

**STUDIES ON HEAVY METAL BIOTRANSFORMATION BY
VIBRIO DIABOLICUS STRAIN EPO3 ISOLATED FROM ENNORE
CREEK, CHENNAI, INDIA**

Submitted in partial fulfilment of the requirements for the award of Bachelor of Science degree in
Microbiology

By

P. DEVADHARSHINI (40300008)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING
SATHYABAMA**

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
Accredited with Grade "A" by NAAC
JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119**

MAY – 2023



SATHYABAMA

not in format

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with "A" grade by NAAC

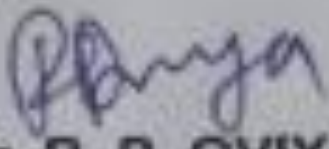
Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai-600119

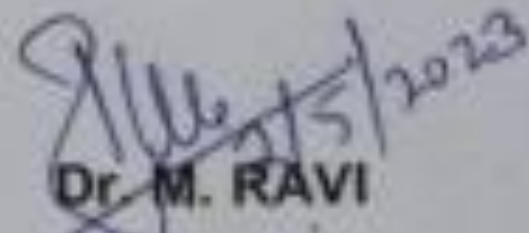
www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

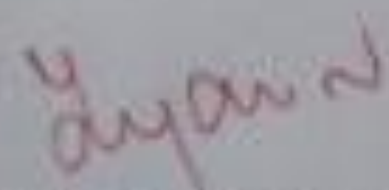
This is to certify that this Project Report is the bonafide work of,
Ms.Devadharshini (40300008) who carried out the project entitled "**Studies of heavy metals biotransformation by *Vibrio diabollicus* strain EP03 isolated from Ennore Creek, Chennai**" India under our supervision from September 2022 to May 2023.


Dr. R. P. OVIYA
Internal Guide


Dr. M. RAVI
External guide


Head of the Department
DR. V. RAMESH KUMAR

Submitted for Viva voce Examination held on 08.05.2023


Internal Examiner


External Examiner

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to Board of Management of **SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to Dr. V. Ramesh Kumar, Head of the Department, Dept. of Biotechnology for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide **Dr. R.P Oviya, Department of Biotechnology and Dr. M. Ravi, Centre of ocean research** for his valuable guidance, suggestions and constant encouragement, which paved way for the successful completion of my project work.

ABSTRACT

Vibrio diabolicus is a polysaccharide-secreting bacterium identified from, Ennore Creek, Chennai. This study focuses on exopolysaccharide (EPS) over production by stressing bacteria by altering temperature, pH, and salinity and adding heavy metals, namely Selenium metal powder, Mercuric sulphate, Lead nitrate, Copper sulphate, Cadmium chloride, and Zinc chloride. Environmental changes influence the generation of EPS. Changes in environmental circumstances alter the quantity and synthesis of new forms of EPSs in bacteria. This reaction is best triggered by abiotic stressors like as drought, heat, and salt. These modifications might be an adaptive mechanism of bacteria in response to these abiotic stress circumstances. The present investigation is aimed to determine correlation between EPS production and tolerance to temperature, pH, Heavy metal and salinity variations.

Keywords: *Vibrio diabolicus*, Polychaetes, Exopolysaccharides, Heavy metals, Abiotic stress

TABLE OF CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
	ABSTRACT	I
	LIST OF ABBREVIATIONS	Vii
	LIST OF FIGURES	Viii
	LIST OF TABLES	Ix
1	INTRODUCTION	
	1.1 MARINE ENVIRONMENT	1
	1.1.1 Polychaetes	2
	1.1.3 Importance of polychaetes	3
	1.1.4 Importance of gut micro biome	5
	1.2 APPLICATION OF EXOPOLYSACCHARIDES	6
	1.2.1 Effects of different stress levels on bacterial growth and exopolysaccharide	7
	1.3 HEAVY METAL POLLUTION	8
	1.3.1 Soil heavy metal pollution	10
	1.3.2 Drinking water heavy metal pollution	11
	1.3.3 Groundwater heavy metal p	13

1.4 IMPACTS OF HEAVY METAL POLLUTION	15
1.4.1 Cadmium heavy metal pollution human health	16
1.4.2 Cadmium heavy metal pollution in Environment	17
1.4.3 Cadmium heavy metal pollution Marine Organism	17
1.4.4 Mercuric heavy metal pollution in human health	17
1.4.5 Mercuric heavy metal pollution in Environment	18
1.4.6 Mercuric heavy metal pollution in marine organisms	19
1.4.7 Lead Nitrite heavy metal pollution in Human health	20
1.4.8 Lead Nitrite heavy metal pollution in Environment	21
1.4.9 Lead Nitrite heavy metal pollution Marine Organisms	21
1.4.10 Selenium heavy metal pollution in Human health	21
1.4.11 Copper sulphate heavy metal pollution in Human health	22
1.4.12 Copper sulphate heavy metal pollution in Environment	23

	1.4.13 Copper sulphate heavy metal pollution in Marine Organisms	23
	1.4.14 Zinc chloride heavy metal pollution in Environment	23
	1.4.15 Zinc chloride heavy metal pollution in Marine Organisms	24
	1.5 Vibrio Diabolicus	24
	1.5.1 Exopolysaccharide production in Vibrio diabolicus	25
2	REVIEW OF LITERATURE	26
3	AIM AND SCOPE OF THE PRESENT INVESTIGATION	
	3.1 AIM	30
	3.2 OBJECTIVES	30
4	MATERIALS AND METHODS	
	4.1 Sampling site	31
	4.1.1 Sampling method	32
	4.1.2 Isolation of Bacterial from Polychaetes	32
	4.1.3 Identification of Bacteria	33
	4.1.4 Gram Staining method	33
	4.1.5 16S rRNA Sequencing method	33
	4.1.6 Biochemical tests	34

	4.2 PHENOL SULFURIC ACID METHOD FOR TOTAL CARBOHYDRATE ESTIMATION	
	4.2.1 Materials	34
	4.2.2 Procedure	34
	4.2.3 Exopolysaccharide Estimation	35
	4.3 DIFFERENT STRESS LEVEL OF pH, TEMPERATURE, SALINITY, HEAVY METALS	
	4.3.1 Different stress levels of pH	35
	4.3.2 Different stress levels of Temperature	36
	4.3.3 Different stress levels of Salinity	37
	4.3.4 Different stress levels of Heavy metals	37
5	RESULTS AND DISCUSSION	
	5.1 Isolation of Polychaetes gut associated microbes	39
	5.2 Characterization of Bacteria	39
	5.2.1 Biochemical characterization	39
	5.2.2 Molecular characterization	40
	5.3 DIFFERENT STRESS LEVELS OF pH, TEMPERATURE, SALINITY, HEAVY METALS	
	5.3.1 Different stress levels of pH	40
	5.3.2 Different stress levels of Temperature	41

	5.3.3 Different stress levels of Salinity	42
	5.3.4 Different stress levels of Heavy metals	43
6	SUMMARY AND CONCLUSSION	50
7	REFERENCE	51

LIST OF ABBREVIATIONS

EPS	- Exopolysaccharides
ZMB	- Zobell Marine Broth
ZMA	- Zobell Marine Agar
HM	- Heavy Metals
HPI	- Heavy Metal Pollution Index
HEI	- Heavy Metal Evaluation Index
CD	- Degree of Contamination
TDS	- Total Dissolved Solids
DO	- Dissolved Oxygen
EC	- Electrical Conductivity
LAB	- Lactic Acid Bacteria
DNA	- Deoxyribo Nucleic Acid
CNS	- Central Nervous System
Mg	- Milligram
ml	- Millilitre
PCR	- Polymerase Chain Reaction
RT	- Room Temperature
HCL	- Hydro Chloric Acid
NaOH	- Sodium Hydroxide
µl	- Micro litre
µM	- Micromole
NaCl	- Sodium Chloride

LIST OF FIGURES

FIGURE NO	TITLE	PAGE NO
4.1	Sample Collection at Ennore Creek	32
4.1.2	Isolation of Gut Contents from Polychaetes	33
4.3.1	Different Stress Levels of pH	36
4.3.2	Different Stress Levels of Temperature	37
4.3.4	Different Stress Levels of Heavy Metals	38
	A) Heavy Metals	38
	B) Different Stress of Heavy Metals	38
5.1	Spread Plate	39
5.2	Characterisation of Bacteria	
	A) Gram Staining	39
5.2.1	B) Biochemical Characterisation	39
5.2.2	Molecular Characterisation	40
5.3.1	Different Stress Levels of pH	41
5.3.2	Different Stress Levels of Temperature	42
5.3.3	Different Stress Levels of Salinity	43
	Different Stress Levels of Heavy Metals	
	A) Cadmium Chloride	44
	B) Lead Nitrite	45
	C) Copper Sulphate	46
	D) Zinc Chloride	46
	E) Mercuric Sulphate	47
	F) Selenium	48

LIST OF TABLES

TABLE NO	TITLE	PAGE NO
5.3.1	Different Stress Levels of pH	40
5.3.2	Different Stress Levels of Temperature	41
5.3.3	Different Stress Levels of Salinity	42
5.3.4	Different Stress Levels of Heavy Metals	
	A) Cadmium Chloride, Lead Nitrite	44
	B) Copper Sulphate, Zinc Chloride	45
	C) Mercuric Sulphate, Selenium	47

CHAPTER 1

INTRODUCTION

1.1 MARINE ENVIRONMENT

Marine environment refers to all the animals, plants and organisms that live in the salt water seas and oceans of the earth. Marine animals are divided into four different taxonomic groups: These include cetaceans (whales, dolphins, porpoises), pinnipeds (seals, sea lions, walruses), sirens (manatees, dugongs), and marine fish pets (polar bears, sea otters). According to the World Register of Marine Species, worms, the total number of marine species we know is about 240,000 (Census 2021). However, there are an estimated 1.4 to 1.6 million marine species on earth. Any worm that lives in a marine environment is considered a marine worm. There are six phylum's containing marine worms: Platyhelminthes (flatworms), Nematode (roundworms), Annelida (worms), Chaetognatha (arrow worms), Hemichordate (fish eggs) and Phoronida (horseshoe worms). Sea worms live under rocks on rocky shores, among seaweed boulders, in mud, silt and sand bristle worm. Of the approximately 9000 species of annelids, over 8000 are polychaetes. These segmented earthworms are one of the most common marine organisms, living in the deep ocean, swimming freely near the surface or burrowing into beach mud and sand. Marine worms are invertebrate mollusks with long, narrow bodies. They have a digestive system, a circulatory system, and a nervous system. Their bodies are covered by an outer covering (epidermis) made of tough but flexible collagen. Polychaetes (meaning hairy) are (usually) brightly colour worms. Polychaetes are multi-segmented worms living in all environments of the world's oceans, occurring from the depths of abysses to shallow estuaries and rocky shores, even swimming freely in open water. They are strictly aquatic annelids, but they are the most abutant and delivered group in the phylum Annelida.

1.1.1 Marine pollution

Marine pollution is defined as the introduction of human substances into the marine environment that leads to harmful effects such as destruction of biological

Resources, risks to human health, impediments to marine activities including fishing, deterioration of sea water use and reduction of facilities. Marine pollution occurs substances used or dispersed by humans, such as industrial, agricultural and domestic waste, small particles, noise, excess carbon dioxide or invasive organisms enter into the ocean and cause harmful effects. The majority of this waste (80%) comes from land-based activities, although shipping also contributes significantly. Since most inputs come from land, through rivers, sewage or the atmosphere, this means that the continental shelf is more susceptible to pollution. Air pollution is also a contributing factor by pulling particles of iron, carbonic acid, nitrogen, silicon, sulphur, pesticides or dust into the ocean. Pollution often comes from non-point sources such as agricultural runoff, wind-blown debris, and dust. These nonpoint sources are largely due to runoff into the ocean via rivers, but wind-blown debris and dust may also play a role, as these pollutants can settle into the oceans. Pollution pathways include direct discharge, inland runoff, and pollution from ships, air pollution and potentially deep-sea mining.

1.1.2 Polychaetes

Polychaetes are marine annelid worms that can contribute to aquaculture diversification. Polychaetes are bristle worms- a class of annelid worms- found in marine environment. 8000 species found worldwide and 153 species in India. Sensitivity to reduced oxygen level and accumulation of heavy metals- pollution indicator. Its culture has been viable, and commercially attempted, but intensive production has progressed only in few countries around the world. In the countries with no production, marine polychaetes are imported or harvested. A strong and sustained research investment provided to a better understanding of the nutritional requirements and reproduction of some species. Recent studies showed new technical improvements, which can lead to an important progress in productivity and give a new impetus to the polychaete production. Some marine worm species were identified as good candidates for integrated multi trophic aquaculture. The development of cost-effective aquaculture technique. Polychaetes are marine multi-segmented worms. They assume great significance from the point of view of ecology and biotechnology. The utility of polychaetes in different fields and their cultural prospects are briefly described. They play a major role in Environment Impact Assessment studies as "stress indicators". They are also proved to be a very good

live feed for aquaculture industry and significant bait for sea angling industry. The diagnostic features and other aspects of polychaetes are elaborated in this article. Polychaetes are widespread across marine ecosystem reaching high density in various seabed habitats, where they play a relevant role in the functioning of benthic communities. Many polychaetes are sessile and tube-dwelling forms, often gregarious, which are able to create primary biogenic structures or to modify the abiotic characteristics of the pre-existing substrate. They act as real builders of forest-like structures that offer new substrates for other benthic species, affect habitat conditions and regulate ecosystem functioning. Thus, polychaetes are ascribed to the engineers of animal forests. In this chapter, the main evidences of polychaetes as habitat formers on both rocky and sedimentary bottoms are reported; the time-scale variations of the biogenic structures are considered and the sensitivity and threats of polychaete forests are exhibited for contributing in decision-making regarding protection measures and management of coastal ecosystems. Polychaetes are common in most marine habitats and dominate many neonatal communities. The functional classification of guilds based on identity and taxonomy linked community structure with ecological function. The functional groups currently include osmotic siboglinids as well as siboglinids, echiurans and myzostomes, which molecular genetic analyses have placed in Annelida. Advances in understanding the mechanisms of encounter clearly link locomotion with feeding regimen. New analyses of burrowing mechanisms explain the prevalence of bilateral symmetry and blur the line between surface and subsurface feeding. The dichotomy between microscopic deposits and suspension and macrophage predators, herbivores and omnivores is further supported by different digestive strategies. Deposit feeding appears to be largely restricted to worms larger than 1 cm, with juveniles and small worms often restricted to digestible organic matter and larger, richer foods, confusing macrophage-microscopic dichotomy applies well to larger worms.

1.1.3 Importance of polychaetes

Polychaetes are important components of the marine environment. Polychaetes are particularly good bio indicators for organic pollution due to their high diversity, abundance and functional importance. Major role in food chains. They are burrowers Polychaetes- burrowing and feeding activity- enhance sedimentary processes- releases hydrophobic organic contaminants. Polychaetes are important

role in organic matter burial, by controlling the amount of organic carbon out of the oceans and atmosphere. Important ecotoxicological testing organisms due to its relatively small size, short life cycles and general ease of maintenance. Polychaetes have long been the obvious choice to serve as representative species. The life history characteristics of polychaetes also make them good candidates for serves as an indicator species. The most important ecological functions performed by estuarine polychaetes were bio turbation of sediment, fragmentation of organic matter, stabilization of sediment, nutrient cycling and secondary production. On the ocean floor, polychaetes help convert organic debris into carbon dioxide, which is transported to the surface dissolved in water. Marine plant plankton take up the carbon dioxide and through photosynthesis produce sugars, or food, releasing oxygen in the process. Also being a food source to over one hundred organisms polychaetes help to provide food for fish and their larva. Because of their important roles polychaete worms aid the fishing industry that provides food for humans, thus symbolically represent a dinner plate to humans in the 21st century. They are an important link in marine food webs. Due to the high calorific value and rich protein content, both the adult and larvae of polychaetes are the main food supply of many economically important fishes. Polychaetes including other benthos greatly response to the environmental pollution and studying about how they resist the polluted habitat would reveal interesting facts about how to overcome these pollutants. An effective strategy for marine pollution control management can be established. Help in nutrient cycling in the marine environment. Polychaetes are the most abundant group of invertebrates in estuarine ecosystems (Rodrigues et al., 2011), also a major component of estuarine and coastal food webs, and a food source. Main species of many fish, crustaceans and birds. The transfer of contaminants to higher trophic levels (Lewis and Watson, 2012; Serrano et al., 2003). Furthermore, because polychaetes are in close contact with sediments, they are also particularly exposed to harmful materials both in the sediment and in the associated pore water (Banta and Andersen, 2003; Scaps, 2002). Polychaetes are considered good models in toxicology studies, being considered important watchdogs in the assessment of contaminated sediments (Dean, 2008). Polychaetes are used as aqua feeds for most aquaculture species and as agents for bioremediation of organic waste. Polychaetes applied to brood stock and crustaceans stimulates the gonadal maturation and endocrine system of white shrimp, thereby overcoming the shortage of young

animals and ensuring the production of the aquaculture crop. Reliable product. Industrial feed dominates the polychaetes culture. The essential nutrients and favourable morphology of the microalgae are ideal for polychaete larval culture. Homemade food is less appealing due to its complex structure, but it can be used as a supplement in the polychaetes' diet. Wastes with high organic matter content are competitive as food for polychaetes, although they may lack appropriate nutrients. The ideal foods for the growth of polychaetes, distinguished by different life stages, are little studied. This review investigates the suitability of microalgae in larval rearing, the nutritional value of commercial and homemade feed as a general feeding option, in the polychaetes diet; and bioremediation benefits from polychaetes waste feeding behaviour.

1.1.5 Importance of gut micro biome

In a healthy state, the gut micro biota has myriad positive functions, including energy recovery from metabolism of non-digestible components of foods, protection of a host from pathogenic invasion, and modulation of the immune system. Most of the intestinal bacteria of the worm originate from the soil adjacent to where the worm is located (Horn et al., 2006; Karsten and Drake, 1995). The content of dominant nutrient elements (carbon, nitrogen, phosphorus, and sulphur) in worm gut was reported to be 2–5 times that of the adjacent soil (Drake and Horn, 2007). As a consequence, the extraordinarily abundant amount of nutrients and bacteria in the worm gut are favourable for the transformation of various pollutants ingested from soil, including heavy metals, organic pollutants, microplastics, and antibiotics, etc. (Drake and Horn, 2007; Wang et al., 2019a, 2019b). It is important to note the reciprocal impact of the pollutants on the bacterial activity and diversity in the worm gut (Chao et al., 2019). The gastrointestinal (GI) micro biome is a complex and dynamic group of microorganisms present in the gastrointestinal tract of an organism that lives in symbiosis with the host and confers benefits to the host with biological functions. Study differently. The GI microbial community is formed by a variety of aerobic, anaerobic, and facultative anaerobic bacteria in aquatic species. In lobsters, the common gastrointestinal microorganisms found in the gastrointestinal tract are *Vibrio*, *Pseudomonas*, *Bacillus*, *Micrococcus* and *Flavo* bacterium, where the structure and abundance of these bacteria varies according to the environment. The gastrointestinal micro flora plays an important role and has a clear influence on the

general condition of lobsters such as the secretion of digestive enzymes (lipase, protease and cellulase) that help digest food, provide nutrition and total Synthesize vitamins necessary for the host system, protect the digestive system. The host resists infection by pathogens and disease by activating immune mechanisms in the gastrointestinal tract. Microorganisms in the water, sediment, and food columns are primarily responsible for modifying, manipulating, and forming gastrointestinal microbial structures and communities. This review also highlights the possibility of isolating the native gastrointestinal micro biota as a potential probiotic strain and introducing it into juvenile lobsters and larvae for better health management.

1.2 APPLICATION OF EXOPOLYSACCHARIDES

Advances in the application of bacterial exopolysaccharides in medicine and biotechnology have seen uses to include bacterial alginate in cell microencapsulation, such as microsphere vectors for drug delivery, making dental impressions, as an active ingredient in absorbent dressings, and anti-reflux therapies .Lactic acid bacteria (LAB) produce exopolysaccharides (EPS), which are crucial in the production of fermented dairy products such yoghurt, drinking yoghurt, cheese, fermented cream, and milk-based sweets. Dextran, like Leu, was the first industrial polysaccharide produced by LAB. It was discovered in sugar cane or beet syrup in 1880, and dextran was found to be responsible for the thickening and gelation of syrup. Although there are some studies on the use of non-LAB EPS (cellulose, pollutant, gellan gum, xanthan gum, etc.) in food packaging, LAB EPS has various bioactivities that can be used in packaging to benefit food products. Although both the composition and the amount of EPS produced by a microorganisms are genetically determined traits, they are highly influenced by media components and cultivation conditions. EPS synthesis is generally driven by the presence of an excess carbon source with limitation by another nutrient (nitrogen, oxygen, etc.). In fact, EPS structure, composition, and viscosity depend on several factors, including:

B. Fermentation conditions such as medium composition, carbon and nitrogen sources, precursor molecules, mineral salts, trace elements, strain type, pH, temperature, oxygen concentration and agitation. Microorganisms used as industrial or technical producers of extracellular polysaccharides are primarily pathogenic bacteria. Xanthomonas, Leuconostoc, Pseudomonas, and Alcaligenes species, which produce xanthan, dextran, gellan, and curdle, respectively, are the best known

and most used industrially. In fact, EPS produced by lactic acid bacteria (LAB) is already recognized as GRAS (Generally Recognized as Safe) and is the most suitable polymer for the food industry. Exopolysaccharides can be used as antigen carriers or as antigens in vaccine formulations. Among the various types of aquatic microorganisms, marine microorganisms account for half of the production of organic matter on earth. So far, many common marine strains capable of producing EPS have been identified, including *Pseudoalteromonas* sp, *Bacillus*, *Alteromonas* sp and *Vibrio* sp.

1.2.1 Effects of different stress levels on bacterial growth and EPS production

Various abiotic stressors such as drought, salinity, temperature and heavy metals are major environmental pressures affecting agricultural productivity and crop yields worldwide. Constant changes in climatic conditions exert selective pressure on microbial ecosystems to produce exopolysaccharides. Apart from soil flocculation, exopolysaccharide (EPS) production affects water permeability, nutrient uptake by roots, soil stability, soil fertility, plant biomass, chlorophyll content, and root and shoot length, and foliage. Not only does it help increase surface area, but it also helps maintain metabolism and physiology. Activity during drought stress. Microorganisms that produce EPS bind to sodium ions in the soil, preventing those ions from reaching the stem, reducing sodium uptake from the soil, and increasing nutrient uptake by the roots, thus helping the plant grow. It can give salt tolerance. Biofilm formation in high-salinity soils enhances cell survival, increases soil fertility, and promotes plant growth and development. A third environmental stressor is the presence of heavy metals in soil due to improper disposal practices of industrial wastes that are toxic to plants. Production of EPS by soil bacteria can lead to bio mineralization of metal ions, thereby endowing plants with metal stress tolerance. Finally, high temperatures can also affect agricultural productivity by reducing plant metabolism, seedling growth, and seed germination. Heavy metal contamination is currently a major concern, and microbial proportions for the biological remediation of metals should be viewed as an efficient and economical strategy. Based on this, we investigated the effects of heavy metal stress on the exopolysaccharide (EPS)-producing agricultural isolate *Pantoea agglomerans*. The production of EPS is a protective response to stress to survive and thrive in metal-contaminated environments. *P. agglomerans* exhibits tolerance and mucus formation in the

presence of heavy metals. Mercury, copper, silver, arsenic, lead, chromium, cadmium. EDX first confirmed metal accumulation, and FTIR identified the functional groups involved in metal binding. ICP-AES identified sites of cell binding and intracellular metal accumulation. Cell surface metal deposits released more Ca^{2+} . Effects on bacterial morphology studied by SEM and TEM revealed sites of metal accumulation as well as possible structural changes. Each heavy metal caused distinct changes and was enriched in intracellular deposits of cell-bound EPS. Therefore, the performance of *P. agglomerans* under metal stress indicated a potential candidate for metal bioremediation.

1.3 HEAVY METAL POLLUTION

Heavy metals normally occur in nature and are essential to life but can become toxic through accumulation in organisms. The term "heavy metal" is now used to refer to metallic chemical elements and metalloids that are harmful to both humans and the environment. A few of the most frequent heavy metals that the environment include mercury, cadmium, arsenic, chromium, nickel, copper, and lead. The term "heavy metals" refers to metallic substances with particularly large densities when from water. An important environmental issue in the modern world is heavy metal pollution. Due to incorrect disposal of industrial waste products into water bodies and land regions, rapid industrialization results in industry expansion, which continuously raises the concentration of metals and generates major pollution issues. (Dixit et al. 2015). The primary components of the earth's crust are heavy metals, which are persistent environmental toxins that cannot be completely biodegraded but must instead be changed into benign forms (Coral et al. 2005). In general, the term "heavy metal" refers to a collection of metals with higher atomic numbers (above 20) and higher densities (5 g/cm^3), including cadmium, lead, mercury, nickel, chromium, arsenic, copper, and zinc. Because of their potent inhibitory effects on biodegradation activities, these metals have a direct relationship with environmental pollution and biological toxicity issues. (Nagajyoti et al. 2010; Gupta et al. 2014). Metals typically serve as cofactors in a variety of enzymatic and metabolic pathways and are required in modest amounts as nutrients. Larger concentrations of these metals, however, can become extremely toxic or suppressive to all life forms, including humans, animals, plants, and microorganisms. Despite the fact that a relatively small number of metals, such as cadmium (Cd),

arsenic (As), and mercury (Hg), are exceedingly deadly at very low quantities (Atlas and Bartha 1993; Nies 1999). When high levels of metals build up in soil, it has a negative impact on fertility and productivity. These may also enter the body through food, water, or the air and bio accumulate over time. (Lenntech 2004; Deeb and Altahali 2009). Lethal metal accumulation in the human body has detrimental effects on health, including aberrant growth and development, carcinogenesis, neuromuscular defects, mental diseases, and metabolic dysfunction. (Thiele 1995; Chandra et al. 2011). The most significant source of toxic metals in the environment comes from a variety of practises and pathways, including combustion and extraction of toxic waste into the atmosphere, as well as excess emissions of wastewaters/effluents containing harmful noxious chemicals and contaminants, which pollute surface waters and soils. It has become damaging for the variety of life on Earth as hazardous metals are continuously used in anthropogenic activities from industrial sectors including electroplating, painting, tanning, textiles and dyes, papermaking, mining, and others. (Dhal et al. 2013; Yadav et al. 2017). These industrial operations frequently discharge hazardous metals at high concentrations (beyond allowed limits), which poses a toxicity concern for environmental contamination. As a result, their removal or remediation is now required for environmental safety. (Dhal et al. 2013; Velma et al. 2009). As a result, biological remediation utilising microorganisms and plants is typically thought of as an environmentally benign, secure, economical, and long-lasting method for the removal of harmful metals when compared to traditional treatment technologies. But compared to biological treatment technologies, which are simple to use and do not produce secondary pollutants, conventional technologies have some drawbacks, such as high operating costs, maintenance requirements, and the production of hazardous by products or inefficiency. (Vargas et al. 2012; Chandra et al. 2011). Bioremediation and phytoremediation are terms used to describe the processes of utilising microorganisms and plants to reduce and detoxify extremely toxic contaminants into harmless and less damaging forms. (Jain et al. 2014). This chapter focuses on the occurrence, uses, and hazardous effects of heavy metals in the environment as well as how they build up in people, animals, and plants and how they affect the environment. A proposed biological method of heavy metal contamination site treatment is also covered in this chapter.

1.3.1 Soil heavy metal pollution

In the soil environment the common pollutants are arsenic (As), cadmium (Cd), chromium (Cr), mercury, lead, zinc, nickel. Food security and human health recently have negative impacts on crop quality. Food security is a high priority issue have both qualitative and quantitative for sustainable global development. Food safety is fundamentally influenced by soil and negatively impacted by heavy metals on crop quality, which poses a concern to human health. Soil is crucial to the safety of food. A heavy metal is any metal or metalloid with a density of 0.5 g/cm^3 . (Oves et al., 2012). Heavy elements include things like mercury (Hg), lead (Pb), cadmium (Cd), and chromium (Cr). Because of its chemical composition and natural behaviour, arsenic (As) is often classified as a heavy metal. (Chen and Zhu, 1999). Heavy metals can form as a result of either natural or artificial processes. The exploitation of natural resources, metal smelting, chemical manufacture, industrial emissions, and sewage irrigation have all been demonstrated to be the main causes of heavy metal contamination (Davies, 1983; Liu et al., 2005; Chen et al., 2014; Zhang et al., 2015). Heavy metal pollution may be harmful to people's health as exposure, for example, can cause cardiovascular and other systemic diseases that can eventually result in cancer (Chen et al., 1995; Mazumder et al., 2000; Huang et al., 2015). Heavy metal pollution may also cause soil quality to decline as a result of decreased soil productivity (Alloway, 2013; Wei et al., 2016). The majority of the 30,000 tonnes of Cr and 800,000 tonnes of Pb that were released into the environment over the previous 50 years settled in soil, causing considerable heavy metal contamination. (Chen et al., 2016). Soil heavy metal contamination is becoming a more serious environmental issue, especially in industrial and agricultural areas, as a result of China's rapid social and economic development. (Chen et al., 2016). The soil quality on cultivated land is particularly concerning, and the issue of the soil environment in former industrial and mining areas was significant, according to the national communique of the soil pollution survey issued by the Chinese Ministry of Environmental Protection and the Ministry of Land and Resources in 2014 (MEP of China, 2014). The Grade II environmental quality standard for soils in China (GB15618-1995) states that the overall soil over-standard rate in China was 16.1%. In instance, the over-standard rates for the five inorganic pollutants Cd, Hg, As, Lead, and Cr were 7.0%, 1.6%, 2.7%, 1.5%, and 1.1%, respectively (MEP of China, 2014). The amount of land that may be used for

agriculture in China's industrial and agricultural sectors has decreased due to heavy metal contamination of the soil. The percentage of polluted companies and the nearby soil reportedly surpassed regulations by as much as 36.3% and 19.4%, respectively, in 2014, according to the MEP of China. China has fought a "war" against soil pollution in an effort to improve soil quality, ensure the calibre of agricultural output, and protect both human and animal health. To prevent and control soil heavy metal contamination, for instance, a national action plan known as the "Soil Ten Chapter" has been implemented (MEP of China, 2016). To measure the heavy metal concentrations at the sample sites, earlier research mainly focused on a selected average area before calculating the quantity of pollution and associated risks. The primary contributors to the rise in soil heavy metals, according to Yang et al. (2017)'s investigation, are Wuhan, China's industry, agricultural, and transportation. Additionally, a study conducted in the central Xinjiang Uygur Autonomous Region of China found that agriculture and industry had distinct effects on soil heavy metal pollution. Several domestic studies have concentrated on industrial and agricultural regions in China, including mining regions in Shaanxi (Xiao et al., 2017), Hunan (Fan et al., 2017; Huang et al., 2017), and other provinces (Liu et al., 2016; Qu et al., 2012). Southeast and northwest China's agricultural regions have been the subject of several studies (Deng et al., 2017; Wang et al., 2017; Wu et al., 2017). Sadly, there aren't many research on the heavy metal contamination of soil in industrial and agricultural areas. A thorough macro-evaluation is desperately needed for the nation. This study's goal is to assess the levels of pollution and associated risks brought on by heavy metals in the soils of China's industrial and agricultural regions. The objectives of this study are to: 1) evaluate the levels of soil heavy metal pollution in China's industrial and agricultural regions; 2) determine the potential health and ecological risks posed by soils contaminated with heavy metals; and 3) formulate policy suggestions for relevant organisations.

1.3.2 Drinking water heavy metal pollution

Heavy metal contamination in drinking water poses a threat to humans and is often the root cause of various severe health issues such as cancer and organ damage. The presence of heavy metal cannot be seen with the naked eye but can be detected with a water test. Water pollution occurs when unwanted materials enter in to water, changes the quality of water and harmful to environment and human

health. Industrial, municipal, and urban runoff frequently contains toxic metals that are dangerous to biotic life and humans. Seema Singh et al. (2011) blame rising urbanisation and industrialization for the rise in trace metal levels, particularly heavy metals, in our rivers. When released into the environment, several hazardous chemical components build up in soil and water body sediments (Abida Begum et al., 2009). Over 50 elements fall into the category of heavy metals, 17 of which are regarded as being extremely hazardous and easily accessible. Characteristically, anions also play a significant role in drinking water; results also indicated that they have an impact on people's health (S. Khan et al., 2012). The level of toxicity is dependent on the type of metal, its biological function, and the kinds of species to which it is exposed. According to Ram S Lokhande et al. (2011) heavy metals have a noticeable impact on aquatic flora and fauna, which through bio magnification penetrates the food chain and eventually affects humans. Lead, iron, cadmium, copper, zinc, chromium, and other heavy metals in drinking water are those most frequently associated with human toxicity. They are needed by the body in modest amounts, but big dosages of them can be hazardous. If present, they make up a significant group of environmentally harmful compounds. Although important trace elements like copper exist, copper can be harmful if present in high concentrations in drinking water. The biological half-life of cadmium in the human body is between 10 and 33 years, and it is exceedingly dangerous even at low quantities. It will bio-accumulate in organisms and ecosystems. Renal damage is also brought on by prolonged cadmium exposure. So, in most nations and international organizations, cadmium is regarded as one of the priority contaminants for monitoring. Water pollution and contamination are closely related. The quality of groundwater and surface water sources must be regularly evaluated (Ehi-Eromosele C.O., Okiei W.O., 2012). Damaged or diminished mental and central nervous system function and decreased energy level are two documented lethal outcomes of heavy metal poisoning in drinking water. They also result in abnormal blood composition and negatively affect important organs including the kidneys and liver (Sher Ali Khan et al., 2011). Multiple sclerosis, Parkinson's disease, Alzheimer's disease, muscular dystrophy, and muscular dystrophy are all neurological diseases that are brought on by prolonged exposure to these metals. Multiple sclerosis is a disease of the nervous system that affects the brain and spinal cord. Lead is one of the most prevalent heavy metals found in drinking water, and when levels exceed the allowable limits,

they can cause general metabolic poisoning and enzyme inhibitors (Gebrekidan et al., 2011). Lead can replenish bone's calcium and create long-term replacement sites. Although important trace elements like copper exist, too much of these metals can be hazardous. Any heavy metal that exceeds its original limitations in drinking water might be toxic if it is present in higher or lower amounts. Many different places, including wells, rivers, lakes, reservoirs, ponds, and so on, provide drinking water. The many water sources represent the biggest threat to people's health because they are contaminated. The main components of water pollution are heavy metals, bacteria, fertilizers, and thousands of harmful organic chemicals. Even though they are present in water in very small amounts, heavy metals are more hazardous to humans. Given the risk that heavy metals in water provide, it was essential to launch this study in order to evaluate the issue and make suggestions for ways to reduce the likelihood that drinking water will become contaminated with deadly heavy metals. After the United Nations International Drinking Water Supply and Sanitation Decade (IDWSSD) was observed from 1981 to 1991, (since June 1991) the beginning of a new era of economic reforms and liberalization, drinking water has been discussed in the spotlight as a commodity to be priced or paid for by the user. Water-related constitutional rights and obligations are hazy within the federal system because all three governmental entities, the federal government, the state government, and local governments at the village (Panchayat) and city (Nagarpalika) levels, are involved in water management (Keshab Das and Neha Panchal, 2006)[7]. Guidelines for the presence of heavy metals in drinking water have been established by many international organizations, such as the USEPA, WHO, EPA, and the European Union Commission, in order to reduce the toxicity of heavy metals in drinking water and to preserve human health.

1.3.3 Ground water heavy metal pollution

Groundwater is a natural resource, the evaluation of groundwater using the long-term water quality helps in developing new strategies. Water scarcity has led to wide use of reclaimed water for irrigation worldwide, which may threaten groundwater quality. Groundwater has become the most important source of water supply for domestic, industrial and agricultural sectors of many countries, including Iran (Rezaei and Hassani, 2017). Iran is located in an arid to semi-arid region where the average annual precipitation is less than one-third of that of the world and the

evaporation rate is extremely high, especially in southeast parts of the country (Baghvand et al., 2010). In many parts of the world demand for freshwater such as groundwater has experienced increase due to population explosion, urbanization, irrigation and domestic activities (MacDonald et al., 2014). Sustainable water resources management is a crucial concern in most countries across the globe (Kalhor et al., 2019). Due to severely deficient sustainable water resources, groundwater is the primary source of water for irrigation as well as human consumption in southeast Iran. Therefore, regular monitoring of groundwater resources coupled with hydro geochemistry study and heavy metals assessment plays a key role in the sustainable management of these resources. Heavy metal pollution is considered to be one of the most pronounced issues threatening water quality. Water quality contamination by heavy metal pollution has severe effects on public health. Heavy metals are well known for their carcinogenic effects and toxicity towards living beings, long residence times (often exceeding decades), and persistent bioavailability, especially when dissolved in ionic form in water (Maanan et al., 2015). Heavy metals exist in water in colloidal, particulate and dissolved phases (Adepoju-Bello et al., 2009) with their occurrence in water bodies being either of natural (e.g. eroded minerals within sediments, leaching of ore deposits and volcanism extruded products) or anthropogenic origin (i.e. solid waste disposal, industrial or domestic effluents) (Marcovecchio et al., 2007). Elevated heavy metals concentrations in water leads to environmental problems making it necessary to centre studies on potential water contamination from metals sources (Rezaei et al., 2017). The hydro chemical evaluation is very important to assess the groundwater quality in any region in which the groundwater is used for both irrigation and irrigation needs (Kumar et al., 2014). Many studies have focused on groundwater quality and pollution sources influenced by anthropogenic and natural processes (Sue et al., 2013; Mehdi et al., 2014; Nasrabadi et al., 2014; Ying et al., 2014; Mohammad et al., 2016; Rezaei et al., 2017). A widely used approach in groundwater metals studies is to employ pollution indices. Pollution indices are useful tools for water quality executives, environmental managers, and decision makers as measures which combine influences of all parameters into one single number (Rezaei et al., 2017 a). When studying heavy metals, pollutants are generally monitored for assessment of water quality by comparing the recorded concentration with allowable heavy metal concentrations recommended by water standards. Knowing concentrations of

pollutants one can develop different pollution indices. Several water quality indices and corresponding applications of index methods have been proposed for estimation of water quality (Edet and Offiong, 2002; Bhuiyan et al., 2010; Chaturvedi et al., 2018). In recent years attention has been vastly given to evaluation of heavy metal pollution in groundwater using the heavy metal pollution index (HPI), the heavy metal evaluation index (HEI), degree of contamination (Cd), and the modified heavy metal pollution (Edet and Offiong, 2002; Prasad and Mondal, 2008; Rajkumar et al., 2015; Chaturvedi et al., 2018). In this study, we have investigated the physicochemical parameters of groundwater such as pH, total dissolved solids (TDS), temperature (T), oxidation- reduction (Eh), dissolved oxygen (DO), and electrical conductivity (EC) in Bazman region to generate an assessment for groundwater quality and its use for agricultural and drinking purposes. Heavy metal pollution indexes (i.e. HPI, HEI, Cd, and m- HPI) have been used to evaluate status of the overall pollution level of groundwater resources of the study area with respect to nine important heavy metals (As, Ba, Cr, Cu, Fe, Mn, Ni, Se, and Zn). To make the results more robust, pollution indices and multivariate statistical techniques (Spearman's correlation matrix and principal component analysis) are collectively used to identify the pollution status and probable sources of pollutants in the Bazman region.

1.4 IMPACT OF HEAVY METAL POLLUTION

1.4.1 Cadmium pollution in human health

At the time of birth, the availability of Cd in the human body is zero but with increasing age, it gets accumulated and the Cd concentration increases in the human body. Cadmium poses a significant health risk even at extremely low concentrations in the body since it cannot be metabolically broken down into less toxic species and has very poor elimination due to the lack of effective chelating agents. Cadmium has been classified as a human carcinogen by the International Agency for Research on Cancer. The body's immune system is hampered by Cd toxicity oxidative stress, which disrupts the antioxidant defence mechanism and produces ROS. ROS attack carbohydrates, enzymes, and proteins, causing enhanced lipid peroxidation of cell membranes. Furthermore, leading to DNA mutation and DNA damage, which may further result in carcinogenesis. Cd also causes alteration in the number and functions of T cells in the body and this adversely affects the immune system. Humans are most exposed to Cd mainly

through inhalation, food, and cigarette smoking. Cd can cause injury of the respiratory tract, anosmia, respiratory stress, emphysema, chronic rhinitis, chronic obstructive pulmonary disease, destruction of the olfactory epithelium, development of bronchitis, and sometimes impairment of lung function. Cadmium can pass through the placenta and mother's breast milk, sometimes causes placental damage, which may adversely affect the foetus. In the male or female reproductive system Cadmium toxicity impact to infertility, prenatal death, abnormal embryonic development, and occasionally structural and functional issues. Some of the Cd-induced damage in the males includes a decrease in sperm motility, damage to the testis manifests, organ degeneration and dysfunction, vacuolization of the seminiferous tubules, and prostate cancer. Cadmium toxicity can cause in females endothelial changes in the blood vessels, ovarian hormonal production, and ovarian progesterone biosynthesis. Cd can accumulate in the renal tissues and bind through metallothionein in the kidney without being eliminated. The renal lesion is the initial sign of tubular damage, resulting in increased urinary excretion containing proteins with low molecular weight i.e., α_2 -, β_2 -, and globulins. In several cases, due to decreased concentration capacity, renal functions are manifested by hyperphosphaturia, aminoaciduria, glycosuria, eventually resulting in nephropathy. Chronic exposure to Cd causes low bone mass, reduced bone density, bone loss, increased bone fractures, and skeletal lesions. Cd hinders the metabolism of magnesium, zinc, calcium, iron, and copper, reduces normal activation of vitamin D, and reduces the phosphate uptake (fibroblast growth factor 23) in bones. Vitamin D (in the kidney activated to calcitriol) plays a major role in the absorption (from the gut) of calcium and calcification of bone. This leads to intense pain in the bones, osteoporosis, osteomalacia, phosphaturia, demineralization of bones, and other bone disorders. Cd toxicity also occurs when there is the consumption of acidic food or beverages that were improperly stored in cadmium glaze containers. The ingested cadmium doses lead to irritation of gastric epithelium and cause tenesmus, vomiting, nausea, pain, abdominal cramps, and diarrhea. In the central nervous system (CNS), Cd enters by altering the permeability of the blood-brain barrier through the process of inhalation. Cadmium affects the CNS by causing oxidative stress and causes a decrease in acetyl cholinesterase activity. Cd toxicity reduces the actions of antioxidants especially superoxide dismutase and glutathione, leading to neurotoxicity and behavioural changes. Cd²⁺ exposure can contribute to lipid

peroxidation leading to disturbances in Na⁺, K⁺ATPase functioning which is responsible for transporting K⁺ and Na⁺ ions into cells in the CNS. Cd exposure decreases membrane potential that can lead to impaired neurotransmitter signalling. The release of neurotransmitters depends on calcium (Ca²⁺) channels, which sometimes gets blocked due to Cd toxicity (Cd²⁺) and thereby interferes with the passage of Ca²⁺ into and out of the cell and disrupt homeostasis. This can later reduce the intracellular influx of Ca²⁺ or a rise in intracellular Ca²⁺ that is released from intracellular stores. A constant rise in intracellular Ca²⁺ can contribute to cell death. During the early development stage, Cd²⁺ exposure alters the activity of specific neurotransmitter systems (including monoaminergic, cholinergic, glutaminergic systems). Cd causes a number of harmful effects on different parts of the human body leading to untreatable diseases which may even cause death.

1.4.2 Impact of Environmental Cadmium Pollution

Cadmium is released into the atmosphere as a result of natural or manmade activities and animals and humans can be exposed to it differently. Cadmium can pollute the aquatic environment that are contribute to absorption, industrial waste, and surface runoff into sediments soil and sediments. Cadmium can make all people sick with contaminated food, air, or water. Cadmium does not have any properties that are helpful for metabolic process and plant growth.

1.4.3 Impact of Cadmium Pollution in marine organisms

Cadmium is one of the most toxic heavy metals for living organisms. Cadmium is found in the hard wall of the earth with the element zinc and has been in the environment for centuries through mining activities to extract zinc resources. Cadmium is also widely distributed in the aquatic environment and its bioaccumulation is well detected by aquatic organisms. The amount of cadmium in the edible part of fish (fish muscle) is generally low, while cadmium accumulates in organs such as the kidneys and liver. Slow, so the organs can be very contaminated and preferably should not be consumed.

1.4.4 Impact of Mercury pollution in human health

Mercury is main effects of humans and the environment are negative for human health. The toxicity of mercury depends whether it is present as an elemental mercury, an organic or inorganic compounds. (Especially, alkyl mercury compounds such as methyl mercury and Ethyl methyl salts and dimethyl mercury). The major source of human exposed to the terms of methyl mercury and dietary ingestion, particularly for seafood and fish. Around 80% of inhaled elementary mercury vapour is retained in the tissue of the lungs where it goes on to penetrate the blood-brain barrier where neurological effects take place. Ingestion of elementary mercury does not always lead to high levels of absorption but deaths have been reported. Inhalation of elementary mercury vapour has been observed to lead to symptoms including tremors, emotional liability, insomnia, memory loss, neuromuscular changes, and headaches as well effects on the kidney and thyroid. High exposures have led to death but the critical effects are neurotoxic and renal. The main route of exposure to inorganic mercury for humans is dietary although for some sub-sections of the population products such as skin lightening creams, soaps and the use in traditional medicine and/ritualistic practices can result in significant exposures to both inorganic an elemental mercury. Methyl mercury is a well-known potent neurotoxin which causes adverse impacts on the developing human brain. It passes readily through the placental barrier and the blood-brain barrier making any exposure during pregnancy of great concern. Methyl mercury is considered as possibly carcinogenic by the International Agency for Research on Cancer.

1.4.5 Impact of mercury pollution in environment

Mercury is an extremely hazardous heavy metal that may be found in biosphere. Due to human activities, it has also become a widespread contaminant and is increasing in the atmosphere. Mercury converts to the highly toxic methyl mercury when in contact with aquatic sediments. Methyl mercury enters the human body through the food chain via fish, seafood, and wildlife, which become contaminated after ingestion of toxic microorganisms. It penetrates the circulation after being absorbed into the human body and causes a variety of neurological problems. A naturally occurring metal with a wide range of shapes and sizes is mercury. The liquid form of metallic mercury is glossy, silver-white, and odourless. When mercury combines with other elements like chlorine, sulphur, or oxygen, it

produces inorganic mercury compounds or salts, which are often white powders or crystal. Tremors, melancholy, and behavioural problems are brought on by the rapid entry of mercury into the brain. At the beginning of time, thousands of years have passed. Mostly, it is employed in the extraction of valuable metals. The paint industry, as well as float valves, mercury switches, mercury relays, manometers, sphygmomanometers, fluorescent lights, and thermometers all employ mercury (Hg). Mercury concentrations above 0.5 ppm were discovered in New Jersey, USA, and are a worry for human health for people who routinely eat fish. Mercury tissue contents greater than 0.3 ppm were found in 48.8% of the population of 36,422 lakes in the USA that were sampled. Mercury, a priority contaminant according to the US EPA, has been the subject of much discussion for a long time. Adsorption holds the greatest promise among the numerous mercury removal techniques discovered over the years since it is straightforward, inexpensive, and effective at purifying water. Over the past four years, we have seen significant advancements in Hg removal in the fields of materials science and adsorptive separation technology. Indicating that the added functional groups' ability to donate electrons may contribute to increasing the adsorption capabilities of the adsorbents, the Hg (II) removal efficiencies rose in the order of ACCI impregnated ACBr-impregnated ACI-impregnated AC. There are a number of potential benefits for the removal of aqueous Hg (II), including quick separation, simple analyte elution, and reusable adsorbent. Due to issues like low surface area and improper distribution of thio/thiol groups, low capacity and moderate affinity for Hg(II), sulphur leaching, and poor stability over a wide pH range, existing adsorbent materials have largely been limited in their effectiveness and efficiency for the removal of Hg(II) from aqueous solutions.

1.4.6 Impact of mercury pollution in marine

Mercury in the environment by agricultural activities (fungicides and seed preservatives), pharmaceutical factories, as paper and paste preservatives, catalysts in the synthesis of organic matter, in the production of batteries and thermometers in the production of metal mixed with mercury and Chlorine is released in caustic soda factories. It is estimated that the annual in flow is 40,000 to 50,000 tons in the atmosphere and approximately 4,000 tons in the sea. The first poisoning by mercury-infected fish was called Mina Mata disease, which after a thorough investigation revealed methyl mercury poisoning. The toxicity of mercury depends on its chemical

form (organic, metallic, and ionic). Mercury is mainly present in fish in its organic form, or dimethyl mercury.

1.4.7 Impact of Lead pollution in human health

All organs are negatively impacted by lead, but the kidneys are the most severely affected. Proximal tubular dysfunction brought on by acute lead nephropathy results in a condition similar to Fanconi. Hyperplasia, interstitial fibrosis, and a trophy of the tubules, together with renal failure and glomerulonephritis, are characteristics of chronic lead nephropathy. Lead is a carcinogenic chemical that damages the DNA repair process, genes that control cellular cancer growth, and chromosomal structure and sequence by producing ROS. By removing zinc from specific regulatory proteins, it alters transcription. Inorganic lead exposure at high occupational (work-related) levels has been the focus of the majority of investigations investigating for a potential connection between lead exposure and cancer. Blood lead concentrations have been observed to be significantly higher in people with heavy workplace exposure to lead than the average blood lead level in the general population. There have been numerous studies looking for a connection between lead exposure at work (mostly among battery workers and smelter workers) and lung cancer. There is a little increase in the risk of lung cancer, according to several of these studies. However, the majority of these studies were constrained in that they neglected to consider other elements that can influence the chance of developing lung cancer, such as smoking or exposure to other heavy metals like arsenic, which is frequently present in industrial environments along with lead exposure. There is a very little risk of lung cancer in persons with higher blood lead levels, according to certain studies that examined lead blood levels in the general population. The risk of stomach cancer was examined in several of these similar workplace studies. The majority of research revealed that higher lead exposure was associated with a higher risk of stomach cancer. The studies did not account for any factors that could have increased the risk of stomach cancer, even though it is doubtful that smoking or exposure to arsenic would have an impact on these findings. The findings of these investigations have been conflicting. While some research did not find any connections, some have. It is obvious that there is a connection between lead exposure and cancer, and additional study is required to clarify this connection's potential impact on various malignancies.

Studies have also investigated potential connections between lead exposure at work and various malignancies, such as brain, kidney, bladder, colon, and rectum tumours.

1.4.8 Impact of environmental pollution in lead

Lead is a non-biodegradable metal that is available in nature and found in relatively low amounts. Atmospheric lead levels are increasing continuously because of the human activities including manufacturing, mining, and fossil fuel burning. Lead is toxic to the human body when exposed to amounts greater than the optimum. Children are at higher risk of lead poisoning; when they come into contact with dust laden with environmental lead, the severity of poisoning increases.

1.4.9 Impact of lead pollution in marine organisms

Lead, as a toxic and dangerous element, enters the natural environment in large quantities through human activities, and despite its low geochemical mobility, the metal has spread throughout the world. Lead is mainly deposited in fish bones and soft tissues such as the heart, gonads and digestive organs do not show high levels of lead. Environmental lead contamination in some parts of the world can be determined by analysing and measuring the amount of lead in fish bones, not muscles.

1.4.10 Selenium heavy metal pollution on human health

Selenium is hazardous in excessive doses, most animals—if not all—need trace amounts of it for proper cellular function. Selenium is a trace nutrient that is crucial for human health. Selenium, for instance, is necessary for the thyroid gland to operate properly. 400 micrograms of selenium per day are the Tolerable Upper Intake Level. Selenosis can result from consumption above that threshold. High amounts that are consumed orally over a brief period of time can make people feel sick and make them throw up and have diarrhoea. Selenosis may result from prolonged oral exposure to high doses. Hair loss, brittle nails, and neurological abnormalities are the main symptoms of selenosis. Breathing difficulties, bronchitis, and stomach problems can all be brought on by short-term exposures to high levels in the air. Long-term exposure might result in coughing, bronchial spasms, and respiratory discomfort.

1.4.11 Impact of copper pollution in Human health

A naturally occurring metallic element called copper has an average concentration of 50 parts per million (ppm) in soil. It is an important nutrient for humans and animals in modest amounts and is found in all animals and plants. Copper mining, smelting, and refining as well as businesses that use copper to make items like wire, pipes, and sheet metal as well as the burning of fossil fuels are the main sources of copper releases into the environment. Copper is frequently used to make water pipes, and copper-containing brass and bronze alloys are sometimes used to make bathroom fixtures. The main source of copper in drinking water is copper leaching from pipes and bathroom fixtures brought on by acidic water. Copper in water can be detected by blue-green stains that are left on bathroom fittings. Utilizing copper in agriculture to combat plant diseases and treating water bodies to get rid of algae are two other ways that copper is released into the environment. Impacts on Health Absorption/Metabolism according to studies looking into copper absorption through food, the amount ranges from 24 to 60 percent. The amount of copper in the diet and competition with other metals found in food, such as iron and zinc, are factors that determine the amount absorbed. Studies evaluating copper exposure through inhalation are lacking. Although the amount of cutaneous absorption is also unknown, a few studies have suggested that it is relatively little. Positive Consequences The regular metabolic processes of humans depend on the presence of copper in numerous enzymes. For adults, 0.9 milligrams (mg) of copper is the Recommended Daily Allowance (RDA). The typical American diet provides between 1 and 1.6 milligrams of copper per day on average. The maximum permissible dosage for a sustained length of time (chronic exposure) is 10 mg per day. Shellfish, organ meats, nuts, legumes, and cocoa are among the foods high in copper. Anaemia, low white blood cell counts, osteoporosis in children and new born, and connective tissue deficiencies causing skeletal issues are just a few of the consequences of copper deficiency. Effects in the Prompt Future Temporary gastrointestinal distress with symptoms like nausea, vomiting, and abdominal pain can result from acute poisoning from consuming too much copper. In levels high enough to cause death, liver damage was seen. Copper exposure at high doses has the potential to destroy red blood cells and lead to anaemia. Effects that Last (Chronically) Animals with efficient mechanisms for controlling their body's copper

storage are typically shielded from high dietary copper levels. However, prolonged overexposure to copper can harm the kidneys and liver at high enough doses. Copper builds accumulated in the liver in Wilson's disease, an inherited (genetic) condition. Jaundice, swelling, and discomfort are among the symptoms of liver damage that typically don't manifest until puberty. Ability to induce cancer, or carcinogenicity although some studies of copper-exposed workers have showed elevated cancer risks, they were also exposed to other substances with the potential to cause cancer at work. Animal studies have not revealed an elevated risk of cancer. The EPA presently classifies copper as a Group D carcinogen (inadequate evidence to classify), and it has not been determined whether copper should be classified in one of the new cancer classification categories. Effects on reproduction and development there are no reports of developmental consequences in humans exposed to high copper levels. In a few studies, animals given large doses of copper showed developmental consequences such as delayed growth and development, delayed bone formation, and lower litter size and body weights.

1.4.12 Impact of copper pollution in environment

Copper is recognized as a vital micro nutrient for living organisms. It has a role in normal physiological functions of plants, such as formation of chlorophyll, photosynthesis, and carbohydrate and protein metabolism. Copper deficiency alters important metabolic processes, and elevated exposure causes toxicity.

1.4.13 Impact of copper pollution in marine

The ability to transport copper in nature is much lower than that of zinc and cadmium. Low-density copper is not toxic to humans and is an essential element for living organisms. Their kidneys accumulate. The average amount of copper in fish muscle is 0.5-0.2 mg / kg of wet weight, while the organs that have more copper include the liver, scales, anal, kidney and gills, respectively. Excess on the aquatic need for copper is stored mainly in the liver.

1.4.14 Impact of zinc pollution in environment

Zinc is a fundamental and omnipresent metal. It is associated with plenty of enzymatic reactions via acting as a cofactor. Zinc toxicity depends on the manner and quantity of exposure. Smelting and mining are major the sources of zinc. A large

amount of zinc emitted into the environment originates from the activities of mineral processing and affects ecosystems as well as living organisms.

1.4.15 Impact of zinc pollution in marine

Zinc, like copper, is an essential element for humans and is a major component of enzymes. Zinc is commonly found in fish and other seafood in milligrams per kilogram, and no amount has been reported in fish edibles that could pose a risk to human health. The average amount of zinc is 3-5 mg / kg based on wet weight, and fresh fish is the most important source of this essential metal for humans.

1.5 VIBRIO DIABOLICUS

Vibrio diabolicus belongs to the genus *Vibrio*, family *Vibrionaceae*. *Vibrio diabolicus*, a marine bacterium originating from deep-sea hydrothermal vents, produces the **HE800 exopolysaccharide** with high value for biotechnological purposes, especially for human health. Its genome was sequenced and analysed; phylogenetic analysis using the core genome revealed *V. diabolicus* is a polysaccharide-secreting bacterium isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. It is facultative anaerobic, heterotrophic, and mesophilic. *V. diabolicus* was originally isolated from the deep sea far away from areas of coastal ecosystems and human activity. *Vibrio diabolicus* was isolated from a sample collected on the polychaete annelid *Alvinella pompejana* from a deep-sea hydrothermal vent on the East Pacific Rise. It is a facultative anaerobic, heterotrophic, and mesophilic organism that is surprisingly easily cultivable under classical laboratory conditions (Raguene et al. 1997). Upon cultivation, this bacterium is able to produce an exopolysaccharide (EPS), designated HE800, composed of a tetra saccharide.

1.5.1 Exopolysaccharide production in *Vibrio diabolicus*

Exopolysaccharides (EPS) are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeated units within the polymer. Bacterial exopolymers are important in the interaction between bacteria and their environment and are chemically diverse. The major organic fractions of the EPS are carbohydrates, proteins and humic

substances (Nielsen and Jahn 1999). A wide range of chemical structures of homo polymeric or hetero polymeric type, made up of sugar and non-sugar components is possible and the range of monosaccharide combinations, together with no carbohydrate constituents and varied linkage types, makes the exopolymers an excellent emulsifying agent and attributes diversity in bacteria (Keene and Lindberg 1983). The bacterial exopolymers are usually acidic heteropolysaccharides possessing the functional groups (e.g. hydroxyl, carboxyl and phosphoric acid) associated with EPS, which exhibits high affinity towards certain metal ions (Mittle man and Geesey 1985). Many interesting physical and chemical properties of microbial exopolysaccharides have found a wide range of applications in the field, e.g. stabilizing, suspending, thickening, gelling, coagulating, film-forming and water-retention capability, e.g. in detergents, textiles, adhesives, paper, paint, food and beverage industries (Sutherland 1996), oil recovery, mining industry and petroleum industries (Sutherland 1998). A wide range of bacteria from various environmental habitats are known to produce complex and diverse EPS occurring as capsular polysaccharides intensively associated with the cell surface or as slime polysaccharides, loosely associated with the cell (Whitfield 1988). Bacterial growth is often accompanied by production of EPS, which have relevant ecological and physiological functions. The nutrient status and growth phase of surface associated bacteria may influence the quality and composition of the EPS produced (Decho 1990). In recent years there has been a growing interest in the isolation and characterization of microbial EPS owing to their importance in adhesion, nutrient sequestration, chelation of heavy metals, detoxification of toxic compounds and protection against osmotic shock (Decho 1990; Hoagland et al. 1993). Despite their importance, very few studies have been carried out on chemical characterization of EPS by marine fouling bacteria (Rodrigues and Bhosle 1991; Majumdar et al. 1999; Muralidharan and Jaya chandran 2003). Keeping in view of the commercial importance of the EPSs, the present investigation deals with the isolation, chemical and physical characterization of extracellular polysaccharides from the bacteria *Vibrio diabolicus* strain HE800.

CHAPTER 2

2. REVIEW OF LITERATURE

The article is a comprehensive review of the use of polychaetes as indicator species of marine pollution. The article discusses the various ways in which polychaetes can be used as indicator of pollution, including their use in monitoring programs and their ability to detect contaminants in sediment and water samples. The author also describes the importance of selecting appropriate species of polychaetes for indicator purposes, and provides examples of their use in various countries around the world. Overall, the article provides valuable insights into the potential use of polychaetes in monitoring and assessing marine pollution. (Dean, Harlan K et al., 2008).

The article provides a comprehensive overview of the alarming threat that heavy metal contamination poses to both the environment and human health. The authors discuss various sources of heavy metal contamination, including industrial activities, agricultural practices, and natural geological processes. They also exposure the negative impacts of exposure to heavy metals, such as respiratory problems, neurological disorders, and cancer. Additionally, the article offers insights into various remediation techniques for reducing heavy metal contamination indifferent environment settings. Overall, the authors emphasize the importance of taking proactive measures to address this pressing issue for sustainable development. (Mishra, S., Bharagava, R. N., et al.,2019).

A review on the effects of heavy metals on aquatic animals suggests that these pollutants can have significant impacts on the health and well-being of marine life. Heavy metals such as have mercury, lead, and cadmium can accumulate in the tissue of aquatic animals, leading to a wide range of adverse effects including reduced growth and reproductive success, impaired immune function and even death. These pollutants can also disrupts the normal functioning of marine eco systems, with potential knock on effects on other species and the overall health of the environments and take steps to reduce pollution in order to protect the health of marine life and, maintain healthy ecosystem.(Vajargah, Mohammad Forouhar et al.,2021).

The article provides a comprehensive review of the current state of heavy metal pollution in soil from industrial and agricultural regions in China. It discusses

various source of contamination, source of exposure, and the associated risks to human health and the environment. The authors also highlight the need for more effective regulation and monitoring of heavy metal pollution in China, and suggests several strategies for reducing pollution and mitigating its impacts. Overall, this article provides valuable insights into the scale and severity of soil heavy metal pollution in China, and offers important guidance for future research and policy efforts. (Yang, Qiangi, et al ., 2018)

The article “Review of heavy metals in drinking water and their effect on human health” by author published in the International Journal of Innovative Research in Science, Engineering and Technology provides an overview of the impact of heavy metals on human health through drinking water. The review discusses the various sources of heavy metals in drinking water, the health effects of exposure to these metals, and methods available for removing them from water. The authors highlight the importance of monitoring and regulating levels of heavy metals in drinking water to prevent adverse health effects. Overall, the article provides valuable insights into the risks associated with heavy metals in drinking water and the strategies to mitigate them. (Mohod, Chaitali V et al., 2013).

The article presents a study on the quality of groundwater in the Bazman basin located in south eastern Iran. The analyses the concentrations of heavy metals in the groundwater and evaluates the pollution levels using different indices such as the heavy metal pollution index (HPI), the heavy metal evaluation index (HEI), and the contamination degree (CD) index. The findings of the study indicate that the groundwater in the region is significantly polluted by heavy metals such as lead, cadmium, and chromium. The study also emphasizes the need to implement effective strategies to reduce the pollution levels in the region and ensure the sustainable use of groundwater resources.(Rezaei, Ali, et al., 2019).

This article analyzes the impact of cadmium pollution on food safety and human health. Cadmium is a toxic heavy metal that can enter the food chain through contaminated soil, water, and air. The authors discuss the sources of cadmium pollution, its accumulation in various food groups, and the health risks associated with its consumption. They also review the current regulatory framework and mitigation strategies to reduce cadmium exposure. Overall, the article emphasizes the importance of monitoring and reducing cadmium levels in food to protect public health. (Suhani, Ibha, et al. 2021)

The article discusses the ways in which heavy metals, such as lead, arsenic, mercury, cadmium can negative impact human health. It explores the various sources of heavy metal exposure , including industrial population contaminated food and water , and occupational hazards .the article goes on describe the specific health effects that heavy metals can cause, including neurological disorders, kidney damage, respiratory problems, and cancer. It also discusses the ways in which heavy metal toxicity can be diagnosed and treated. Overall, Mahurpawar and Manjus article highlights the importance of awareness and prevention when it comes to heavy metal exposure, in order to protect public health and prevent serious health consequences. (Mahurpawar, Manjus et al., 2015).

The researchers conducted a comparative genomic analysis of *Vibrio diabolicus* and six other species that were previously classified as synonyms. By analysing the genetic makeup of these bacteria, the researchers were able to gain insights into the distribution and diversity and this expanded species. Ultimately, their findings suggest that *Vibrio diabolicus* may be more widespread than previously thought and that there is a significant amount of genomic diversity within this group of bacteria. (Turner, Jeffrey W., et al., 2018).

The article describes the discovery of a new species of bacterium, *Vibrio diabolicus* that was found to secrete a polysaccharide. The bacterium was isolated from a deep sea hydrothermal vent polychaete annelid known as *Alvinella pompejana*. The article provides a detailed description of the new species and its characteristics, including its morphology, physiology, and genetic makeup. The findings of this study shed light on the diversity of life that can be found in extreme environments such as deep sea hydrothermal vents.(Raguene, Gerard, et al., 1997).

The article explores the environmental impact of heavy metals in various industrial processes. The authors discuss how heavy metals such as lead, zinc, and copper can be released into the environment during mining, smelting, and manufacturing activities. They also cover the negative effects of these metals on the environment, including air and water pollution, soil degradation, and harm to wildlife and human health. The article concludes with a discussion on the importance of reducing heavy metal pollution and implementing sustainable practices in industry. (Khalef, Riyam N et al., 2022).

This article provides a brief overview of the potential health hazards associated with exposure to heavy metals. They explain how many metals can accumulate in the body and cause damage to organs and tissues. The authors describe the various ways in which humans can be exposed to heavy metals, including through contaminated food, water, air. Finally, the article highlights some of the health impacts that have been linked to heavy metal exposure, such as neurological disorders, cancer, and cardiovascular disease. Overall, the paper provides important information regarding the potential risks associated with exposure to heavy metals and the need for continued research in the area.(Martin, Sabine et al., 2009).

The article discusses the harmful effects of heavy metals on environment. The authors explore the source of heavy metal pollution, the effects of heavy metals on living organisms, and the various methods used to minimize their negative impact. The article also discusses the role of regulations and policies in controlling heavy metal pollution. Overall, the article provides an informative overview of the dangers of heavy metal pollution and the importance of taking steps to mitigate its effects.(Rajeswari, T. Raja Nambuuru Sailaja et al ., 2014).

CHAPTER 3

3.1 AIM

To study the heavy metals bio transforming ability of *Vibrio diabolicus*.

3.2 OBJECTIVES

- To Isolate and characterize gut bacteria from marine polychaete worm from Ennore estuary, Chennai, India.
- To extract and analyze exopolysaccharides isolated from *Vibrio diabolicus*.
- To investigate the effects of different stress levels on the growth of *Vibrio diabolicus* and exopolysaccharide production.
- To study the heavy metal bio transforming ability of the *Vibrio diabolicus*.

CHAPTER 4

4. MATERIALS AND METHODS

4.1 SAMPLING SITE

Ennore estuary (lat. 13°13'05.24"N and long. 80°19'01.524"E) situated on the east coast of India, is about 15 km north of Chennai. It runs parallel to the sea coast and extends over a distance of 36 km. The temperature and salinity of this estuary ranged between 26–30°C and 25–29 ppt respectively. This estuary was chosen as the test (contaminated) site as it receives untreated sewage from Royapuram sewage outfall, untreated/treated industrial effluents from Manali Industrial Belt, which houses many chemical industries like fertilizer, oil refineries, sugar, chemicals, etc. In addition to that, fishing and navigational activities take place in the area. The dredging activities in this area result in changes in the landscape, sediment transport, and dust pollution to the coast by quarrying process (Padmini and Usha Rani, 2010). The rapid development of Chennai City in the last two decades has put additional stress on the local aquatic environment. The main source of metal input to Ennore creek is via the discharge of waste water effluents, leachates, chemicals, paints, fertilizers and petroleum refining industries located in the northern part of the city limits. A major portion of the effluent input is also from a coal-powered thermal power plant situated very close to the creek which drains the effluents directly into it (Periakali and Padma, 1998). This estuary is contaminated by heavy metals like lead, cadmium, mercury, zinc, iron, etc. to a significant extent (Padmini and Vijaya Geetha, 2007). The offshore region is normally less-polluted due to natural phenomena such as circulation pattern. In this study, the fish collected from the off shore region (13°13'05.24"N and 80°20'03.402"E) designated as unpolluted to compare with the polluted Ennore estuary.

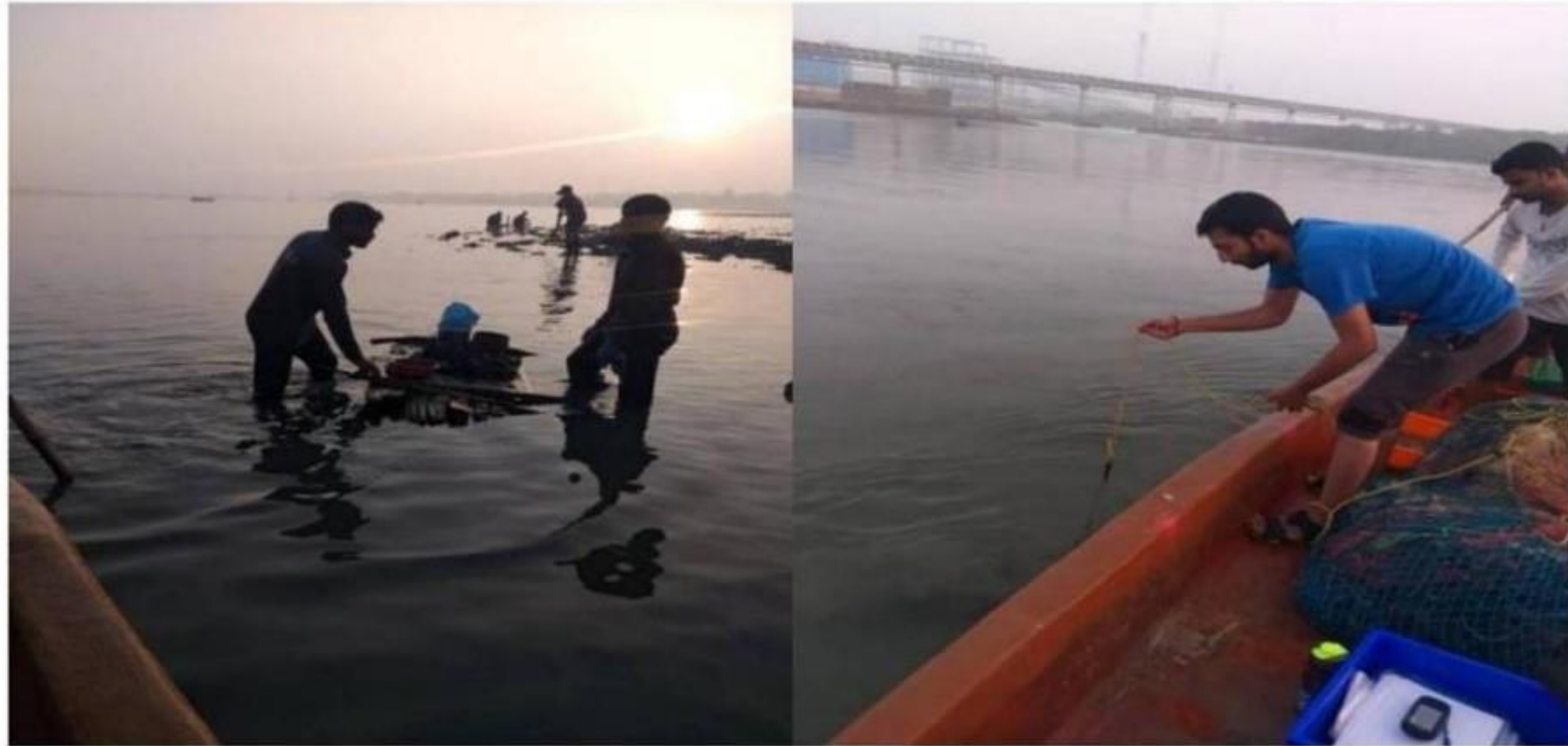


Fig 1. Sample collection at Ennore Creek

4.1.1 Sampling Method

The polychaete samples is usually found in the sediments at the mangrove area. Five samples of polychaetes were collected during post monsoon at low tide in the sediment. The sediments were shovelled and the worms were pulled out slowly so a whole shape will be obtained, otherwise the polychaete would be cut off if pulled aggressively. Next, the polychaetes were washed with seawater to wash away the leftover sediments and were placed into a zip lock bag with a damp paper towel to mimic its environment's humidity. The polychaetes were placed in an icebox and transported to the laboratory. The polychaetes were kept in a -20°C to preserve it until further analysis.

4.1.2 Isolation of Bacteria from polychaetes

The frozen polychaetes were defrosted first by rinsing them with distilled water and dried using tissue paper. The workbench was sterilized using 70% alcohol. Next, all five polychaete samples were dissected along its body (midsection). Sterile cotton swabs were used to swab the polychaetes gut fluid, and the gut fluid sample was streaked on the Zobell Marine Agar (ZMA) plates. The plates were incubated at 37°C for 24 h. After 24 h, the mixed colonies were obtained. Next, a sub-culture is performed to get a single colony of bacteria. The pure isolated bacteria were used for subsequent analysis.

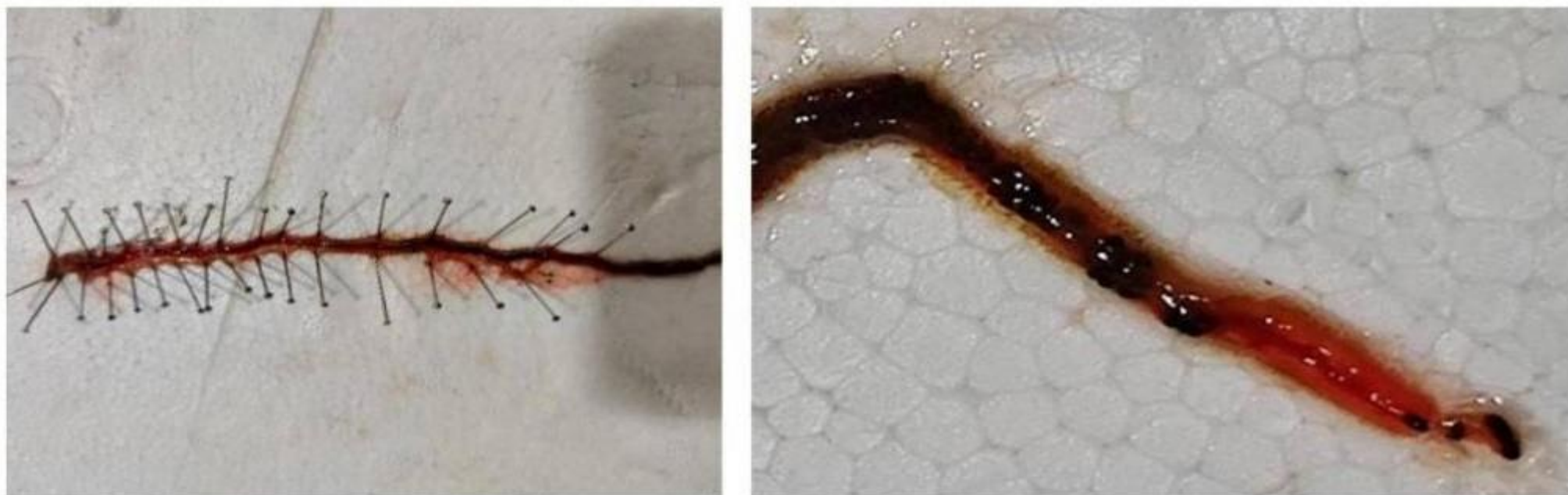


Fig 2. Isolation of gut contents from polychaetes

4.1.3 Identification of Bacteria

Identification and characterization of selected bacterial isolates were based on macroscopic and microscopic observations. The morphology of the bacteria isolates was observed using the Gram staining technique and further identified using 16S rRNA sequencing.

4.1.4 Gram Staining Method

The staining procedures started with a uniform smearing bacterial film made on a clean glass slide and heat-fixed. Then, one drop of crystal violet solution was dropped approximately in a minute on the entire bacterial smear. Then the slide was washed under a slow flow of water and later, a few drops of iodine solution were flooded onto the colour stain and left for a minute. Alcohol 95% was then used to decolorize the stain. After that, the slide was flooded with safranin counterstain for about 30 to 60 s and then rinsed with running tap water. The glass slide was air-dried, and then it was further examined under a light microscope with oil immersion. Gram-positive bacteria showed a purple cell, while Gram-negative bacteria showed a red cell (Isaac & Jennings, 1995).

4.1.5 16S rRNA Sequencing Method

The 16S rRNA sequencing was performed to further identification of the bacteria isolates. The bacteria were grown on nutrient agar for 24-48 h and then the extraction of the bacterial DNA was conducted using Vivendi's Nucleic acid Extraction Kit manufactured by Vivendi's Technologies Sdn. Bhd. The extracted DNA was stored in -20°C to further amplify by Polymerase Chain Reaction (PCR).

4.1.6 Biochemical tests

Biochemical tests were performed catalase mediates breakdown of hydrogen peroxide into oxygen and water. Bacterial isolate can produce catalase enzyme, a small inoculum of bacterial isolate is mixed into hydrogen peroxide solution 3%. Use a loop to transfer a small amount of colony growth in the surface of clean, glass slide. Observe the evolution of oxygen bubbles.

4.2 PHENOL SULFURIC ACID METHOD FOR TOTAL CARBOHYDRATE ESTIMATION

4.2.1 Materials

1. Phenol 5%: Redistilled (reagent grade) phenol (50g) dissolved in water and diluted to one liter.
2. Sulfuric acid 96% reagent grade.
3. Standard glucose: Stock- 100mg in 100ml of water.
4. Working standard- 10ml of stock diluted to 100ml with distilled water.

4.2.2 Procedure

1. Weigh 100mg of sample into a tube.
2. Hydrolyse by keeping it in boiling water bath for 3hrs with 5ml of 2.5 N-HCL and cool at room temperature.
3. Neutralize it with solid sodium carbonate until the effervescence ceases.
4. Make up the volume to 100ml and centrifuge.
5. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of tubes.
6. Pipette out 0.1ml and 0.2 ml of the sample solution in two separate test tubes. Make up the volume in each tube to 1ml with water.
7. Set a blank with 1ml of water.
8. Add 1ml of phenol solution to each tube.
9. Add 5ml of 96% sulphuric acid to each tube and shake well.

10. After 10 minutes shake the content in the tubes and place in a water bath at 25-30°C for 20 minutes.
11. Read the colour at 490nm.
12. Calculate the amount of total carbohydrate present in the sample solution using the standard graph.

4.2.3 Exopolysaccharide Estimation

1. The polysaccharide was produced as previously described using a 2-L fermenter containing 1 L of marine 2216 broth medium supplemented with glucose, at atmospheric pressure, a temperature of 25 °C and at pH 7.2.
2. After 48 h, the viscosity of the medium reached a stable value of 35 Pa s. Cells were removed from the medium by high speed centrifugation at 20,000g for 2 h.
3. The exopolysaccharide was precipitated from the supernatant by adding EtOH to a final concentration of 30% alcohol and washed with 70-100% EtOH water.
4. The polysaccharide was then dissolved in water and subjected to ultrafiltration using a Millipore system with PM-10 membrane (10,000 Da cut-off), washed with water, concentrated, and finally lyophilized.
5. The yield was 2 g of polysaccharide, which was stored at room temperature (RT).

4.3 DIFFERENT STRESS LEVELS OF pH, SALINITY, TEMPERATURE, HEAVY METALS

4.3.1 Different stress levels of pH

The media was prepared in 700ml distilled water and 36g marine broth. pH 4, 6, 7.5, 8, 9 is pH values. Different pH values adjusted by addition of HCL and NaOH. In 5 conical flask add each flask added 50ml distilled water. Control add distilled water and marine broth. Then transfer the syringe bottles from pH values of 50ml in each bottles. pH stress was generated by addition of 50 ml of marine broth stabilized at different pH values by addition of HCl or NaOH 24hr, 48hr, 72hr at pH values 4, 6, 7.5, 8, 9 and control. Controls were obtained by in the marine broth. pH values 4, 6, 7.5, 8, 9 was inoculated in 50µl at bacterial culture. After 24hr, 48hr, 72hr incubation in the presence of the different pH values 24hr, 48hr, 72hr at environmental shaker

at the following pH values 4, 6, 7.5, 8, 9 and control. Then centrifuge for 8000rpm in 10 minutes and then collect the supernatant. The pellets are stored at -20° refrigerator. Then carbohydrate analyse method is phenol sulphuric acid method was done in standard stock solution and pH samples in eppendorf tubes in add 50µl pH sample, and blank 50µl, 50µl phenol, 125µl sulphuric acid. Put the tubes for incubator in 30 minutes. Then immediately add the Elisa plates for 100µl. Then read the absorbance for 490nm. Measurements were performed graph immediately thereafter.



Fig 3: Different stress levels of pH

4.3.2 Different stress levels of temperature

The media was prepared in 700ml distilled water and 36g marine broth. Incubation temperatures 24°C, 27°C, 30°C, 32°C, 35°C. Then transfer the syringe bottles from temperature values of 50ml in each bottles. Then autoclave in 121°C for 20 minutes. The different temperature was inoculated in 50µl at bacterial culture. After 24hr, 48hr, 72hr incubation in the presence of the different temperature values 24hr, 48hr, 72hr at environmental shaker at the following temperature values 24°C, 27°C, 30°C, 32°C, 35°C. Then centrifuge for 8000rpm in 10 minutes and then collect the supernatant. The pellets are stored at -20° refrigerator. Then carbohydrate analyse method is phenol sulphuric acid method was done in standard stock solution and temperature samples in eppendorf tubes in add 50µl temperature sample, and blank 50µl, 50µl phenol, 125µl sulphuric acid. Put the tubes for incubator in 30 minutes. Then immediately add the Elisa plates for 100µl. Then read the absorbance colour for 490nm. Measurements were performed graph immediately thereafter.



Fig 4: Different stress levels of temperature

4.3.3 Different stress levels of salinity

The media was prepared in 900ml distilled water and 36g marine broth. Salinity prepared in Sodium chloride (NaCl) 0.5%, 1%, 2.5%, 5%, 7%, control. Take a 6 test tubes add each tubes of 2gram marine broth and 0.5%-0.250g of NaCl, 1%-0.500g NaCl, 2.5%-1.25g of NaCl, 5%-2.50g of NaCl, 7%-3.5g of NaCl, and control no added salt. Then transfer the syringe bottles from values of 50ml marine broth in each bottles. Then autoclave in 121°C for 20 minutes. The different salinity was inoculated in 50µl at salinity culture. After 24hr, 48hr, 72hr incubation in the presence of the different salinity values 24hr, 48hr, 72hr at environmental shaker at the following salinity values 0.5%, 1%, 2.5%, 5%, 7%, control. Then centrifuge for 8000rpm in 10 minutes and then collect the supernatant in another folcen tubes. The pellets are stored at -20° refrigerator. Then carbohydrate analyse method is phenol sulphuric acid method was done in standard stock solution and salinity samples in eppendorf tubes in add 50µl salinity sample, and blank 50µl, 50µl phenol, 125µl sulphuric acid. Put the tubes for incubator in 30 minutes. Then immediately add the Elisa plates for 100µl. Then read the absorbance colour for 490nm. Measurements were performed graph immediately thereafter.

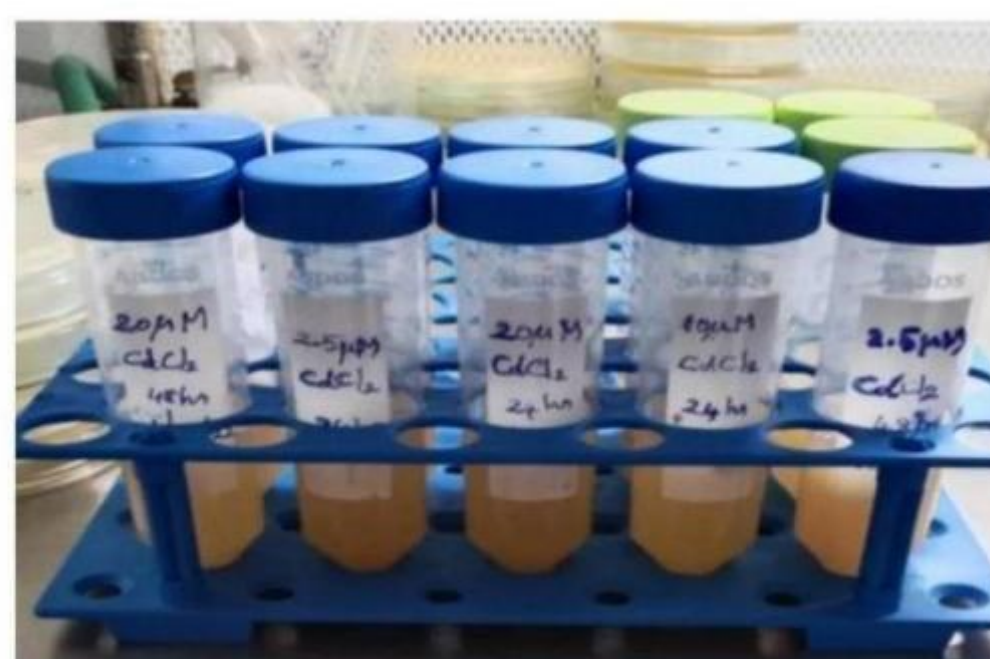
4.3.4 Different stress levels of heavy metals

The media was prepared in 700ml distilled water and 36g marine broth. Heavy metals are Cadmium chloride, Mercuric sulphate, Lead nitrite, Selenium, Copper sulphate, Zinc chloride was prepared in test tubes. Transfer the syringe bottles from 20ml marine broth in each bottles. Then autoclave in 121°C for 20 minutes. The heavy metals are transfer into bottles. The different heavy metals are 2.5µM, 10µM,

20µM was inoculated in each bottles at 24hr, 48hr in 5µM, 20µM, and 40µM in micro pipette. After then 24hr, 48hr inoculated the heavy metals samples are inoculated into 200µl in *Vibrio diabolicus* bacteria. After 24hr, 48hr, in the presence of the different heavy metals values are 2.5µM, 10µM, 20µM 24hr, 48hr at environmental shaker at the following heavy metals. Then centrifuge for 8000rpm in 15 minutes and then collect the supernatant. The pellets are stored at -20° refrigerator. Then carbohydrate analyse method is phenol sulphuric acid method was done in standard stock solution and heavy metal samples in eppendorf tubes in add 100µl heavy metal sample, and blank 100µl, 100µl phenol, 250µl sulphuric acid. Put the tubes for incubator in 30 minutes. Then immediately add the Elisa plates for 100µl. Then read the absorbance colour for 490nm. Measurements were performed graph immediately thereafter.



Fig 5: A. Heavy metals



B. Different stress of heavy metal

CHAPTER 5

5. RESULTS AND DISCUSSION

5.1 Isolation of polychaete gut associated micro organisms



FIG: 6 Spread plate method

5.2 Characterisation of Bacteria

5.2.1 Biochemical characterisation

The gram staining result for single colony picked from the marine agar plates was found to be Gram negative bacteria and the catalase test showed positive which emphasised the isolated bacteria is Catalase positive and Gram negative rod shaped bacteria.

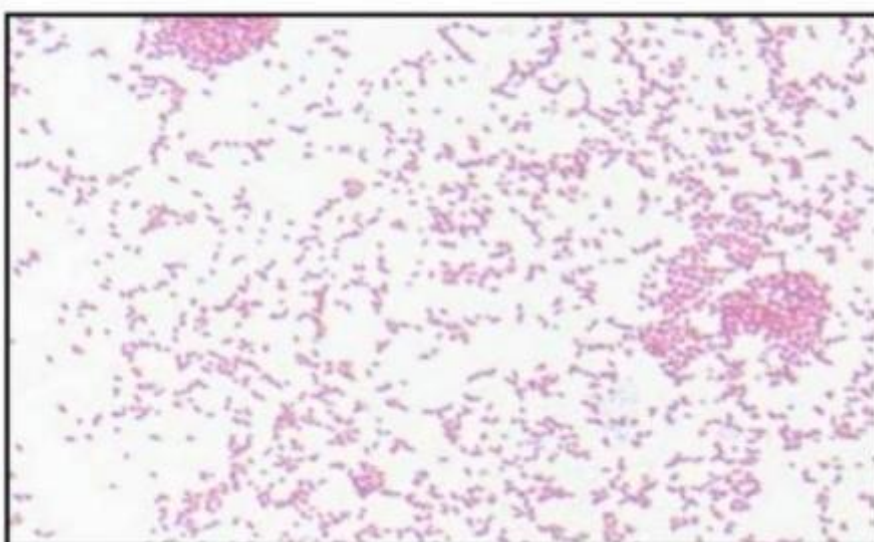
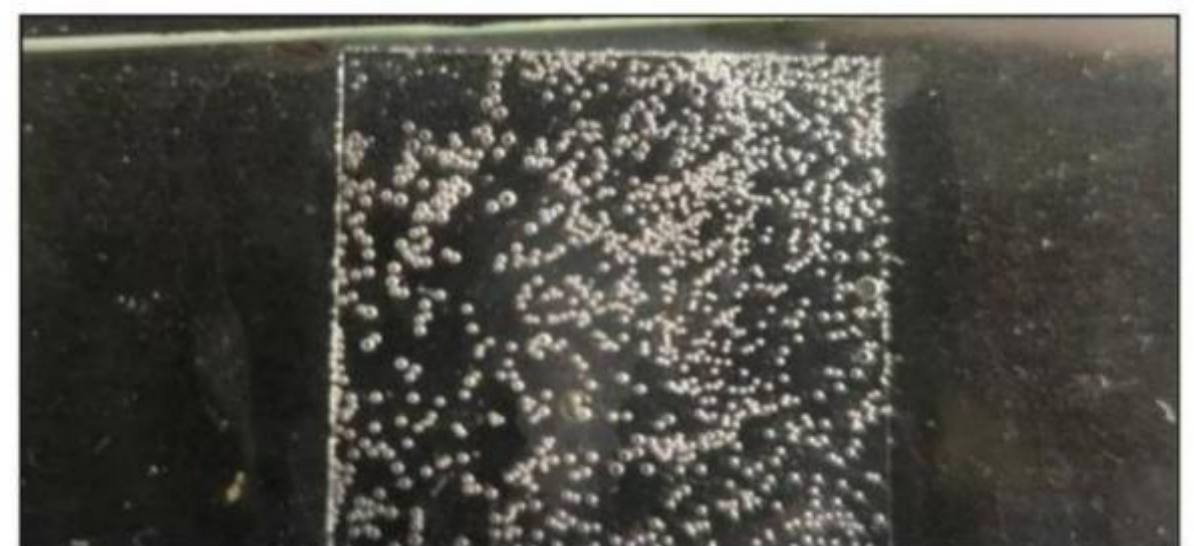


Fig7.A. Gram-stained microscopic slide shows Gram negative bacteria



B. Bubble formation in the slide shows catalase positive

5.2.2 Molecular characterisation

16srRNA sequencing of the bacteria revealed that the isolated strain is *Vibrio diabolicus*.

The screenshot displays a BLAST search interface. On the left, the 'Job Title' is 'Nucleotide Sequence'. The 'RID' is 'Y3TES68R013' with a search expiration time of '02-08 12:47 pm'. The 'Program' is 'BLASTN', the 'Database' is 'nt', and the 'Query ID' is 'lcl|Query_30923'. The 'Description' is 'None', the 'Molecule type' is 'dna', and the 'Query Length' is '1149'. On the right, the 'Filter Results' section shows 'Organism' set to 'only top 20 will appear' and 'exclude' checked. The 'Percent Identity' is set to 'to', 'E value' is 'to', and 'Query Coverage' is 'to'. The 'Filter' and 'Reset' buttons are visible. Below the search results, a table titled 'Sequences producing significant alignments' shows a single result for 'Vibrio diabolicus strain FA3 chromosome 1, complete sequence' with a 'Scientific Name' of 'Vibrio diabolicus', a 'Max Score' of 2084, a 'Total Score' of 22526, a 'Query Cover' of 99%, an 'E value' of 0.0, a 'Per. Ident' of 99.56%, an 'Acc. Len' of 3256558, and an 'Accession' of 'CP042451.1'.

Fig 8: 16srRNA sequencing of the bacteria

5.3 DIFFERENT STRESS LEVELS OF pH, SALINITY, TEMPERATURE, HEAVY METALS

5.3.1 Different stress levels of pH

Optimum pH is found to be pH 6 for the production of exopolysaccharide. The production of total sugars is high at 48h at pH 6 when compared to the control which is the pH of Zobell Marine Broth.

pH LEVEL	Concentration of carbohydrate (mg/ml) at different time intervals		
	24hr	48hr	72hr
pH 4	0.604	0.3110	0.897
pH 6	0.549	2.322	0.550
pH 7.5	0.338	0.340	0.862

pH 8	0.968	0.654	0.830
pH 9	0.795	0.514	0.786
Control	0.675	0.765	1.355

Table 1: Different stress levels of pH

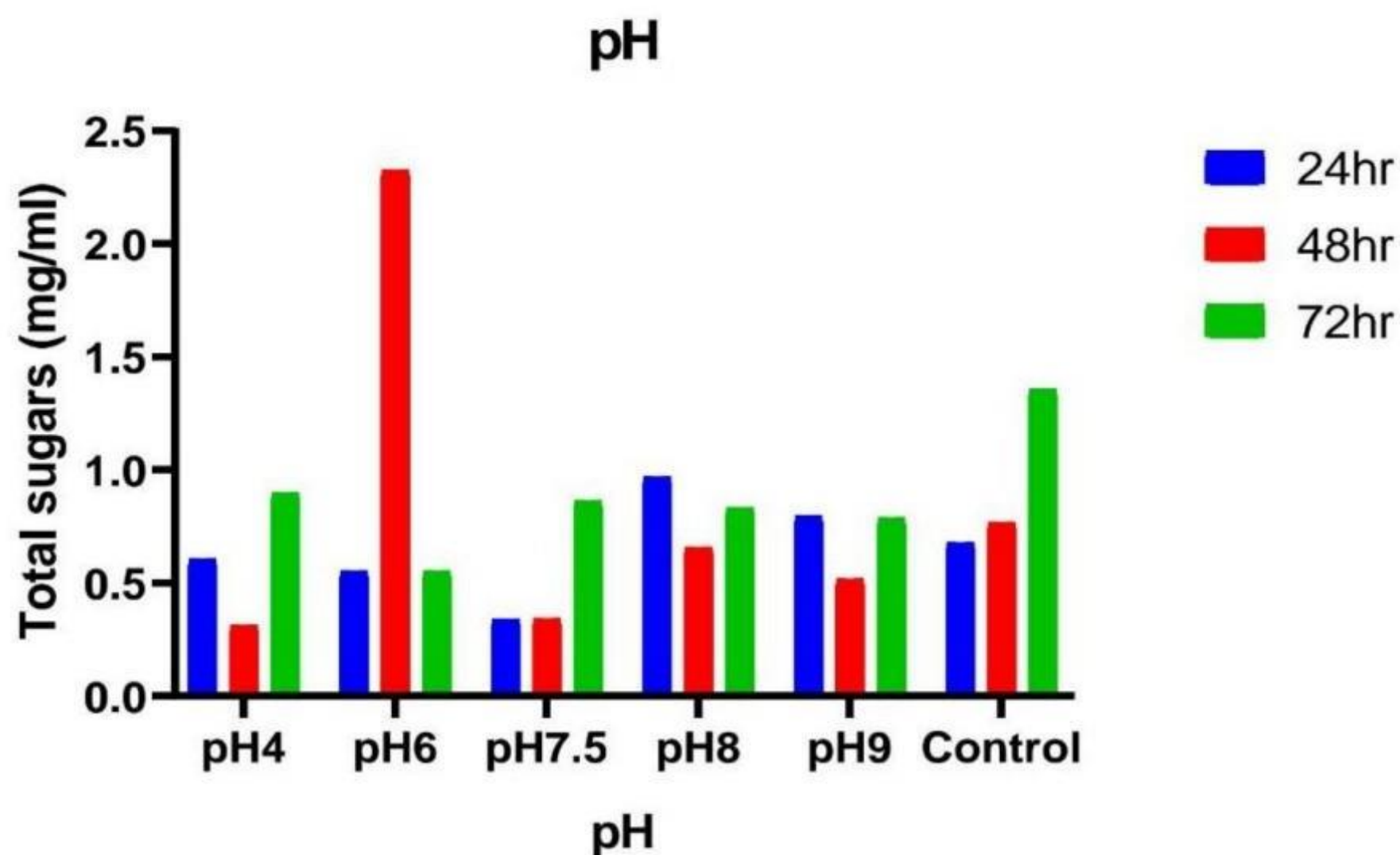


Fig 9: Stress levels of pH stress

5.3.2 Different stress levels of temperature

Optimum temperature for exopolysaccharide production is 24°C.

TEMPERATURE	Concentration of carbohydrate (mg/ml) at different time intervals		
	24hr	48hr	72hr
24°C	3.276	1.830	1.520
27°C	0.220	0.300	0.372
30°C	0.226	0.125	0.180
32°C	0.161	0.157	0.187
35°C	0.205	0.294	0.467

Table 2: Stress levels of temperature

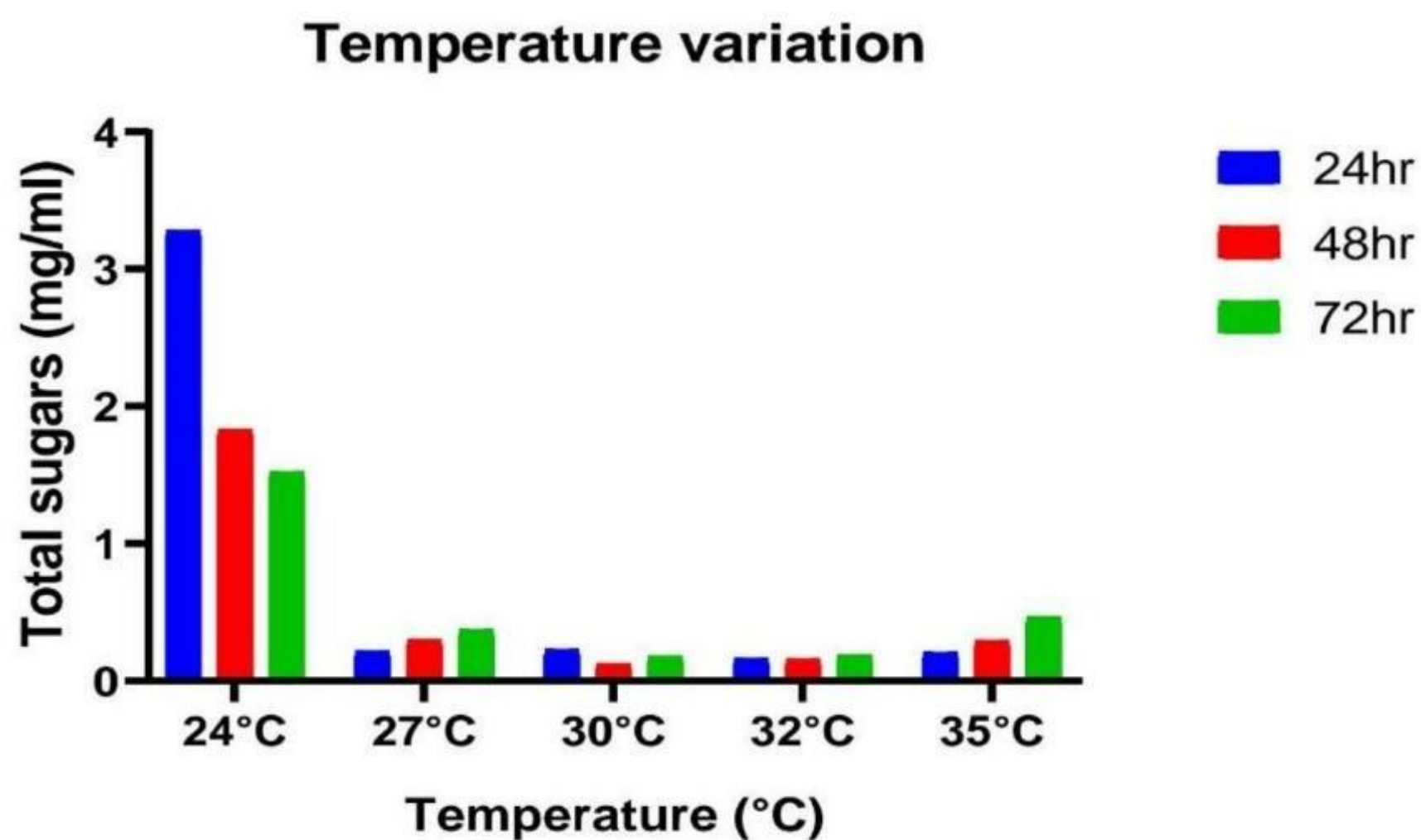


Fig 10: Stress levels of temperature

5.3.3 Different stress levels of salinity

Optimum salinity is found to be the salinity already present in the Zobell marine broth. (19.5g/l or 0.3324M) or 19.522274909 ppm.

SALINITY	Concentration of carbohydrate (mg/ml) at different time intervals		
	24hr	48hr	72hr
0.5%	0.611	0.443	0.199
1%	0.641	0.135	0.896
2.5%	0.533	0.591	0.529
5%	0.487	0.842	0.538
7%	0.297	0.337	0.217
Control	0.983	1.226	0.885

Table 3: Stress levels of Salinity

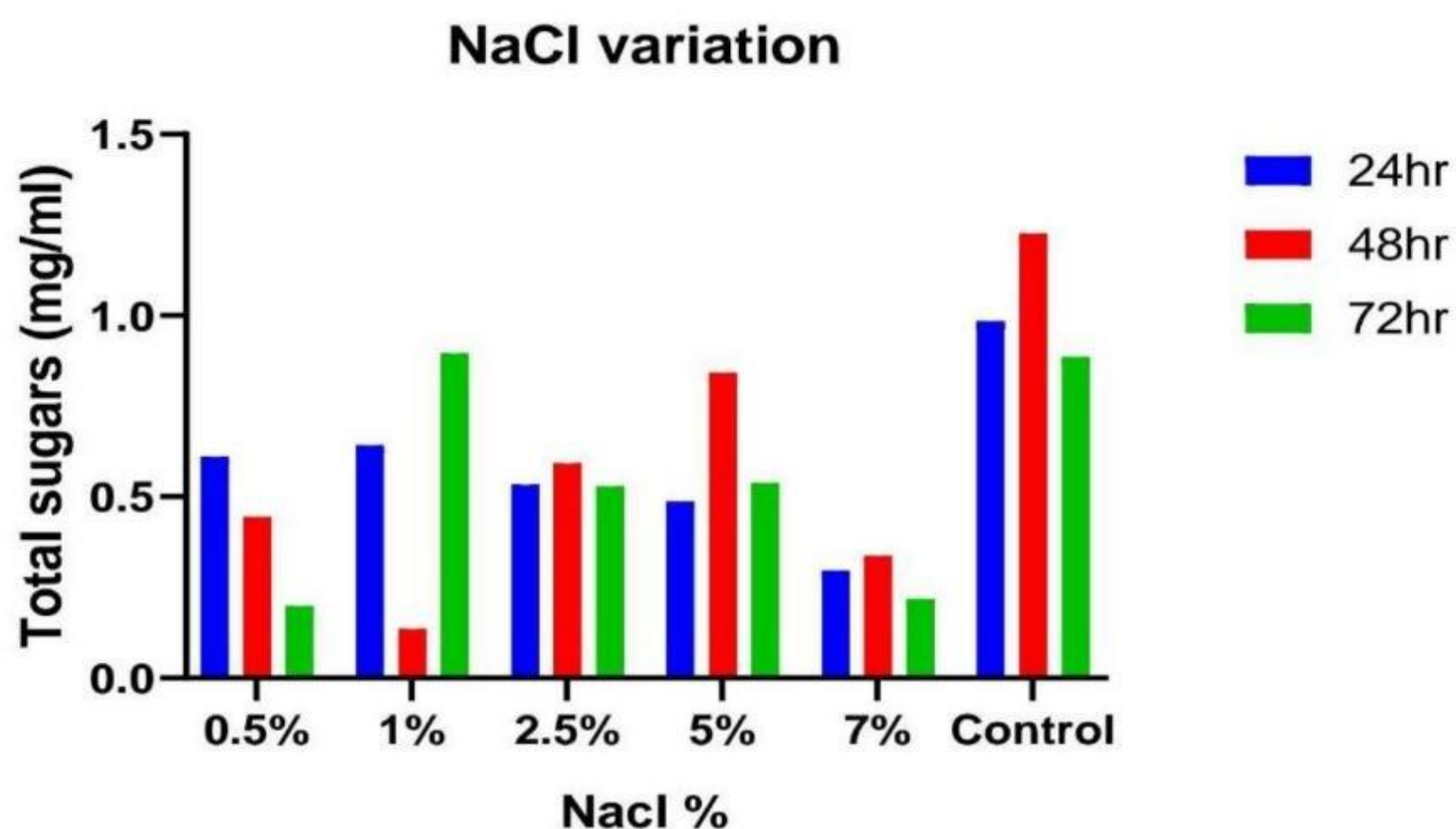


Fig 11: Different stress levels of salinity

5.3.4 Different stress levels of heavy metals

There may be some positive influence on EPS production from Selenium and HgSO_4 as total sugars is as high as 4.3 mg/ml at 2.5microM on 48h culture in case of selenium. The production of EPS is greater than 2 mg/ml compared to the rest of heavy metals.

HEAVY METALS	Concentration of carbohydrate (mg/ml) at different time intervals	
	24hr	48hr
CdCl₂		
2.5μM	2.193	1.667
10μM	1.318	1.811
20μM	1.872	2.136
Pb(NO₃)₂		
2.5μM	1.923	1.793
10μM	2.062	1.790
20μM	2.819	1.860

Table 4: A) Stress levels of Cadmium Chloride and Lead Nitrite

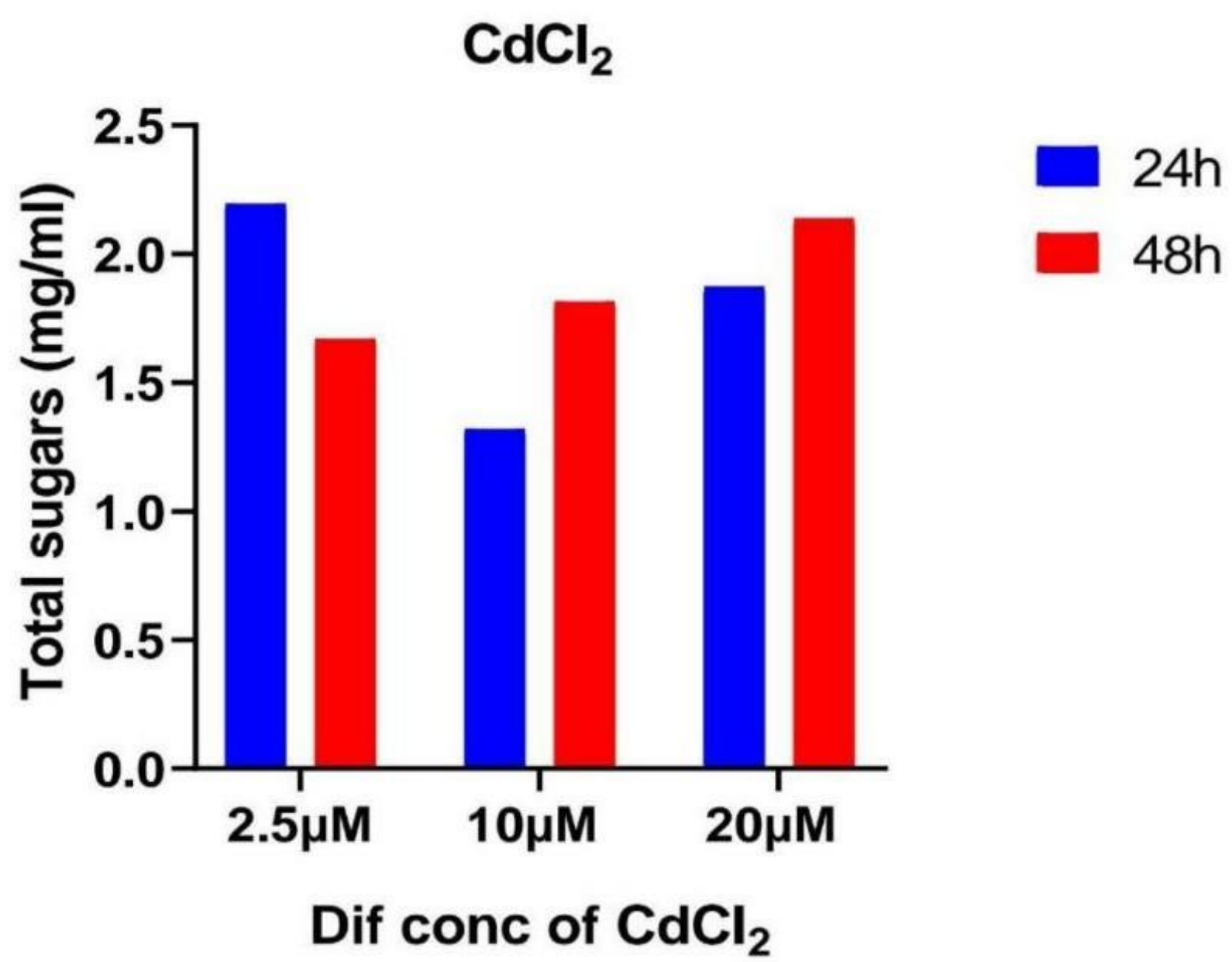
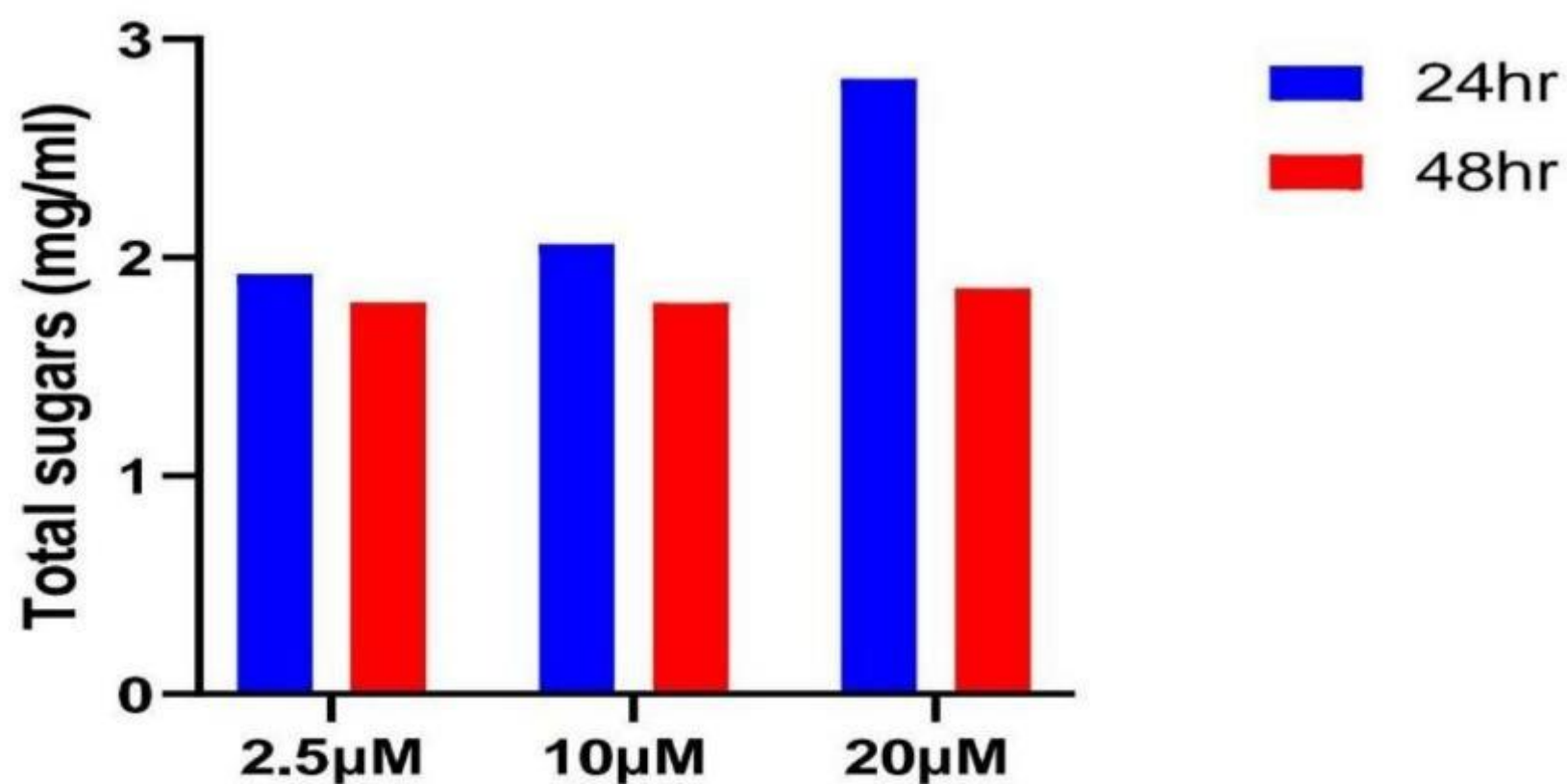


Fig 12: A) Cadmium Chloride

Lead Nitrite

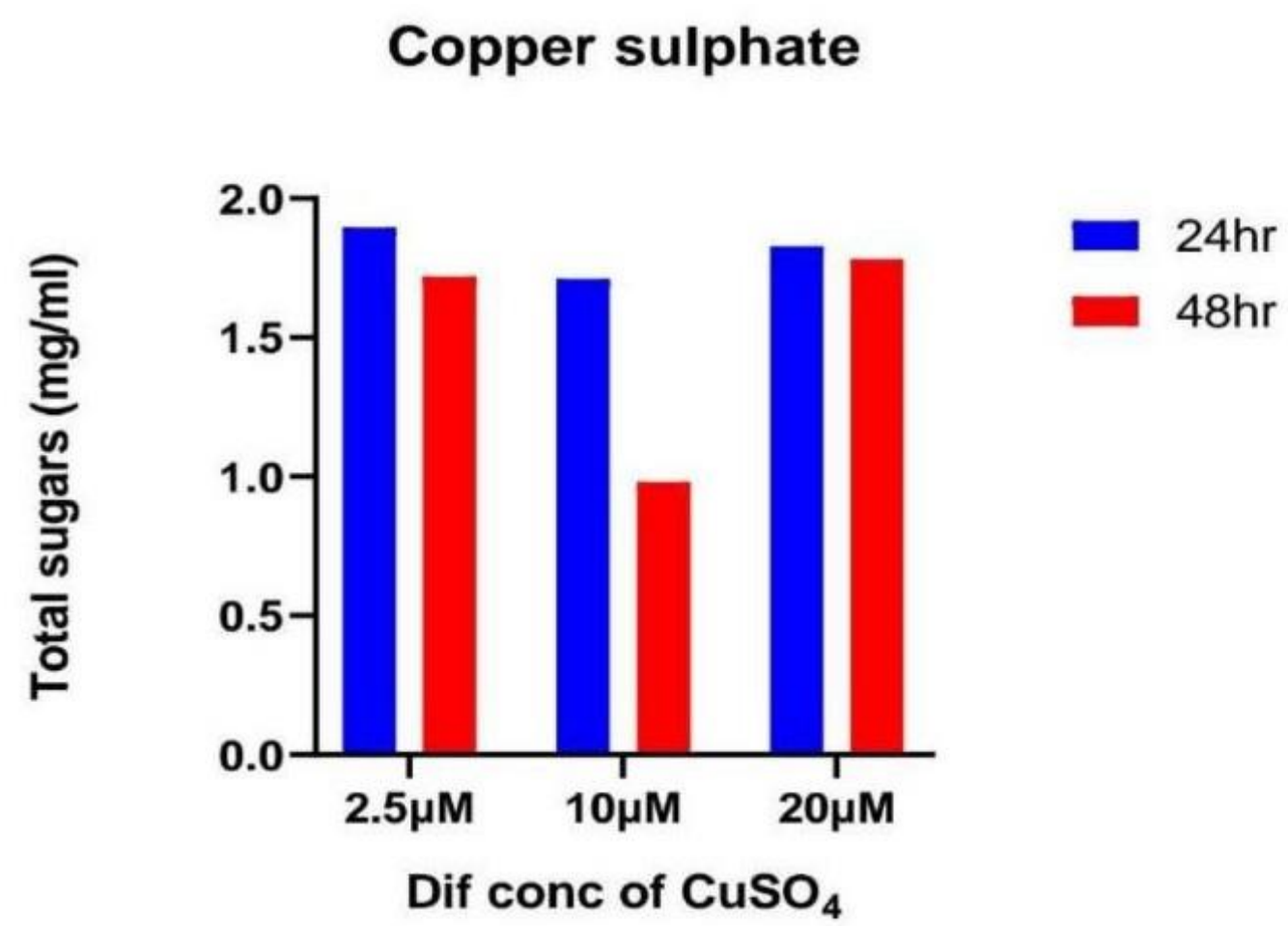


Dif conc of $Pb(NO_3)_2$

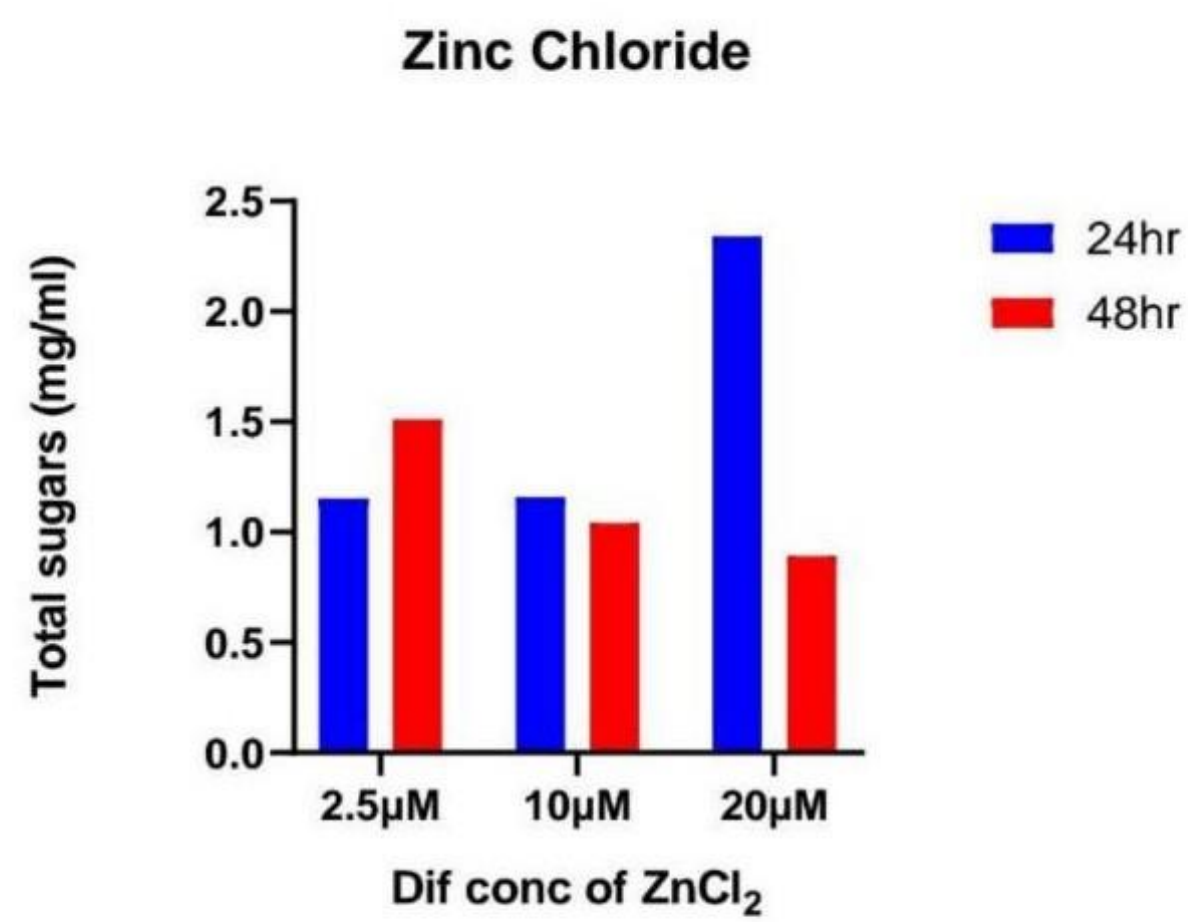
B) Lead Nitrite

HEAVY METALS	Concentration of carbohydrate (mg/ml) at different time intervals	
	24hr	48hr
CuSO₄		
2.5µM	1.895	1.798
10µM	1.790	0.981
20µM	1.830	1.780
ZnCl₂		
2.5µM	1.152	1.517
10µM	1.157	1.040
20µM	2.340	0.891

B) Copper Sulphate, Zinc Chloride



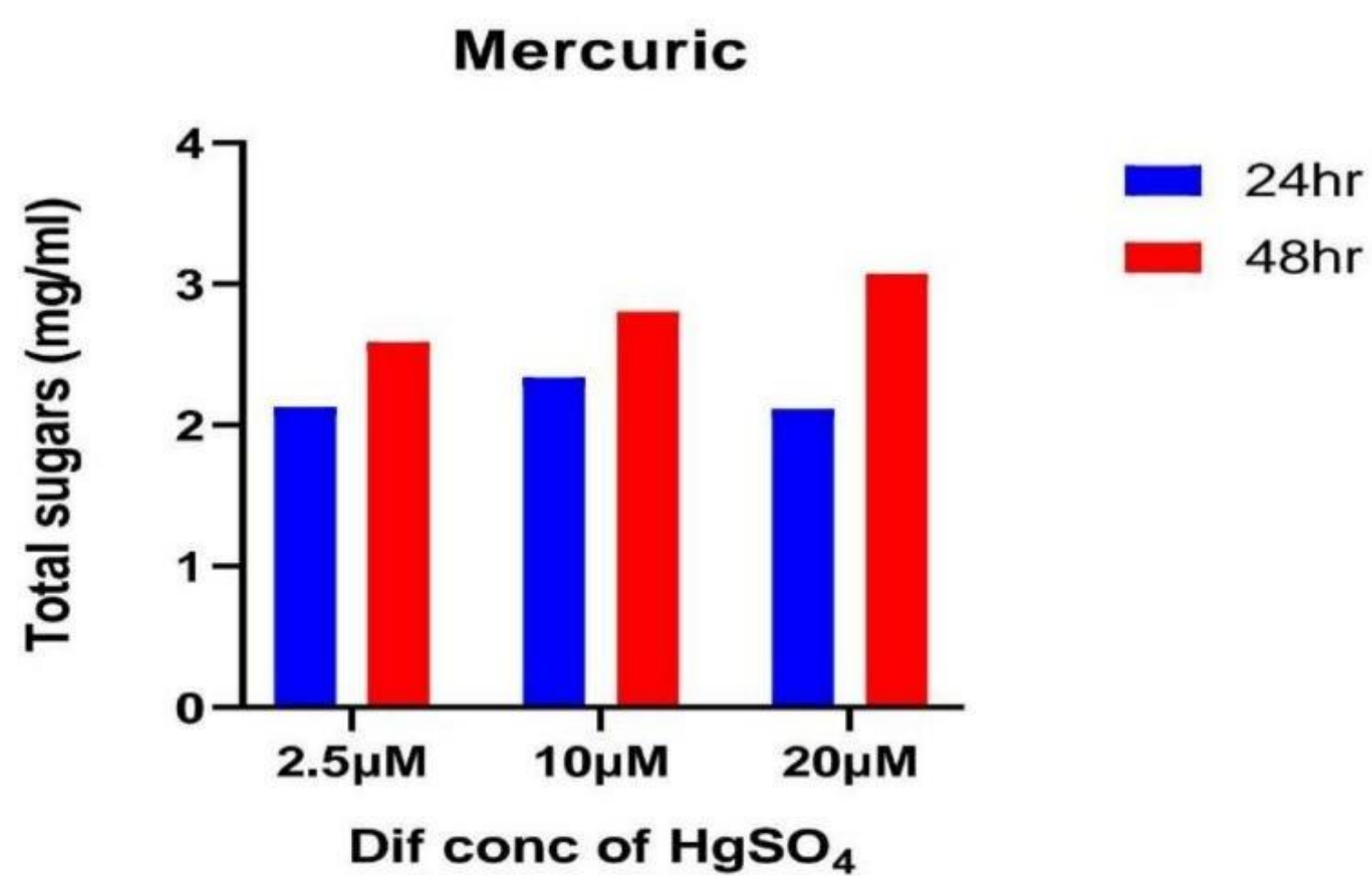
C) Copper Sulphate



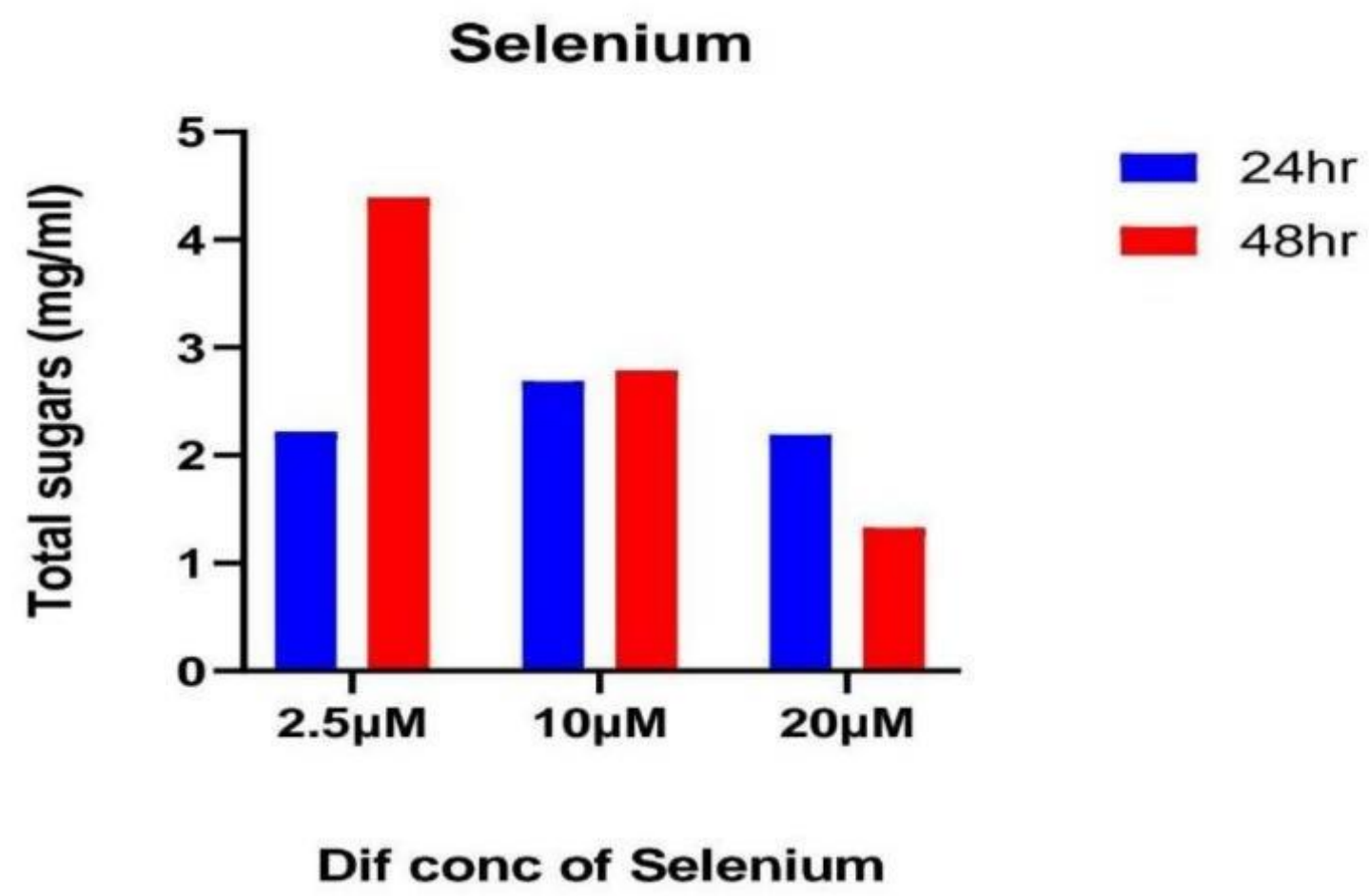
D) Zinc Chloride

HEAVY METALS	Concentration of carbohydrate (mg/ml) at different time intervals	
	24hr	48hr
HgSO₄		
2.5µM	2.128	2.589
10µM	2.340	2.806
20µM	2.116	3.071
Selenium		
2.5µM	2.221	4.388
10µM	2.686	2.790
20µM	2.195	1.326

C) Mercuric Sulphate, Selenium



E) Mercuric Sulphate



F) Selenium

Discussion

This study focuses on heavy metal degrading bacteria from gut contents of polychaetes taken from Ennore creek. The isolated single colony bacteria were found to be *V. diabolicus* by molecular characterisation. It is EPS producing bacteria which was first isolated from a hydrothermal vent polychaete named *Alvinella pompejana*. The various stress levels induced to bacteria could lead to reduced or increased production of EPS. From the results, at temperature 24°C they produced high number of EPS and at pH 6, the EPS production was as high as ~2.5mg/ml. The optimum salinity was found to be 19.522274909 ppm which is the salinity present in Zobell marine broth. Further increase in salinity did not yield increased production of EPS. The heavy metals Se, Lead nitrate and Mercuric sulphate positively influenced the EPS production.

CHAPTER 6

Summary and Conclusion

In this work, we aimed to quantify and characterize the EPS synthesized by *V. diabolicus* EP03 during growth under abiotic stress conditions such as increased salinity, pH, and Temperature. Strain EP03 could tolerate matrix stress of pH 6-9, salinity up to 19.52 ppm and temperature 24°C indicating the presence of stress responsive mechanisms. The total yield of EPS produced by strain GAPP45 was greatly influenced by all the three stress factors and production increased with increasing stress levels. Many different environmental stresses increase production of extracellular carbohydrates. An increase in the production of EPS with increasing matrix stress has been reported in *Pseudomonas spp.* strain before. Sheng et al. reported that the number of EPS produced by *Rhodo pseudomonas acidophila* increased at high NaCl concentrations and high temperature induced changes in exopolysaccharide production of heat resistant mutants of *Rhizobium sp.* And *Xanthomonas campestris* was observed respectively. Moreover, under stress conditions, microorganisms generate EPS to act as diffusion barrier between the cell wall and extreme environments, a stress responsive mechanism.

In the present study, we put forward an indigenous EPS-producing marine isolate, *V. diabolicus*, for its heavy metal accumulation potential and response to metal stress. *V. diabolicus* tolerated the heavy metal presence, namely Hg, Cu, Cd, Se, Pb, and Zn selected based on their ecological significance. The heavy metal stress consequences were discussed concerning the effect on EPS production and growth of the bacteria. Se and Hg stress increased the EPS production. The future studies aim at discovering the effect of these HM on protein synthesis and morphology of the bacterial cells. One benefit of using these organisms is that significant amounts of EPS that was responsible for their superior ability to accumulate heavy metals also protect the bacterial cells. Hence, the performance of *Vibrio diabolicus* indicated that they would be the potential candidate for bioremediation processes in the metal-contaminated area. Exploration of EPS-producing *Vibrio diabolicus* for metal remediation is further needed, confirming its utility for habitual use under multi metal stress condition and use of its purified E

Reference

1. Bramhachari, P. V[†], and S. K. Dubey. "Isolation and characterization of exopolysaccharide produced by *Vibrio harveyi* strain VB23." *Letters in applied microbiology* 43.5 (2006): 571-577.
2. Dean, Harlan K. "The use of polychaetes (Annelida) as indicator species of marine pollution: a review." *Revista de Biología Tropical* 56.4 (2008): 11-38.
3. Drake, H. L., & Horn, M. A. (2007). As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. *Annu. Rev. Microbiol.*, 61, 169-189.
4. Duboc, P., & Mollet, B. (2001). Applications of exopolysaccharides in the dairy industry. *International dairy journal*, 11(9), 759-768.
5. Duboc, Philippe, and Beat Mollet. "Applications of exopolysaccharides in the dairy industry." *International dairy journal* 11.9 (2001): 759-768.
6. Goudenège, David, et al. "Genome sequence of *Vibrio diabolicus* and identification of the exopolysaccharide HE800 biosynthesis locus." *Applied microbiology and biotechnology* 98 (2014): 10165-10176.
7. Khalef, Riyam N., Amal I. Hassan, and Hosam M. Saleh. "Heavy Metal's Environmental Impact." (2022).
8. Mahurpawar, Manju. "Effects of heavy metals on human health." *Int J Res Granthaalayah* 530 (2015): 1-7.
9. Martin, Sabine, and Wendy Griswold. "Human health effects of heavy metals." *Environmental Science and Technology briefs for citizens* 15 (2009): 1-6.
10. Mishra, S., Bharagava, R. N., More, N., Yadav, A., Zainith, S., Mani, S., & Chowdhary, P. (2019). Heavy metal contamination: an alarming threat to environment and human health. *Environmental biotechnology: For sustainable future*, 103-125.
11. Mohod, Chaitali V., and Jayashree Dhote. "Review of heavy metals in drinking water and their effect on human health." *International Journal of Innovative Research in Science, Engineering and Technology* 2.7 (2013): 2992-2996.
12. Mtanga, A., and J. Machiwa. "Assessment of heavy metal pollution in sediment and polychaete worms from the Mzinga Creek and Ras Dege

- mangrove ecosystems, Dar es Salaam, Tanzania." *Western Indian Ocean Journal of Marine Science* 6.2 (2008).
13. Nguyen, Phu-Tho, et al. "Exopolysaccharide production by lactic acid bacteria: The manipulation of environmental stresses for industrial applications." *AIMS microbiology* 6.4 (2020): 451.
 14. Raguénès, Gérard, et al. "Vibrio diabolicus sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*." *International Journal of Systematic and Evolutionary Microbiology* 47.4 (1997): 989-995.
 15. Rajeswari, T. Raja, and Namburu Sailaja. "Impact of heavy metals on environmental pollution." *Journal of Chemical and Pharmaceutical Sciences* 3 (2014): 175-181.
 16. Rezaei, Ali, et al. "Evaluation of groundwater quality and heavy metal pollution indices in Bazman basin, southeastern Iran." *Groundwater for sustainable development* 9 (2019): 100245.
 17. Sall, Mohamed Lamine, et al. "Toxic heavy metals: impact on the environment and human health, and treatment with conducting organic polymers, a review." *Environmental Science and Pollution Research* 27 (2020): 29927-29942.
 18. Suhani, I., Sahab, S., Srivastava, V., & Singh, R. P. (2021). Impact of cadmium pollution on food safety and human health. *Current Opinion in Toxicology*, 27, 1-7.
 19. Sun, Mingming, Huizhen Chao, Xiaoxuan Zheng, Shaopo Deng, Mao Ye, and Feng Hu. "Ecological role of earthworm intestinal bacteria in terrestrial environments: a review." *Science of the Total Environment* 740 (2020): 140008.
 20. Turner, Jeffrey W., et al. "Comparative genomic analysis of *Vibrio diabolicus* and six taxonomic synonyms: a first look at the distribution and diversity of the expanded species." *Frontiers in Microbiology* 9 (2018): 1893.
 21. Vajargah, Mohammad Forouhar. "A review on the effects of heavy metals on aquatic animals." *J ISSN* 2766 (2021): 2276.

22. Yang, Qianqi, et al. "A review of soil heavy metal pollution from industrial and agricultural regions in China: Pollution and risk assessment." *Science of the total environment* 642 (2018): 690-700.