Optimization of metal leaching bacteria and to characterize their protein expression/pathways using *in silico* approach

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

B. Tech-M. Tech (Integrated)

In Biotechnology Submitted by

Shivangini Singh (161811)

Guide - Dr. Sudhir Kumar

Co-Guide – Dr. Jata Shankar

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, Waknaghat,

Solan-173234, Himachal Pradesh

May- 2021

ACKNOWLEDGEMENT

Presentation, inspiration and motivation have always played a key role in the success of any venture. This journey would never have been completed without the support, well wishes and encouragement of many people. I would like to acknowledge all those who contributed to this project and helped me to reach this destination.

First and foremost, I express my heartfelt gratitude to the ***Almighty God*** who blessed me with all the favorable circumstances to go through the work.

My deepest gratitude to my mentor "***Dr. Sudhir Kumar" Professor and Head, Department of Biotechnology and Bioinformatics***. It was only due to his solemn efforts, resolute guidance, steadfast encouragement, meticulous supervision and positive attitude that I have reached here. His patience and motivation encouraged me throughout the work. He was always there with his vision, encouragement and advice to proceed through the work and complete it.

I also express my heartfelt gratitude to my co-guide "Dr. Jata Shankar" who kept on motivating me in my entire journey. This work would never have been done completely without his guidance and motivation.

I also owe my sincere thanks to **JUIT** administration, ***Vice Chancellor, Prof. (Dr.) Vinod Kumar, Director and Academic Head, Prof. (Dr.) Samir Dev Gupta and Registrar Maj. Gen. Bassi*** for all the support so that we can complete our work on time.

Last but not the least, I would like to pay out deepest gratitude to my parents whose love and support helped a lot to reach here. Their unconditional love, guidance, care, support, innumerable sacrifice and trust have always encouraged me in every step of life.

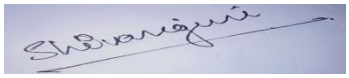
CERTIFICATE

Candidate's Declaration

I hereby declare that the work presented in this report entitled **"***Optimization of metal leaching bacteria and to characterize their protein expression/pathways using in silico approach*" in partial fulfillment of the requirements for the award of the degree of Masters of Technology in biotechnology submitted in the department of biotechnology and bioinformatics, Jaypee University of Information Technology, Waknaghat is an original record of my own work carried out over a period from July 2020 to May 2021 under the supervision of Dr Sudhir Kumar (HOD, Department of biotechnology and bioinformatics) and Dr. Jata Shankar.

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

(Student Signature)



Shivangini Singh
(161811)

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

Guide: Prof (Dr.) Sudhir Kumar

Signature

Co-Guide: Dr. Jata Shankar

Signature

Department name: Department of Biotechnology and Bioinformatics
Date: June 17, 2021

Contents

1. Abstract

The present study focusses on electronic waste as a useful resource for the extraction of precious metals, also highlights the importance of eco-friendly techniques which needs to be used for the proper disposal of electronic waste. Further, it presents about the diversity of microorganisms which have been reported in different studies for their application in extracting precious as well as toxic metals. Various *in silico* approaches have been used to do the structural analysis of the protein and these tools include UniProt, ExPASy-ProtParam and ExPASy SIB Bioinformatics SOPMA tool. Hydrogen Cyanide synthase has been studied in this work and its study includes physical parameters, primary structure analysis and secondary structure analysis. The isoelectric point (pI) of this protein ranges between 4.99-10.43 which indicates the diverse alkaline and acidic nature of the protein. The Average molecular weight of the protein was calculated to be the 46675.24 Da. Instability index range lies between 26.69-50.66, which indicates the diverse structure of the protein. Aliphatic index of the protein lies between 83.07-101.59 which shows that the protein has higher range of temperature stability. *Streptomyces lavendulae subsp. Lavendulae* shows the highest thermostability for this protein with a value of 101.59. Out of all the 23 retrieved sequences, 20 sequences seem to have hydrophilic and non-polar nature. The 5 dominant amino acids were found to be Leucine (10.3%), Alanine (9.8%), Glycine (9.3%), Valine (6.8%) and Glutamic acid (6.3%). The maximum mean value of random coils i.e., 38.86% clearly demonstrates that this protein structures do not have the regular secondary structure and is not characterized by any regular H-bonding patterns, followed by α-helix 37.15%, extended strand 17.49% and β-turns 8.08%.

2. Introduction

A global issue in form of e-waste is alarming in the different parts of the world. Countries are showing a tremendous growth in the accumulation of this waste because of the lack of proper disposing facilities [1]. There has been an exponential increase in the generation of e-waste due to the high demand and use electronic applications. E-waste contains various toxic and hazardous metals (lead, cadmium, mercury, beryllium etc.) which has raised various health and environmental concerns and to their disposal also [2]. E-waste is considered as an issue of great concern due to its complex composition and increasing volumes. Therefore, e-waste recycling is an important subject for both- waste management as well as recovery of valuable metals [3]. Increasing value of this complex is of great concern because e-waste is considered to be the fastest growing source of waste worldwide [4].

The recent development in the field of Science has led to a substantial increase in electrical and electronic euipments (EEE). PCBs accounts for about 3-5% of total WEEE which is collected each year. They consist of about 40% metals, 30% ceramics and 30% plastics. The fraction of metal includes Cu (20%), Al (5%), Ni (1%), Pb (1.5%), Zn (2%) and Sn (3%) (w/w) [5, 6,7].

There are various recycling techniques which are used for its proper disposal which includes hydrometallurgy, hydrometallurgy and bio-hydrometallurgy (bioleaching). Hydrometallurgy and pyrometallurgy are considered to be the fastest methods as they consume less time and their metal extraction is also rapid as compared to the bioleaching. But these two methods do have some disadvantages which make bioleaching more preferable and their disadvantages include high toxicity, high metal loss, extensive energy requirement and high investment cost. In comparison to them, bioleaching is an eco-friendly and cost-effective method which has been employed by various researchers [2].

Enzymes are considered to be one of the powerful tools which helps to maintain an eco-friendly environment by using itself in the management of waste. Bioremediation using enzymes is potentially a faster method for the removal of waste and also extraction of metals without causing any damage to the environment [8]. Therefore, the present work has been carried out to study the enzymes that have shown their potential in extracting metals from Printed Circuit Boards (PCBs). *In silico* tools have been used to study the bacterial Hydrogen Cyanide Synthase.

The physical parameters, primary and secondary structures of bacterial Hydrogen Cyanide Synthase were analysed by studying different physiochemical parameters viz. molecular

weight, instability index, aliphatic index, grand average of hydrophobicity (GRAVY), no. of amino acids and its composition, α-helices, β-turns, extended strands and random coils. Tools which have been used to study are - UniProt data bank, ExPASy-ProtParam, ExPASy SIB Bioinformatics SOPMA tool [9,10,11].

The project entitled "*Optimization of metal leaching bacteria and to characterize their gene expression/pathways using in silico approach*" is concerned with three major objectives.

- The first objective is to characterize the potent microorganisms involved in the process of bioleaching.
- Second objective is to determine the cyanide lixiviant and to optimize different parameters like pH, temperature and pulp density.
- And the last objective is to identify or characterize genes or pathways involved in bioleaching using in silico approach.

3. Review of Literature

1. E-waste – An issue of great concern.

E-waste has become an issue of worldwide concern because of the rapidly growing technology and shorter lifespan of electrical products. This is a new challenge for all the business organizations and policy makers. The volume is increasing on a large scale every year and it is also believed that this is one of the most critical problems of twenty-first century to deal with [12]. It is a type of waste which consists of waste that is generated from electronic equipment and various appliances that are used in houses which are no more fit for the use in homes and regarded as waste. These devices and appliances include most commonly cell phones, computers, laptops, televisions sets, ACs and coolers and many more. E-waste has 1000 of different substances stored in it and many of the substances are toxic in nature which can cause harm to the environment as well as to human beings who are involved in recycling them for the sake of their livelihood. Those toxic components include mercury, lead, cadmium, Brominated flame retardants etc and they require to be handled safely as they may cause harm to the life of a person involved in recycling. The development of technology as well as rapid innovations has contributed towards increasing of e-waste across the world. This rapid development and innovation in technology has actually resulted in the short life span of the product (less than 2 years). The obsolescence of the product is also one the important causes in increasing e-waste. Along with this one more cause for accumulation of e-waste is lack of responsibility towards maintaining it and handling it, both by the people as well as government.

Lack of laws which needs to made compulsory every citizen of the country is the main reason behind recycling of e-waste in informal sectors and it leads to the release of highly toxic substances in a higher amount which is basically a result of not handling the waste properly. The absence of proper disposal techniques and recycling facility has led e-waste to find its way to the informal sectors for example – scarp dealers, various local dismantlers etc. The existing infrastructure does not have the proper recycling equipments while the informal sector has all the dismantling facilities [13]. As most of the e-waste goes directly into the landfills and its exposure to the environmental toxins get increased, the risk of getting cancer and other neurological disorders become higher [12].

According to the UN's Global e-waste monitor 2020, 53.6 Mt e-waste was generated in 2019 - directly 21% higher in just 5 years and predictably it will reach 74 Mt by 2030. Only 17.4% of e-waste has been recycled properly in 2019 and also Asia is holding first place in generation of e-waste in 2019 which is 24.9 Mt followed by America (13.2 Mt) and Europe (12 Mt) [14].

Around 80% electronic products are in storage because of not knowing about how to use it and manage it, they can be called as unattended waste which gets mixed up with the other household wastes and ultimately goes to the landfills. It is not happening at household level only but also at industrial level there are no such rules and management polices to handle e-waste and minimize it. Management steps can be taken on industrial level at the time of its generation. There can be various ways to minimize its quantity - for e.g., modifications can be done in its manufacturing processes, adopting the way of inventory management, recover the old ones that are stored and reuse it. In the process of inventory management, a proper control on the materials used is there in order to use the hazardous metals in less quantity. Reduction in the quantity of hazardous metals and the raw materials lead to lessen the quantity of e-waste to an extent [15].

2. Inflow of e-waste

According to the current statistics about 44.7-50 Mt of electronic wastes are generated worldwide every year which is equal to around 6.1 kg per inhabitants. E-waste is occupying 1-3% of municipal waste on a global scale and continuously increasing with a rate of 3-5% every year. By the year of 2021, it is estimated to reach up to 52.2 Mt which equals 6.8 kg per inhabitants [16,17,18,19,20,21]. Tracking the amount of e-waste transported across different countries is a challenging task [16,18]. Perkins et al. reported that around 25% of e-waste is documented and recycled officially on a global scale and the remainder goes to the informal

sectors. By another report, around 20% i.e., 8.9 Mt of total e-waste is documented while remaining 80% i.e., 35.8 Mt is undocumented. The EU, U.S and the U.K. are the leading exporters to the developing countries like India, Nigeria and China, getting benefits from the low-cost disposal and labor [16,18,22,23].

Bakhiyi et al. has shown that e-waste management involves various challenges which includes growth of e-waste continuously, lack of harmony in defining e-waste, complex recycling procedures, fragility of formal recycling sectors and destruction of informal recycling sectors which are combined with unforeseeable and complex patterns of illegal trade of e-waste [1,2]. Similar to this, Tansel has argued that despite of increasing markets for e-waste recycling, it is still a challenging process. These challenges include absence of proper infrastructure for disposal, unawareness of people about its safe handling and absence of the accounting mechanisms for international transports [2].

53.6 Million tons (Mt) of total e-waste was generated across the world – 21% up in 5 years. According to the current statistics given in Fig-1 and Table-1, it is predicted that it will reach 74 Mt by till 2030 if not managed properly. Out of all these only 17.4% has been properly documented and recycled. In 2019, the e-waste data has been represented in fig-2 and table-1. Among all Europe is one of the continents with highest documented e-waste i.e., 5.1 Mt (42.5 %) out of 12 Mt [7].

India lies in the South Asian region and India is the only country in South Asia which has established legislation for the proper management of e-waste. India is showing growth and improvement as the management laws have already been introduced there in 2011 [7].

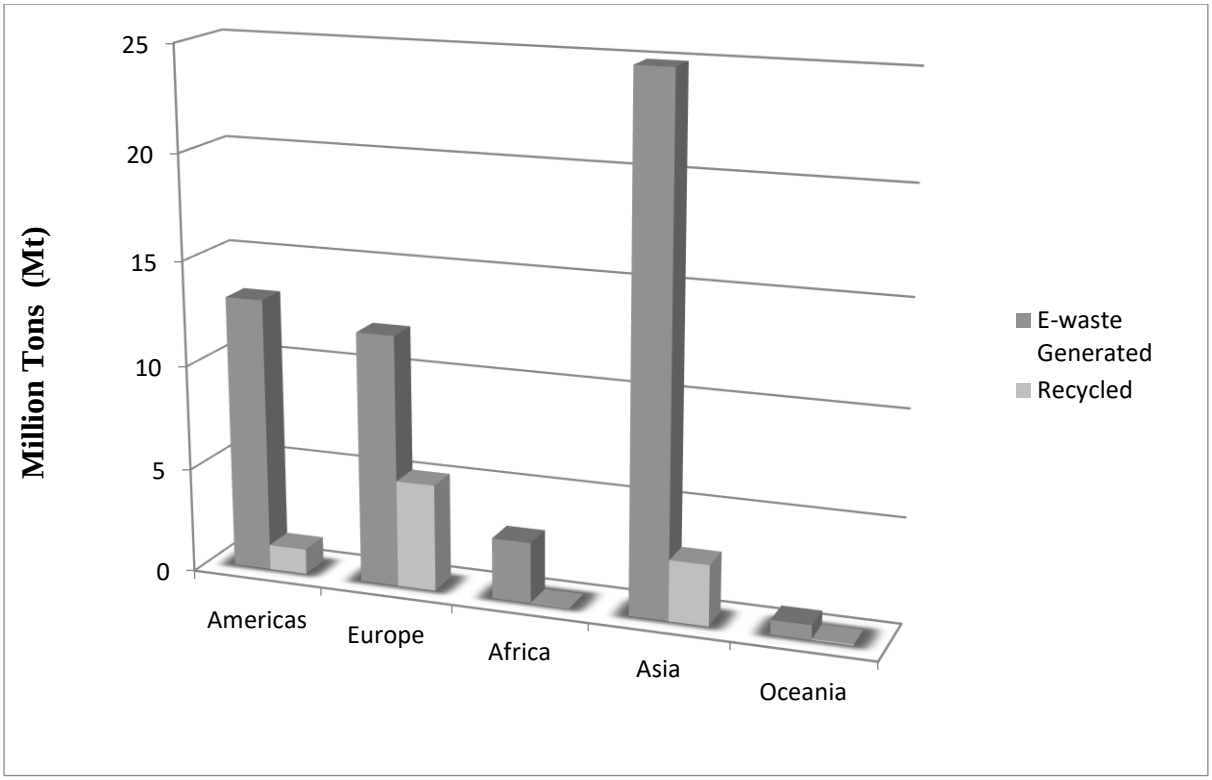


Fig 1 – Total e-waste generated worldwide till 2019 [14].

Continents	**Total Generated Amount (Million Tons)**	**Total Recycled Amount (Million Tons)**
Americas	13.1	1.2 (9.4%)
Europe	12	5.1 (42.5%)
Africa	2	0.03 (0.9%)
Asia	24.9	2.9 (11.7%)
Oceania	0.7	0.06 (8.8%)

Table 1 – Depicting the amount of e-waste generated worldwide till 2019 [14].

3. Microorganisms involved

A large number of studies have been published, that have given description about the phylogenies and physiologies of the acidophilic microorganisms in detail. The main role of the microorganisms in the process of bioleaching is to catalyse regeneration of protons and ferric ions, by sulphur oxidation and from ferrous iron respectively. The most efficient and important microorganisms in bioleaching process that operates from the ambient temperatures to approximately 40°C are considered as a consortium of sulphur and iron-oxidizing *Acidithiobacillus ferrooxidans,* sulphur-oxidizing *Acidithiobacillus caldus and Acidithiobacillus thiooxidans* and iron-oxidizing *Leptospirillumferriphilum* and

Leptospirillumferrooxidans. In moderately thermophilic environment *A. caldus, Sufobacillus, L.ferriphilum, -like* bacteria. In the category of archaea, *Ferroplasma* seems to dominate. The Thermophilic consortia are typically dominated by archaea with species of, *Metallosphaera, Sulfolobus and Acidianus* being the most prominent. Some major micro-organisms are explained below and the diversity of microbes playing its potential role in bioleaching is listed in the Table - 2 along with their applications.

1.Chromobacterim violaceum

It is a facultative anaerobe and gram negative in nature. They are also non-pathogenic to the humans and are to be found in subtropical and tropical areas of various continents. Sometimes it behaves like an opportunistic pathogen for both humans as well as animals and they also cause septicemia which is a kind of bacterial infection in the various parts of the body like skin, lungs and also can enter the bloodstreams. This bacterium also holds a capacity to exploit energy resources with the help of appropriate reductases and oxidases. this characterstic allows it live both aerobically and anaerobically. *Chromobacterium violaceum* is considered to be effective for the dissolution of gold through biological processes. The bacteria follow cyanide-associated mechanism to dissolve metals. Cyanide production by the microorganism has an advantage of inhibiting the competing organisms [24].

In the genome of *C. violaceum*, an operon for HCN synthase (hcnA, hcnB & hcnC) is present which encode two amino acid oxidases and formate dehydrogenase and they are directly involved in the synthesis of cyanic acid. In the process of cyanide synthesis, HCN synthase produces 4 electrons which are transferred on Oxygen. Low oxygen is needed for these reactions. Cyanide is produced as secondary metabolite by these bacteria. There is one more feature about these bacteria which puts a great impact on biology and that is its cyanide degrading capacity. According to the previous studies, bacillus megaterium and *Chromobacterium violaceum* tends to synthesize b-cyanoalanine synthase enzyme which plays its role in converting cyanide to b-cyanoalanine and these reactions take place in the late stationary and the death phase [24].

2.Thiobacillus sp.

It is one of the most active bacteria in the process of bioleaching. They are gram-negative in nature and also non-spore forming. They require aerobic conditions for their growth. Most of

the thiobacilli are chemolithotrophic in nature and uses carbon dioxide for their carbon source in order to synthesize cell material. These bacteria usually need pH between 1.5-3 to carry out bioleaching process and this is the ph value at which most of metal ions remain in the solution. That's why *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* are considered to be really important in the process of leaching out metals. Other organisms of this genus are also helpful in bioleaching but they work under higher pH and also they are not able to maintain the metal ions in the solution [11].

Acidithiobacillus thiooxidans holds a significant importance in the rapid oxidation of elemental sulfur. The reduced compounds of sulfur are also used in the solution and produces sulfuric acid. And the intensive production of sulfuric acid helps in the decomposition of the rocks very rapidly in order to have the acid-soluble metals in the solution as sulfates. Sulfuric acid also helps in lowering the ph from 1.5-1. After that, the most important role in bioleaching is played by *Acidithiobacillus ferrooxidans* which was isolated by Colmer and Hinkle in 1947. The cells of *ferrooxidans* are identical to *thiooxidans* morphologically but differ in the course of oxidation, *ferooxidans* have much slower oxidation rate in comparison to the *thiooxidans.* The most important difference between *ferrooxidans* and the other thiobacilli is in their process of obtaining energy through oxidation of reduced sulfur compounds. When the oxygen is absent, *ferrooxidans* are still able to show growth which is by the reduced inorganic sulfur compounds and use ferric ion as an electron acceptor [25].

3.Leptospirillum sp.

This bacterium was isolated by Markosyans in Armenia from mine waters. It is also an acidophilic bacterium with a characterstics of chemolithotrophic ferrous iron oxidation. These microorganisms are capable of tolerating lower pH and metals with higher concentrations like silver, molybdenum and uranium. But these are more sensitive towards copper and also, they are not capable of oxidizing sulfur compounds or sulfur. And this is the reason behind not attacking the mineral sulfides, it can only be done with the help of *Acidithiobacillus thiooxidans* or *Acidithiobacillus ferrooxidans* [25].

Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans and *Leptospirillum ferrooxidans* are known to be the mesophilic bacteria and grows at the temperature of 25-35°C [25].

4.Fungi

- Along with these bacteria explained above there are various species of fungi which are also involved in the leaching of metals mainly those belonging to the genera of *Penicillium* and *Aspergillus.* They were found to be successfully involved in leaching of various heavy as well as valuable metals which shows their potential in the bioleaching mechanisms. Fungal leaching mainly follows four mechanisms to solubilize the metals and extract them out and those are as follows [26]-
- **Acidolysis** – It is considered to be one of the most important mechanisms which is being carried out by solubilizing the metals which is resulting from the productions of different organic acids [26].
- **Complexolysis** – It is the mechanism in which metals are able to form complexes with organic acids produced [26].
- **Redoxolysis** – In this mechanism, the organic acids that are produced mediates the reduction of metals [26].
- **Bioaccumulation** – in this type, mycelium is utilized as a sink for metal ions. In short here the mycelium is used so as to collect all the metals ions and then extract it out [26].

Domain	**Microorganisms**	**Metals extracted**	**Applications**	**References**
Bacteria	***Acidithiobacillus ferroxidans***	Fe	The extracted iron plays an effective role as Fenton's catalyst in the degradation of hebicides.	Bhaskar *et al* (2021)
		Cu & Zn	Upscaling of existing bioleaching applications for metal extraction.	Sajjad *et al* (2018)
		Cd, Cu, Cr, Ni, Pb, Zn	The study used a mixed culture of bacteria and yeast in order to enhance the process of bioleaching.	Camargo *et al* (2018)
	Roseovarius tolerans	Au	An alternative to Cyanidation which is highly toxic to	Kudpeng *et al* (2020)

	Roseovarius mucosus		environment. Iodide leaching can be applied as a modern alternative as it makes more stable complexes in comparison to cyanide and less toxic also.	
	Roseospira sp.	Mn & Ag	Mining waste can also be an appreciable source of metals and should be treated with biotechnological extraction process.	Rosas *et al.* (2020)
	Shingomonas sp.			
	Roseovarius sp.	Au	Alternative to Cyanide - Other halogens like chloride, bromide and iodide can also leach gold with low impact.	Khaing *et al.* (2019)
	Chromobacterium violaceum	Cu, Au, Zn, Fe, Ag	Comparison between the metal leaching efficiencies of mixed and pure culture has demonstrated mixed culture to be more efficient as its leaching capabilities were quite high. As a result, it has presented an application of using mixed culture.	Pradhan & Kumar *et al.* (2012)
	Pseudomonas aeruginosa			
	Pseudomonas fluroscens			
	Sulfobacillus thermosulfidooxidans	Cu	This work emphasized that it is possible to set up conditions to enable bioleaching even at high fluoride concentrations.	Rodrigues *et al.* (2018)
	Leptospirillum ferriphilum	Cu & Zn	Compared to pure culture, mixed culture shows more pronounced attachments and prove mixed culture to be the more efficient in comparison to the pure one.	Sajjad *et al.* (2018)
	Leptospirillum ferrooxidans			

Fungi	***Aspergillus niger MH378446***	Hg & Pb	Mycoremediation - Treatment of heavy metal - polluted soil using indigenous metallotolerant fungi.	Khan *et al.* (2019)
	Aspergillus fumigatus FJ011537			
	Aspergillus terrius MH378451			
	Cladosporium halotolerans	Mn	The study demonstrates the laccase activity which is one class of enzyme and catalyze the Mn oxidation and its removal. Overall, it helps in bioremediation on Mn contamination in water.	Mota *et al.* (2020)
	Hypocrea jecorina			
	Aspergillus Niger	Al	In this study, the ability to acts as chelating agents form stable metal complexes.	Shah *et al.* (2019)
	Penicillium simplicissimum			
	Penicillium chrysogenum	Cd, Cu, Pb, Zn	Efficient in bioleaching heavy metals from soil.	Deng *et al.* (2018)
Yeasts	***Pichia pastoris***	Au & Pd	Genetically engineered yeasts give an advantage of easy-to-use automation, inexpensive growth requirement and investment, simple scaling ups, high biomass yield, time/cost effectiveness.	Elahian *et al.* (2019)
	Meyerozyma guilliermondae	Cd, cr, Cu, Ni, Pb, Zn	Adding yeast along with bacterial culture can increase the rate of bioleaching. Yeasts produce	Camargo *et al.* (2018)

			biosurfactants which improves the metal solubilization.	

Table 2- Potential role of microorganisms in the extraction of metals.

3.4 Sequence Analysis and protein function analysis

Sequence analysis was carried as described by Raman and Shankar (2015) [27]. In this tool, statistical techniques are used in order to determine likelihood of the particular alignments between the sequences or the sequence region arising by chance showing size composition and size of database being searched.This was carried out by constructing general global multiple sequence alignment, after which highly conserved regions are isolated and used to construct a set of profile matrices [27].

3.5 Next Generation Sequencing (NGS)

3.5.1 Use of next generation sequencing technology in the assessment of bioleaching microbial community

Microorganisms participating in the process of bioleaching are of great importance as they have a potential application of extracting out metals from minerals. These metals are basically a metabolic requirement for the microbes at a certain concentration, but beyond that it becomes toxic to it. It starts to denature the protein molecules. Understanding and assessment of the microbial community is highly important so as to improve the process of bioleaching. It is very much clear from the previous studies that bioleaching is quite a complex technology to extract metals and highly concerned with the type of relationships between the environment and microbes and also with the interactions between the organisms. In that case of microbial interactions among themselves as well as with the environment, there is a huge diversity of microbes that exist in the bioleaching systems. And it is a challenging process to understand this relationship among them so as to enhance the process of bioleaching. In order to address that challenge, there has been various molecular technologies which are used and those are – denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), real time PCR, stable isotope probing (SIP) and many more related technologies like proteomics

and microarray. They all are discovered to assess the structure of microbial community and diversities among genes in different environments [28].

3.5.2. NGS to address the challenge of microbial ecology in the bioleaching environments

RNA-sequencing is an application of High throughput technology and is recently developed in multiple laboratories across the world. It is also known as whole transcriptome sequencing. It basically utilizes NGS technologies for the profiling of RNA through sequencing of cDNA. From the past few years, this approach has been used to reveal a huge information on a transcriptome level from yeasts to human. This technique has been emerged as one of the promising tools to study the bacterial transcriptomes, also it has shown higher sensitivity towards genes which are expressed either at very high or very low levels and this characterstics of RNA sequencing shows that it has higher accuracy and dynamic range in both quantization and quantification. One more advantage of this technique is that it has higher reproducibility for technical as well as biological replicates [28].

3.5.3 NGS in Genome Analysis

To understand the microbial ecology of the complex environments and their interactions with the microbes, studies of model ecosystem in necessary. In case of bioleaching, acid mine related environments have been taken as model ecosystem and a broad research has been done as these areas has shown their importance in the field of biomining. NGS has led to the discovery of techniques that enables the genome analysis which recovers information about their characteristic and their metabolic potential. Till 2016, total 157 genomes of acidophiles were included in the public databases [28].

3.6 Hydrogen Cyanide Synthase and its role in metal extraction

Hydrogen Cyanide Synthase is an enzyme that produces Hydrogen Cyanide (HCN) and Carbon Dioxide (CO_2) from glycine. It transfers 4 electrons to respiratory chain, molecular oxygen acting as terminal acceptor. Along with that, this enzyme is highly sensitive to the molecular oxygen and as a result, becomes inactivated rapidly when oxygen is present [29]. In the bacterium *P. aeruginosa*, HCN is not produced under the complete anaerobic conditions, when terminal electron acceptor is nitrate. The Optimal expression of Hydrogen Cyanide Synthase occurs from exponential to stationary phase and in low oxygen levels. In these, *HCNabc* Gene

cluster have played the major role [30]. Along with *HCNabc* gene clusters, there are several other genes which encodes for Hydrogen Cyanide Synthase given in Table-3 [9].

Submitted name of the Protein	**Microorganisms**	**Genes**	**Accession IDs**
Hydrogen cyanide synthase subunit HcnC	*Pseudomonas aeruginosa strain ATCC 15692*	hcnC	G3XD12
Hydrogen cyanide synthase	*Pseudomonas putida*	QV12_10475	A0A0D1PB34
Cyanide-forming glycine dehydrogenase subunit HcnC	*Pseudomonas brassicae*	hcnC	A0A6B3NVU9
Glycine/D-amino acid oxidase	*Pseudomonas fuscovaginae*	PF66_04508	A0A0N0E2F5
Hydrogen cyanide synthase	*Pseudomonas sp. MF4836*	MF4836_18470	A0A1T1HY48
HcnC	*Pseudomonas fluorescens (strain F113)*	hcnC	G8QBD3
Hydrogen cyanide synthase subunit HcnC	*Pseudomonas protegens (strain DSM 19095)*	hcnC	O85228
Hydrogen cyanide synthase	*Chromobacterium sp. LK11*	VK98_01745	A0A0J6NS71
Hydrogen cyanide synthase HcnC	*Chromobacterium violaceum (strain ATCC 12472)*	hcnC	Q7NXE5
Hydrogen cyanide synthase	*Thalassomonas actiniarum*	SG35_27440	A0A0D8CPL7
Hydrogen cyanide synthase	*Pseudoalteromonas rubra*	TW77_20355	A0A0F4QHD9
Hydrogen cyanide synthase	*Marinomonas sp. S3726*	TW85_12690	A0A0F4R1V2

Hydrogen cyanide synthase HcnC	*Pseudoalteromonas citrea DSM 8771*	PCIT_01900	U1KWD8
Hydrogen cyanide synthase	*Vibrio neptunius*	TW84_06645	A0A0F4P9C6
Hydrogen cyanide synthase HcnC	*Pseudoalteromonas luteoviolacea DSM 6061*	N475_20400	A0A161XTZ8
Hydrogen cyanide synthase	*Salinivibrio sp. KP-1*	WN56_14570	A0A0F5AN00
Hydrogen cyanide synthase subunit HcnC	*Pseudovibrio axinellae*	hcnC_2	A0A165VZK3
Hydrogen cyanide synthase HcnC	*Vibrio caribbeanicus ATCC BAA-2122*	VIBC2010_04559	E3BK35
Hydrogen cyanide synthase	*Piscirickettsia litoralis*	BGC07_05105	A0A1E3G9X6
Hydrogen cyanide synthase subunit HcnC	*bacterium HR25*	hcnC	A0A2H5YN88
Hydrogen cyanide synthase subunit HcnC	*Streptomyces sp. F-1*	hcnC_1	A0A1K2FTK3
Hydrogen cyanide synthase subunit HcnC	*Streptomyces lavendulae subsp. lavendulae*	hcnC2	A0A2K8PK79
Hydrogen cyanide synthase subunit HcnC	*Streptomyces sp. AVP053U2*	hcnC	A0A1D2IH12

Table 3 – Genes encoding Hydrogen Cyanide Synthase [9].

3.7 Role of Hydrogen Cyanide Synthase in metal extraction

Leaching by the cyanogenic microbes generally takes place through the indirect process along with the microbial production of various metabolites. The role of these metabolites is to dissolve the metals from the minerals and it takes place by making metallic complexes which are soluble in nature. An example of Au dissolution in the cyanide solution is shown equations (1,2,3) below [24] -

$4Au + 8CN^- \longrightarrow 4Au(CN)_2^- + 4e^-$ …….. (1)

$O_2 + 2H_2O + 4e^- \longrightarrow 4OH^-$ …….. (2)

$4Au + 8CN^- + O_2 + 2H_2O \longrightarrow 4Au(CN)_2^- + 4OH^-$ ….. (3)

In these reactions, cyanide lixiviant is produced by the microorganisms and then it reacts with gold as shown in equation (1-3), in order to complete the process of bioleaching. This cyanide lixiviant is produced by the HCN which is derived from the glycine with the help of the enzyme HCN synthase. Cyanide is present as free cyanide in the solution which includes the production of both HCN (non-dissociated hydrocyanic acid) and cyanide ion (CN^-). At physiological pH, the pKa value of hydrogen cyanide is 9.3 at temperature 26°C and that is the reason why cyanide is present in the more volatile HCN. This pKa value can decrease up to 8.3 in the presence of the salts and thus volatility is also reduced. Generally, cyanide has the tendency to react with almost all the transition metals except actinides and lanthanides and form quite good complexes with these metals and also, they are stable and have high solubility [24].

Leaching through cyanide is one of the efficient methods for the recovery of the metals but along with that they have some serious impacts on the human health as well as environment. In that case, an alternative is highly needed and that alternative is the capacity of the bioleaching microbes to not only produce cyanide but to consume it also. With this alternative of consuming cyanide from the solution, at least some of its negative impacts can be controlled [24].

3.8 E-waste health issues and their possible solutions

The important thing to be noted about e-waste is that it contains a large number of precious metals and considered as valuable from an economic point of view. But along with that it contains various toxic metals also which can cause health issues to not only the environment but to the human beings also who are involved in their recycling. People working under high temperature and also the inhaling of gases emitted during the leaching experiment can cause various health problems like asthma, respiratory diseases, eye problems, wheezing, chest pain, coughing etc [31].

Example- when the PVCs are burnt, it releases Hydrogen Chloride. When it is inhaled and reaches the lungs, mixes with water and forms hydrochloric acid. And it starts to affect the lung tissues and causes several respiratory disorders. Various e-waste health hazards are given in Table 3 [31].

E-waste sources	Constituents	Effects on health
Semiconductors and Chip resistors	Cadmium	• Teratogenic. • Gets accumulated in liver and kidney. • Neural damage is also caused.
Motherboard	Beryllium	• Cancer (mainly lungs). • Skin disease like warts. • Berylliosis caused by inhaling fumes and dusts.
CRTs (Front panel)	Barium	• Weaknesses in muscles. • Heart, liver and spleen is also damaged.
Plastic housing in circuit boards and electronic equipments.	Brominated flame retardants (BFR)	• Endocrine system becomes disrupted.
Computer housing and Cabling	Mainly plastics and PVCs	• Reproductive problems can be caused. • Immune system is also damaged.
Gaskets and glass panels of computer monitors	Lead	• Both central and peripheral nervous system is damaged. • Affects kidney also.

		• Brain development of children is affected.
PCBS (printed circuit boards)	Mercury	• Brain can be damaged. • Skin and respiratory disorders can also be there.
Galvanized steel plates	Hexavalent Chromium	• Damage in DNA. • Asthma problems.

Table 3 – Constituents of e-waste responsible for health issues [31].

3.9 Possible solutions to these problems-

- Import of e-waste should be banned totally [31].
- Safe disposal of domestic waste should be addressed and carried out as well on an individual level [31].
- Investment in this sector should be attracted [31].
- There should be awareness programs on e-waste [31].
- Recycling business should be made viable on an economic level [31].
- Laws should be made mandatory [31].

3.10 Pros and Cons of biological extraction

3.10.1 Pros

1. It is a simple process [32].
2. It is also an inexpensive technique in comparison to other traditional techniques [32].
3. There is no emission of poisonous sulphur dioxide as happens in smelters [32].
4. High pressure is also not required [32].
5. It is an environment friendly technique [32].

3.10.2 Cons

1. It is a time-consuming technique, takes about 6-24 months or more than this [32].
2. Yield is also very low [32].
3. Large area is required for the treatment [32].
4. Contamination risk is also higher [32].
5. Yield can be inconsistent as the microbes do not grow uniformly [32].

4 Materials and methods

4.1 Sequences retrieved

Total 23 different sequences of Hydrogen Cyanide Synthase of bacterial domain were retrieved from UniProt data bank (https://www.uniprot.org/). These 23 sequences were selected on the basis of microorganisms studied in my literature survey. Percent identity was also considered as the basis for selection. Accession numbers of respective proteins are given in the Table-3 above [9,33].

4.2 Determining the physical parameters of proteins

The physical parameters of bacterial Hydrogen Cyanide Synthase were analysed by studying different physiochemical parameters viz. isoelectric point, molecular weight, instability index, aliphatic index, grand average of hydrophobicity (GRAVY) and no. of amino acids and its composition. The tool used to study parameters was ExPASy-ProtParam (https://web.expasy.org/protparam/)[10,33].

4.3 Analysis of Primary Structure

In order to analyse the primary structure of the Proteins; viz. the amino acids composition present in the polypeptide chain of the bacterial Hydrogen Cyanide Synthase were studied and recorded. Tool used to analyse this was ExPASy-ProtParam [10,33].

4.4 Analysis of Secondary Structure

In order to analyse the secondary structure of the retrieved bacterial Hydrogen Cyanide Synthase α-helicies, β-sheet, extended strand, β-turns and random coils were studied and recorded. This analysis was performed by ExPASy SIB Bioinformatics SOPMA tool. To use this tool and analyse the results, sequences were submitted in the FASTA format [11,33].

5 Results and Discussions

5.1 Sequences retrieved

There was total 23 bacterial sequences retrieved of Hydrogen Cyanide Synthase from the UniProt databank. All the bacterial species were taken on the basis of study done. Hydrogen Cyanide Synthase has been chosen to study as it plays a major in the extraction of metals reported by Liu et al. (2016). This study has led to the collection of more information about Hydrogen Cyanide Synthase along with the genes involved [9,33].

5.2 Determining the physical parameters of proteins

Studying the physical parameters of any protein is an important step as it defines the uniqueness of any protein on the basis of values calculated for the respective sequences taken. The physical parameters determined in this study were - molecular weight, instability index, aliphatic index and grand average of hydrophobicity (GRAVY). The pI of this protein ranges between 4.99-10.43 which indicates the diverse alkaline and acidic nature of the protein as shown in the Fig 2. The Average molecular weight of the protein was calculated to be the 46675.24 Da shown in Fig 3. Instability index is calculated to examine the stability of protein structure, value above 40 depicts the instability in the structure of protein while below 40 indicates that the protein structure is highly stable. In this study, 16 out of 23 retrieved sequences had their instability index value below 40 while remaining 7 were above 40 shown in Fig 4. The range lies between 26.69-50.66, which indicates the diverse structure of the protein. Aliphatic index is defined as the relative volume of a protein occupied by aliphatic side chains. Aliphatic index of the protein lies between 83.07-101.59 which shows that this protein has higher range of stability in terms of temperature. Greater the aliphatic index, more the thermostability of the protein. *Streptomyces lavendulae subsp. Lavendulae* shows the highest thermostability for this protein with a value of 101.59 shown in Fig 5. GRAVY stands for grand average of hydropathy. Hydropathy is defined as the relative hydrophilicity or hydrophobicity of the amino acids which are present in the sequence. Negative value of GRAVY indicates that this protein is non polar in nature as well as interacts with water which means it is hydrophilic. Out of all the 23 retrieved sequences, 20 sequences seem to have hydrophilic and non-polar nature i.e., negative GRAVY value shown in Fig 6 [9,33].

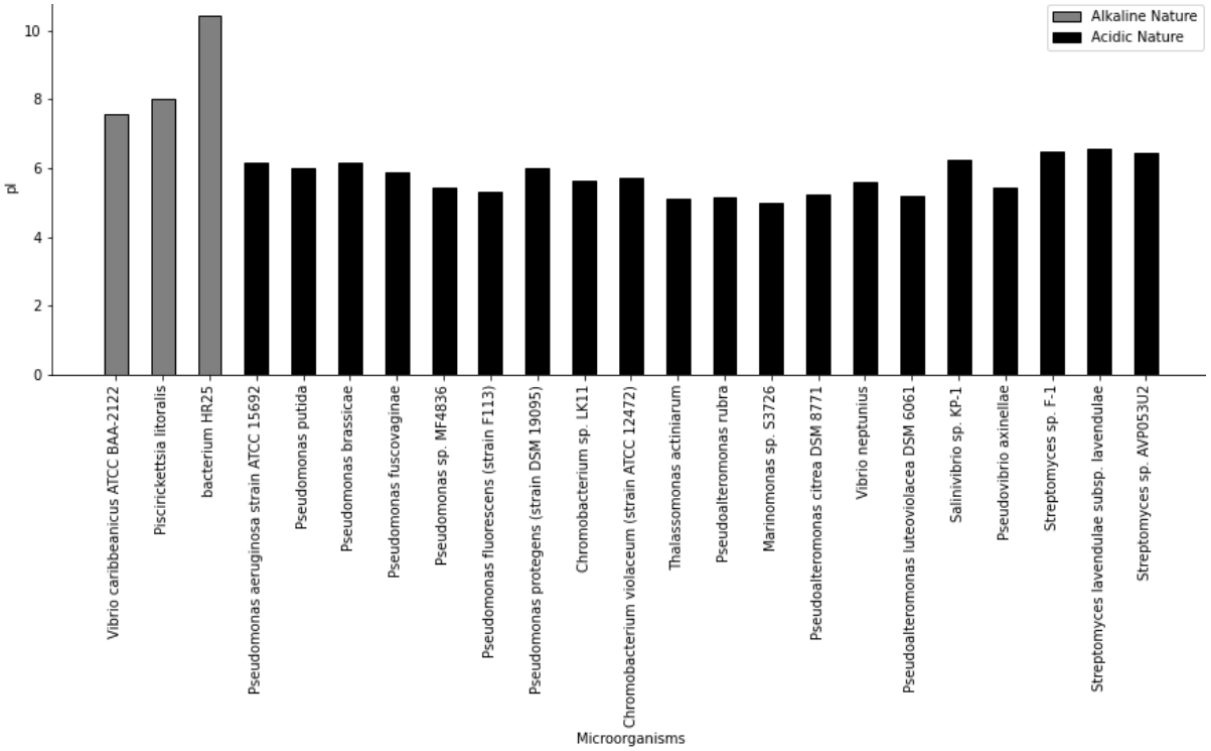


Fig 2 – Isoelectric point of the protein [10].

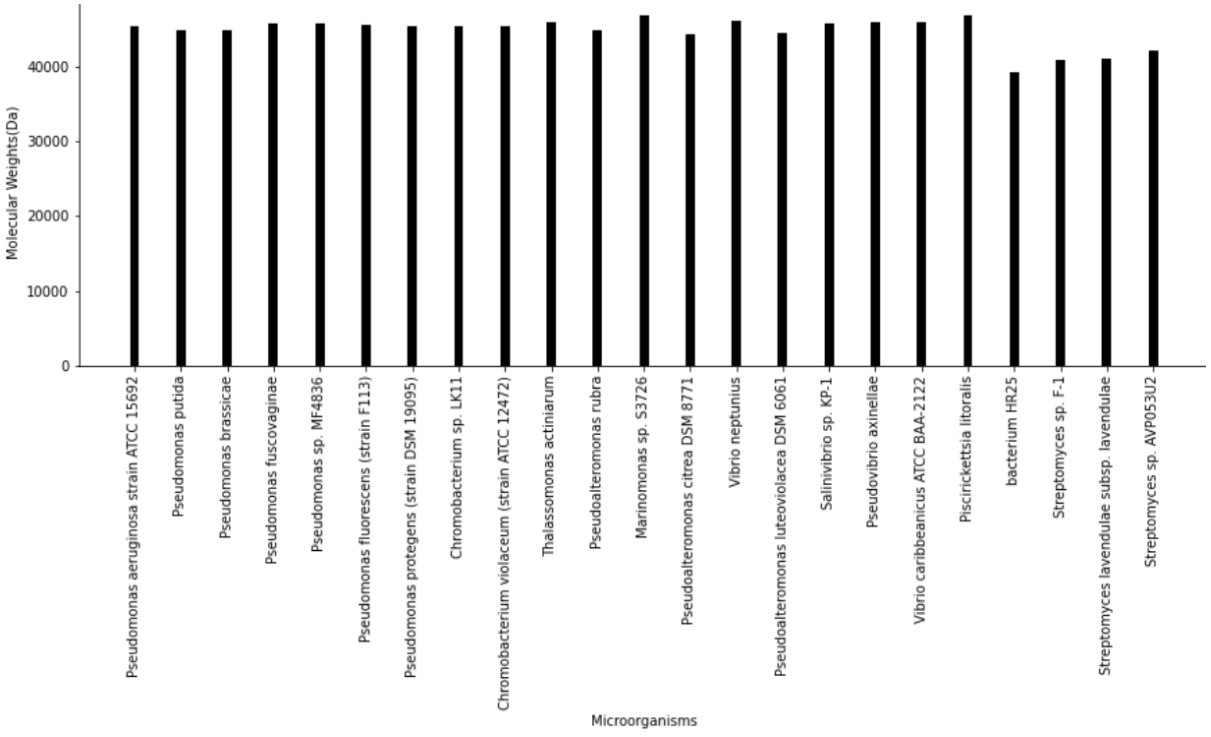


Fig 3 – Molecular weight of the retrieved protein sequences (Da) [10].

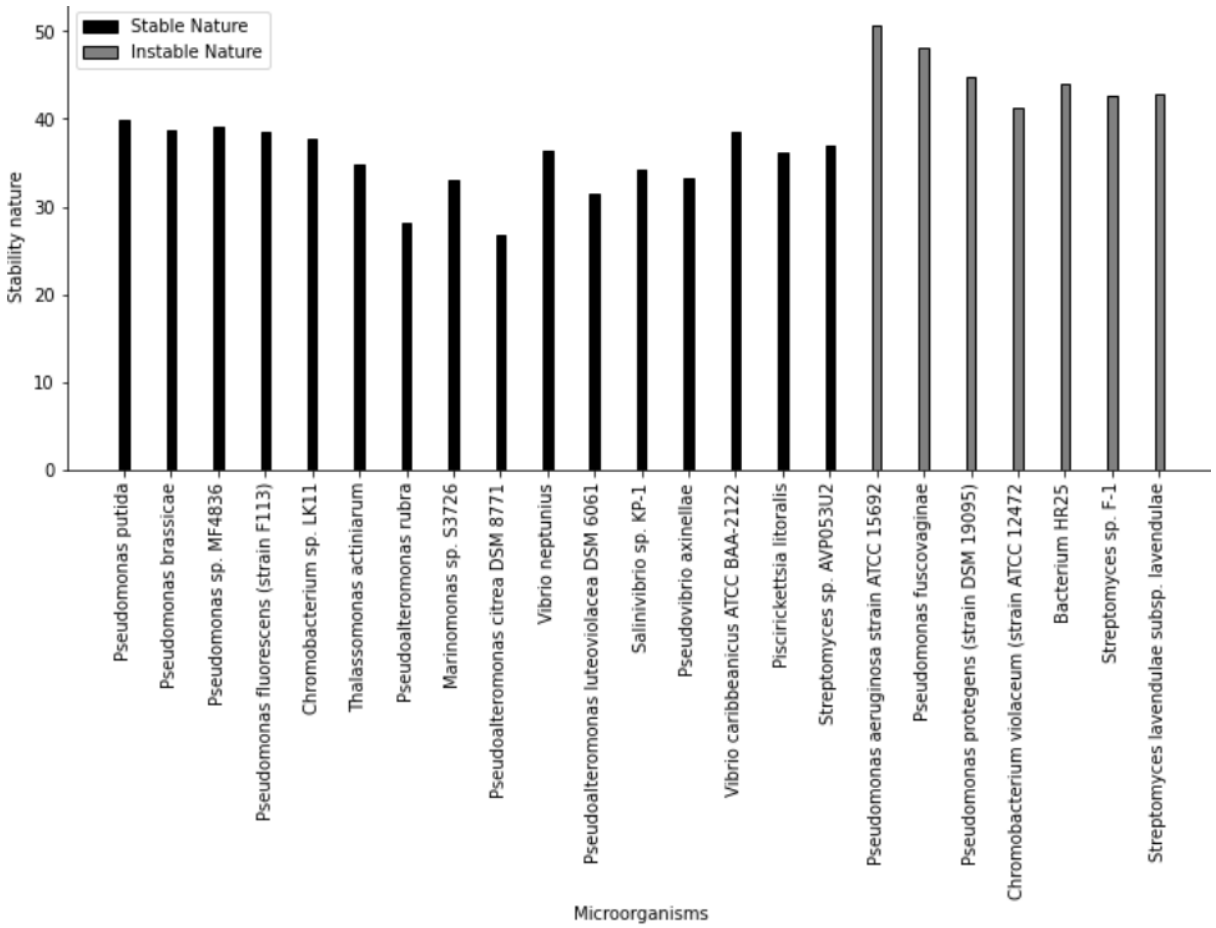


Fig 4 – Instability index of the retrieved sequences [10].

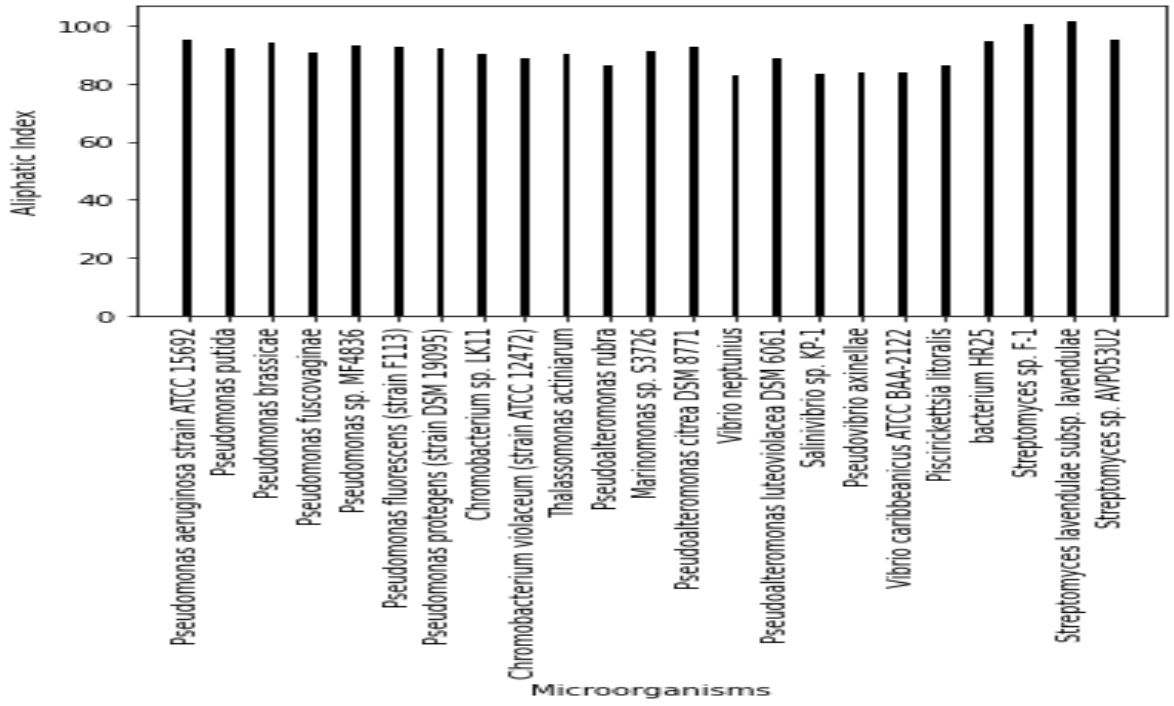


Fig 5 – Aliphatic index of the retrieved sequences.

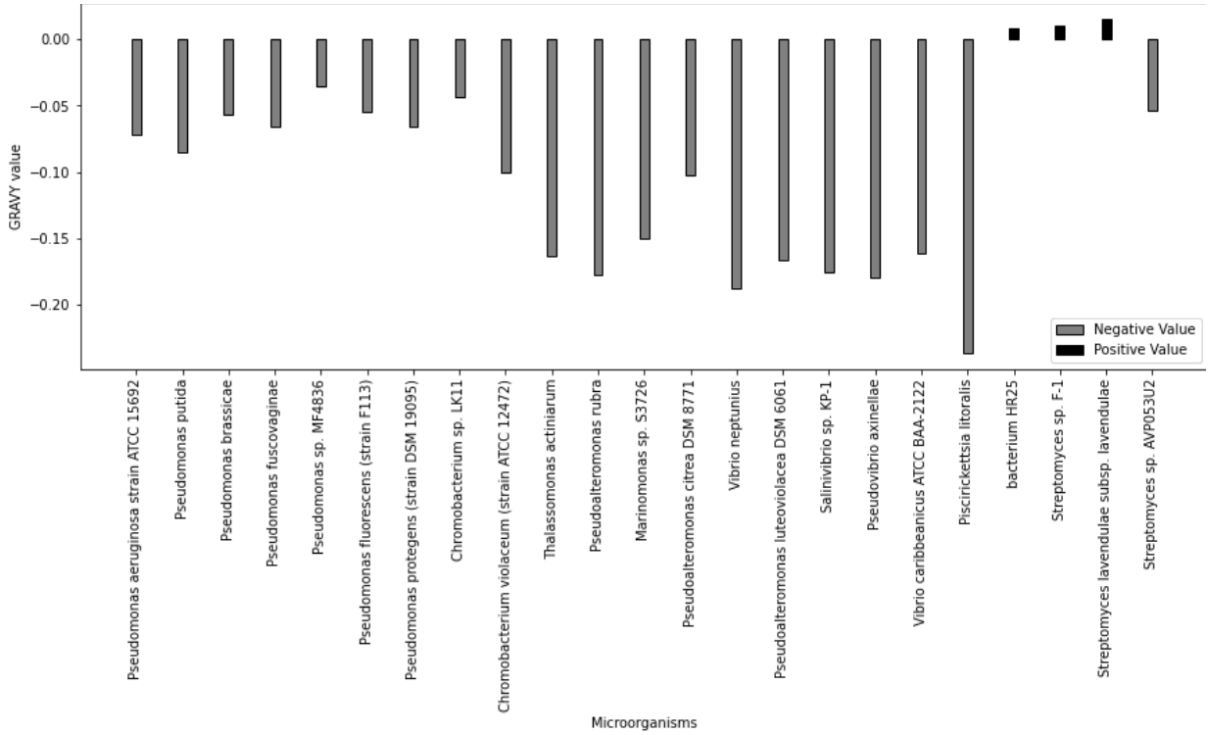


Fig 6 – GRAVY values of the retrieved sequences [10].

5.3 Analysis of Primary Structure

For the primary structure analysis of the protein, amino acid composition was determined as shown in Fig 7. The 5 dominant amino acids were found to be Leucine (10.3%), Alanine (9.8%), Glycine (9.3%), Valine (6.8%) and Glutamic acid (6.3%). Amino acids that are hydrophilic in nature are in contact with the aqueous phase. Leucine and Alanine, both are hydrophobic in nature and buried inside the folded proteins. Glycine is the simplest amino acid with no side chains and found on the surface of the protein and provides good flexibility to polypeptide chain. Valine is also a hydrophobic amino acid, related to leucine. It is found in the interior of proteins. Glutamic acid is hydrophilic in nature is found on the surface of proteins and come in contact with water [10,33].

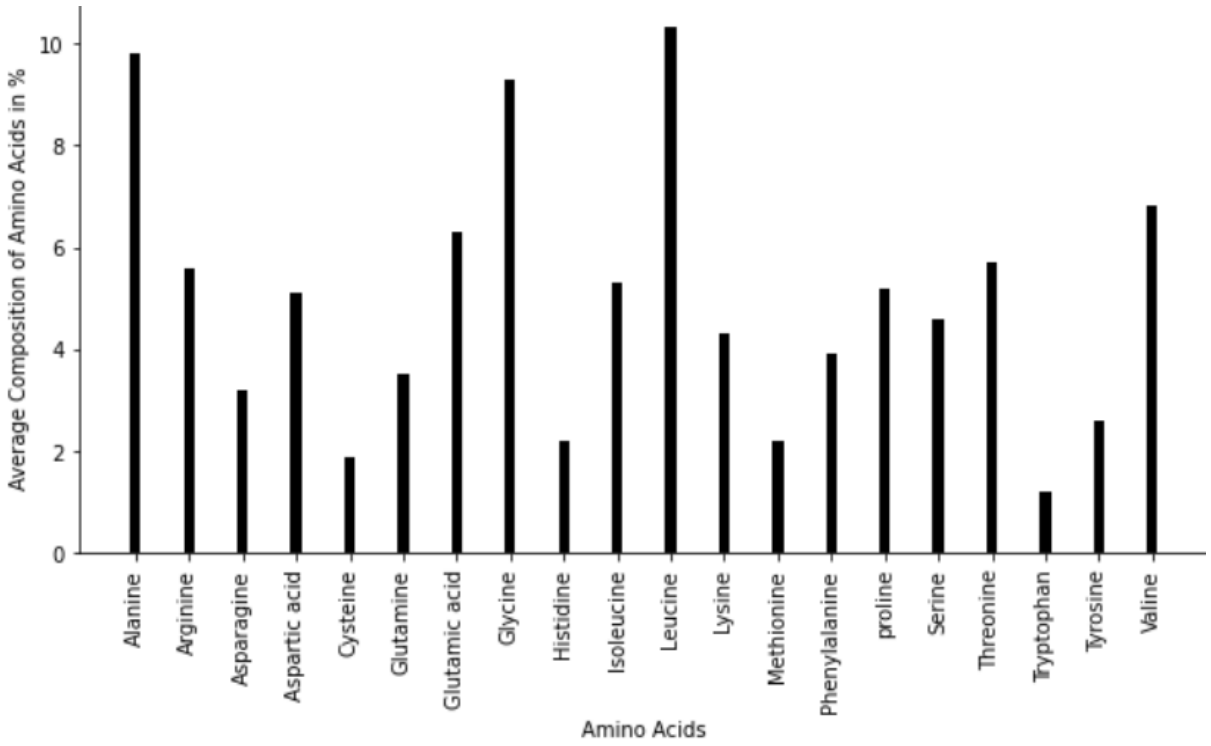


Fig 7 – Average (%) of amino acids present in the retrieved sequences [10].

5.4 Analysis of Secondary Structure

In order to analyse the secondary structure of the retrieved bacterial Hydrogen Cyanide Synthase α-helix, β-sheet, extended strand, β-turns and random coils were studied and recorded. The mean value was calculated for all the four regions. The maximum mean value of random coils i.e., 38.86% clearly demonstrates that this protein structures do not have the regular secondary structure and is not characterized by any regular H-bonding patterns, followed by α-helix 37.15%, extended strand 17.49% and β-turns 8.08% [10,33]. The analysis is shown in fig 8.

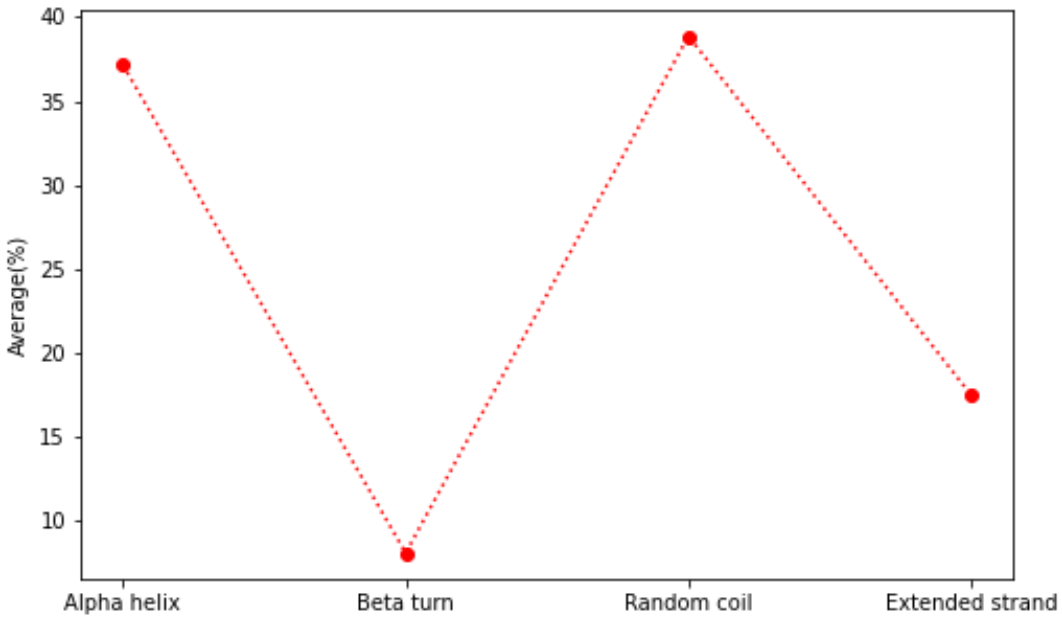


Fig 8 – Analysis of α-helix, extended strand, β-turns and random coils.

6 Conclusions

The present study shows the importance of e-waste being recycled in an eco-friendly manner. E-waste is considered as an issue of great concern due to its increasing volumes and complex composition. Therefore, e-waste recycling is important subject for both- waste management as well as recovery of valuable metals. Further, it presents about the diversity of microorganisms which have been reported in different studies for their application in extracting precious as well as toxic metals. Various *in silico* approaches have been used to do the structural analysis of the protein and these tools include UniProt, ExPASy-ProtParam and ExPASy SIB Bioinformatics SOPMA tool. Hydrogen Cyanide Synthase being reported in numerous studies for their metal binding capabilities has been studied in this research. The physical parameters which include molecular weight, aliphatic index, instability index and GRAVY has been analysed. Along with this, primary and secondary structures are also examined. For the primary structure analysis of the protein, amino acid composition was determined - Leucine (10.3%), Alanine (9.8%), Glycine (9.3%), Valine (6.8%) and Glutamic acid (6.3%) are the five dominant ones. Further, the analysis of secondary structure with maximum mean value of random coils i.e., 38.86% clearly demonstrated that protein structures do not have the regular secondary structure

and is not characterized by any regular H-bonding patterns, followed by α-helix 37.15%, extended strand 17.49% and β-turns 8.08%. This *in silico* study is important in terms of doing further study of bacterial Hydrogen Cyanide Synthase in wet lab so as to establish the more helpful data, further tertiary structure analysis and functional analysis may be suggested.

7 References

1. Lakshmi S, Raj A & Jarin T; (2017); "A Review Study of E-waste management in India", Asian Journal of Applied Science and Technology (AJAST); vol-1.
2. Kumar A, (2017), "Bioleaching of gold and silver from waste printed circuit boards by Pseudomonas balaerica SAE1 by an e-waste recycling facility", Current microbiology, vol-75.
3. Sahni A, (2016), "Chemo-biohydrometallurgy – "A hybrid technology to recover metals from obsolete mobile SIM card, environmental nanotechnology, monitoring and management", vol-6.
4. Perkins DN, Drisse M, Nxele T and Sly DP, (2014), "E-waste: a global hazard, Annals of global Health", vol-80.
5. Argumedo-Delira R, Gomez-Martinez J M and Soto J B, (2019), "Gold Bioleaching from Printed Circuit Boards of Mobile Phones by *Aspergillus niger* in a Culture without Agitation and with Glucose as a carbon Source" vol-9.
6. Hall J. W and Williams T. P, (2007), "Seperation and recovery of materials from Scrap printed circuit boards", Resource Conservation and Recycling, vol-51.
7. Zhou Y and Qui K, (2010), A w2new technology for recycling materials from waste printed circuit boards, Journal of Hazardous Materials, vol-175.
8. Jadhav U and Hocheng H, (2015), "Enzymatic bioleaching of metals from printed circuit board", Clean Technologies and Environmental Policy, vol-17.
9. https://www.uniprot.org/
10. https://web.expasy.org/protparam/
11. https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html
12. M. Khurrum, S. Bhutta, A. Omar and X. Yang (2011), "Electronic Waste: A Growing Concern in Today's Environment", vol. 2011.
13. Jadhav S. (2013), "Electronic Waste: A Growing Concern in Today's Environment Sustainability", Vol.2.
14. V. Forti, C.P. Balde, R. Kuehr and G. Bel (2020), "The Global E-waste Monitor 2020".
15. S. Lakshmi, A. Raj and T. Jarin (2017), A Review Study of E-Waste Management in India, vol. 1.

16. Shevchenko T, Laotaloa k and Danko Y, (2019), "Understanding Consumer E-waste Recycling Behaviour: Introducing a New Economic Incentive to Increase the Collection Rates", Sustainability, vol-11.
17. Wang F, Huisman J, Stevels A and Balde C P, (2013), Enhancing E-waste estimates: Improving data quality by multivariate Input-Output Analysis", Waste Management, vol-33.
18. Balde C P, Forti V, Gray V, Kuehr R and Stegmann P, (2017), "The Global E-waste Monitor -2017.
19. Borthakur A and Govind M, (2017), "Emerging disposal behaviour and awareness: A worldwide overview with special focus on India", Resource Conservation and Recycling, vol-117.
20. Agamuthu P, Cooper J and Herat s, (2012), "Dilemma in reuse and recycling of waste electrical and electronic equipment, Waste Management Resources, vol-30.
21. Schulep M, Hageleuken C, Kuehr R, Magalini F, Maurer C, Meskers C, Mueller E and Wang F, (accessed on 6 May 2019), "Recycling – from E-waste to Resources", United Nations Environmental Programme & United Nations University.
22. Perkins D N, Brune Drisse M N, Nxelle T and Sly P D, (2014), "E-waste – A Global hazard", Annals of Global Health, vol-80.
23. Chi X W, Streicher-Porte M, Wang M Y L and Reuter M A, (2011), "Informal Electronic Wsate Recycling: A sector review with special focus on China", Waste Management, vol-31.
24. Liu R, Li J and Ge Z; (2016), "Review on Chromobacterium violaceum for Gold bioleaching from e-waste", vol. 31.
25. Bosecker K, (1997), "Bioleaching: metal solubilization by microorganisms", vol. 20.
26. P. Rosoulnia, S.M. Mousavi, S.O. Rastegar and H. Azargoshasb; (2016); Fungal leaching of valuable metals froma power plant residual ash using Penicillium simplicissimum: Evaluation of thermal pretreatment and different bioleaching methods; vol. 52.
27. R. Thakur, and J. Shankar, (2016), In silico analysis revealed high-risk single nucleotide polymorphisms in human pentraxin-3 gene and their impact on innate immune response against microbial pathogens. *Frontiers in microbiology*, *7*, p.192..
28. S. Zhou, M. Gan, J. Zhu, X. Liu and G. Qui; (2018); "Assessment of Bioleaching Microbial Community Structure and Function Based on Next-Generation Sequencing Technologies" vol. 8.

29. Blumer C and Haas D, (2000), “Iron regulation of the hcnABC genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator Gac A in Pseudomonas fluorescens CHAO, Microbiology Society, vol-146.
30. Laville J, Blumer C, Schroetter V C and Gaia V, (1998), “Iron regulation of the hcnABC genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator Gac A in Pseudomonas fluorescens CHAO”, Journal of Bacteriology, vol-180.
31. Shagun, A. Kush and A. Arora; (2013); Proposed solution of e-waste management; vol. 2.
32. Md. Osaid Alam, Bioleaching and biomining.
33. Dutta B, Banerjee A, Chakraborty P and Bandopadhyay R, (2018), “In silico studies on bacterial xylanase enzyme: structural and functional insight”, Journal of Genetic Engineering and Biotechnology.