviant produced by bacteria is more available to other metals too in the waste P4. Whereas, in the waste P5, it is mainly consumed by Cu and hence the low mobilization of precious metals. The results of the study are consistent with literature reports in which higher metals bioleaching was recorded for e-waste containing low metals concentrations i.e. e-waste dust [24, 45], as compared to the e-waste containing higher concentration [18, 30, 40].

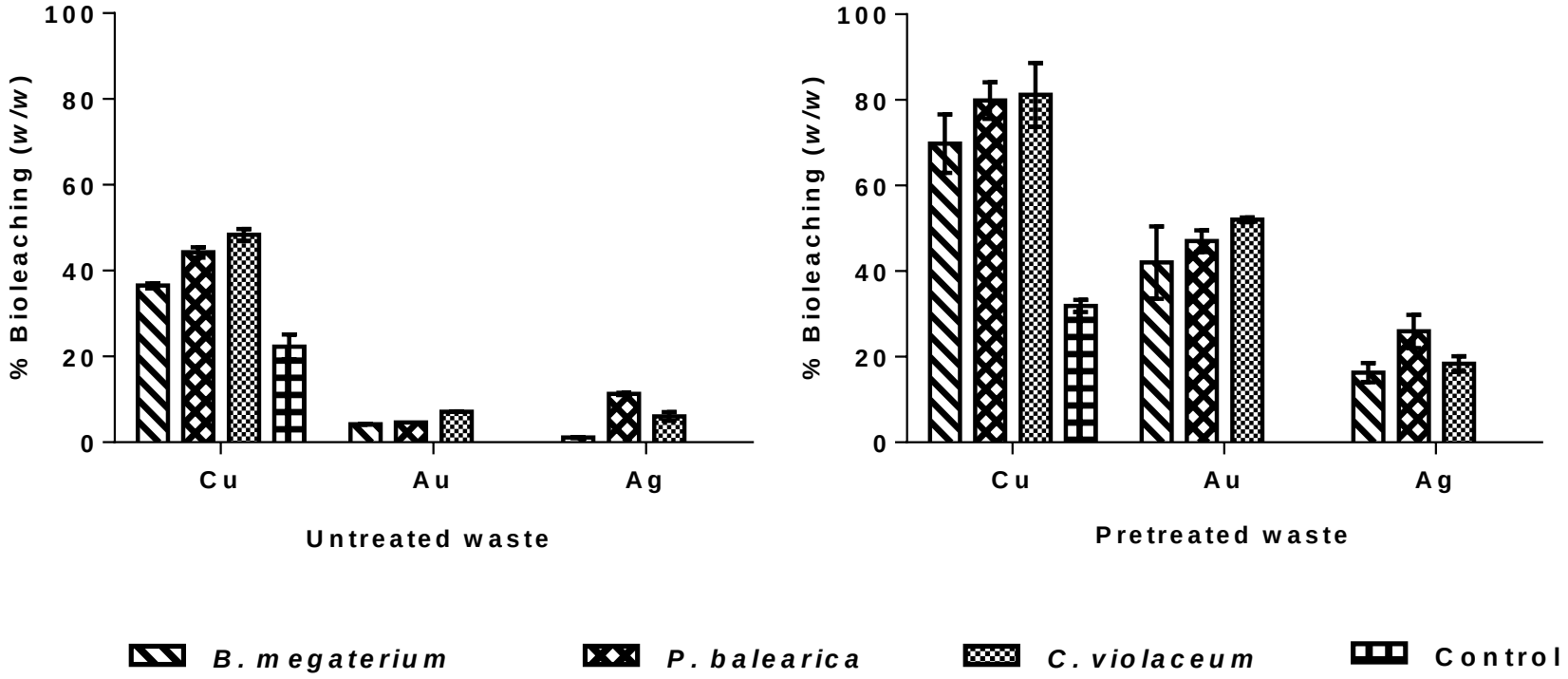


Figure 4.22: Comparative bioleaching of Cu, Au, and Ag from untreated and pretreated waste P5 using cyanogenic microorganisms

Table 4.14: Comparative bioleaching assessment among waste P4 and P5 using cyanogenic microorganisms

Bacteria	P4 (% bioleaching)			P5 (% bioleaching)		
	Cu	Au	Ag	Cu	Au	Ag
C. violaceum	87.5±7	73.6±4	13.02±2	48.3±1.4	7.1±0.1	6.0±0.9
P. balearica	77.4±7	68.5±5	33.8±4	44.2±1.2	4.7±0.2	11.2±0.3
B. megaterium	72.7±5	66.6±6	15.3±3	36.3±0.5	4.2±0.07	1.13±0.05

4.16 Final disposal of the waste CPCBs

The residual CPCBs P4 and P5 after bioleaching were characterized for metal content using aqua regia digestion method. The metallic composition of both the residual wastes was presented in Table 4.15. The Cu content in residual waste P4 was 0.15% compared to the initial 2.34%. The Fe (0.56%) content was maximum among all the metals analyzed. This may be because Cu has high affinity towards cyanide, which forms a water-soluble complex during cyanogenic bioleaching. The precious metals Au and Ag in residual waste P4 were 0.002 and 0.03% compared to the initial 0.008 and 0.04%, respectively. Other metals like Ni, Co, Zn, and Cr were 0.004, 0.001, 0.009, and 0.07%, respectively, were also present but in significantly low quantities. Although bioleaching using cyanogenic microorganisms have recovered metals from waste CPCBs yet complete recovery of metals were not attained. Similarly in the residual waste P5, Fe (0.65%) content was maximum than Cu (0.5%). The total Cu and Fe present in the P5 was 9.8% and 4.0%. The significant amount of metals were leached from P5 through a combination of chemical (pretreatment using HNO_3) and biological methods (bioleaching using cyanogenic bacteria). Therefore, the final waste needs special attention and safe disposal. In our case we have collected the residual e-waste for our industrial collaborator Exigo Recycling Pvt. Ltd. to safely dispose it off at their treatment storage and disposal facility (TSDF).

We have also consulted the experts/scientists working on bioleaching of metals from e-waste that how do they dispose off their residual e-waste after bioleaching? They hand over

the residual e-waste to authorized recyclers. It is recommended to handover the residual e-waste to TSDF site of the region/state.

Table 4.15: The metals content of residual (after bioleaching) waste CPCBs

Metals	**Concentration (% *w/w*)**	
	P4	**P5**
Cu	0.15 ± 0.02	0.5± 0.01
Fe	0.56 ± 0.06	0.65±0.1
Ni	0.004± 0.006	0.08±0.02
Co	0.001 ± 0.01	0.001±0.001
Cr	0.07 ± 0.005	0.08±0.02
Zn	0.009 ± 0.001	0.002±0.002
Ag	0.03 ± 0.005	0.05±0.009
Au	0.002 ± 0.007	0.01±0.002

SUMMARY & CONCLUSION

The findings of present work have provided an environmentally agreeable and sustainable solution i.e. bioleaching for treatment and metals recovery from e-waste; an important global issue. The major findings are summarized as follows:

The metal content of different size fraction of waste CPCBs showed a large heterogeneity. The amount of Cu (29.3%) was maximum in waste CPCBs ≥0.71 mm, while Au (0.008%) and Ag (0.04%) were highest in the CPCBs of ≤0.15 mm particle size fractions. Overall, the Cu content varied among different particle size fractions from 2.3% (≤0.15 mm) to 29.3% (≥0.71 mm). The metals content of CPCBs also showed variation when digested for a time period of 1, 2, and 3 h, respectively, using aqua regia. The metallic composition of waste CPCBs-P5 (unprocessed/virgin; ≤0.15 mm) was higher than P4 (processed/dust; ≤0.15 mm); for example, the Au and Ag content in P4 was 0.008±0.001% and 0.04±0.004%, while in P4 it was 0.14±0.01% and 0.03±0.002%, respectively. The significant quantities of metals in the e-waste both in dust and unprocessed/virgin waste makes it a suitable resource to extract metals.

Indigenous bacterial strains from abandoned gold mine and e-waste recycling facility were isolated and employed for bioleaching of waste CPCBs. Initial screening for bioleaching of Cu, Au, and Ag from waste CPCBs showed four prominent bacterial strains viz. *P. balearica*, *B. megaterium*, *Bacillus* sp. and *L. sphaericus*. However, *C. violaceum* a reference strain of known bioleaching potential showed higher bioleaching activity during initial screening. All the bacterial strains showed cyanide producing ability which infers the mechanism of Cu, Au, and Ag leaching is cyanide-based. *P. balearica* and *L. sphaericus* are first time applied for bioleaching of Cu, Au, and Ag from the e-waste (CPCBs in our case).

The toxicity assessment and dose-response analysis showed higher EC_{50} values for *P. balearica* SAE1 followed by *Bacillus* sp. SAG3, *B. megaterium* SAG1 and *L. sphaericus* SAG2 and *C. violaceum* were Log 2.5 (325.7 g/L), 2.1 (128.9 g/L), 1.9 (98.7 g/L), 1.9 (90.8 g/L), 1.9 (83.70 g/L) respectively. The higher resistance of indigenous bacterial strains towards e-waste toxicity confirms the viable operation range and technological feasibility of metals bioleaching from waste CPCBs. The toxicity assessment of e-waste on bacterial strains is first time investigated and reported in the study.

To maximize precious metals dissolution; optimization was conducted using both the OFAT and CCD-RSM approach. The process parameters such as pH, pulp density, temperature, and precursor molecule (glycine) were optimized to enhance metal mobilization. The maximum metals recovery under OFAT optimized conditions occurred at 10 g/L pulp density, 9.0 pH, 5 g/L glycine, and 30°C temperature by *C. violaceum*; extracted 87.5% and 73.6% of Cu and Au, respectively. Whereas, Ag (33.8%) mobilization was maximum by *P. balearica*. The kinetic modeling results showed that bioleaching using cyanogenic microorganisms followed the first-order reaction kinetics, where the rate of metal solubilization from CPCBs depends upon microbial lixiviant production. The CCD-RSM optimization extracted 81.7, 73.9 and 41.6% of Cu, Au, and Ag by *P. balearica* at pulp density 5 g/L, glycine concentration 6.8 g/L, initial pH 8.6, and temperature 31.2°C, respectively. The CCD-RSM proposed three polynomial quadratic models which can be used as an effective tool to predict bioleaching of Cu, Au, and Ag from e-waste using cyanogenic microorganisms. A combination of chemical and biological methods showed significant metals mobilization from the unprocessed waste of CPCBs containing higher metals concentration. The residual CPCBs after bioleaching was sent back to the authorized recyclers i.e. Exigo Recycling Pvt. Ltd. for final disposal through TSDF.

In conclusion, the indigenous bacterial strains highly resistant to e-waste toxicity may become a suitable candidate for resource recovery from e-waste at industrial scale bioleaching operations. Our findings suggest that bioleaching of metals from e-waste is significant when used to treat waste of low metallic load i.e. e-waste dust. However, for e-waste with higher metallic load, a combination of chemical and biological methods can be adopted for metals extraction. The safe disposal of residual e-waste after resource recovery is still a major cause of concern and needs the attention of the scientific community. These research efforts will surely pave the way towards pursuing the recovery of material at high supply risk i.e. precious metals and REEs, conservation of primary/natural resources, prevent environmental degradation, and significantly promote the concept of circular economy. Further, current research offers convincing evidence on bioleaching for its possible application to recover metals from e-waste at industrial scale operations.

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APPENDIX

A.1 *Bacillus megaterium* SAG1, 16S ribosomal RNA gene, partial (KU163234)

TGCAGTCGAGCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAA
CACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCGGATA
GGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCC
CGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTT
TCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGA
CCGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTTATACGTAGGTGG
CAAGCGTTATCCGGGATTATTGGGCGTAAAGCGGGCCCCAGGCCGTTTCTTAAGTCTTATGT
GGAAGCCCACGGCTCCCCCGGGGGAAGGGCCTTTGGAAACTGGGGAAATTGAATTCCGGAGA

A.2 *Lysinibacillus sphaericus* SAG2, 16S ribosomal RNA gene, partial sequence (KU163235)

GAGTGGAGGTGGCGAGGCGACTATCTGGTCTGTACTGACACTGAGGCGCGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGG
GTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCA
AGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGTTGACCACTGTAGAGATATGGTT
TCCCCTTCGGGGGCAACGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCAC
TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACGATACAAACGGTTGCCAACTCGCGAGA
GGGAGCTAATCCGATAAAGTCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAA
GCCGGAATCGCTAGTAATCGCGGATCACCATGCCGCGGTGAAT

A.3 *Bacillus* sp. SAG3 16S ribosomal RNA gene, partial sequence (KU163236)

CGACTTTCTGGTCTGTACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTG
CTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
CTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTG
ACAAACCGGAGGAAGGTGGGGATGAGGTCAAATCATGCCGGCCCTCGTGACCAGGGCTACGC
ACGTGGTACAATGCAGAGTACTGGGGGGTGCAGGACCGCGAGGTGGATCT

A4. *Bacillus amyloliquefaciens* SAG4 16S ribosomal RNA gene, partial sequence (KU163237)

TCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTG
CAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT

ACCAGGTCTTGACATCCTCTGACAATCCTAGAGATGGACGTCCCCTTCGGGGGCAGAGTGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTGACTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CTACAATGGACAGAACAAAGGGCAGCGAAACCGCGGGGTTAAGCCAATCCCACAAATCTGTT
CTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAG
TTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTGGGACAGA
TGATTGTGTG

A5. *Bacillus* sp. SAG5 16S ribosomal RNA gene, partial sequence (KU163238)

TCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTG
CAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT
ACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTG
TTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAG
AGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAAGTGGGACA
GATGATTGGGGTGAAGTCG

A6. *Brevibacterium frigoritolerans* SAG6, 16S ribosomal RNA gene, partial sequence (KU163239)

TTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGC
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCCTTCGGGGGACAGAGTG
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACAC
GTGCTACAATGGATGGTACAAAGGGCTGCAAACCTGCGAAGGTAGCGAATCCCATAAAGCCA
TTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAG
AGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTCATGGAGCCAGCCGCCTAAGTGGG

A7. *Chryseomicrobium amylolyticum* SAG7, 16S ribosomal RNA gene, partial sequence (KU163240)

TCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCA
GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCCCGCCGACCGCCCAGGAGACTGGGCCTTCCCTTCGGGGACGGCGGTGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG

CGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGATGGTACAAAGGGCTGCGAACCCGCGAGGGGGGCCAATCCCATAAAACCAT
TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGTGG
ATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGA
GTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTATGGAGCCAGCCGCCGAAAGTGGGACAG
ATGATT

A8. *Bacillus safensis* SAG8, 16S ribosomal RNA gene, partial Sequence (KU163241)

TGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGTGAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
GGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGG
TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT
ACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCAATCCCACAAATCTGTTCTC
AGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTT
GTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAAGTGGGACAGATGA
TTGGG

A9. *Pseudomonas balearica* SAE1, 16S ribosomal RNA gene, partial sequence (KU053282)

CTGGGCTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGC
TAACGCATTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
GGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCA
ACCCTTGTCCTTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGGAGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTG
CTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGAT
CGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAA
TCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCTCCAGAAGTAGCTAGTCTAACCTTCGGGGGGACGGTTACCACGGAGTGATTCAT
GACTGGGGTGAA

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1. **A. Kumar** and S. Kumar (2015) Accession No.:- KU053282, KU163234, KU163235, KU163236, KU163237, KU163238, KU163239, KU163240, KU163241.