BACTERIAL POPULATION OF *Pteris vittata* WITH POTENTIALS FOR BIOREMEDIATION OF ARSENIC RICH SOILS AND PLANT GROWTH PROMOTION

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by

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LIST OF ABBREVIATIONS

ARS	Arsenic-rich soil
NMS	Natural mineral soil
OUT	Operational Taxonomic Unit
PGPR	Plant growth-promoting rhizobacteria
PGPM	Plant growth-promoting microorganisms
PSB	Phosphate solubilizing Bacteria
PSM	Phosphate solubilizing Microorganisms
KSB	Potassium solubilizing Bacteria
PHs	Phytohormones
IAA	Indole-3-acetic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AR	Arsenate reductase
As	Arsenic
As (III)	Arsenite
As (V)	Arsenate
ASB	Arsenobetaine
MMA (III)	Monomethylarsenite
MMA (V)	Monomethylarsonate
DMA	Dimithylarsenic acid
DMA (III)	Dimethylarsenite
DMA (V)	Dimethylarsonate
TMAO (V)	Trimethylarsenic oxide
TMAO (III)	Trimethylarsine oxide
DARPs	dissimilatory As (V)-reducing prokaryotes
ABA	Abscisic Acid
ET	Ethylene
Gas	Gibberellins
CKs	Cytokinins

HPLC	High-Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ROS	Reactive oxygen species
SOD	Superoxide dismutase
CAT	Catalase
et al	et alia (and others)
pH	Potential of hydrogen (hydrogen ion concentration)
sp.	Species
OD	Optical density
A600	Absorbance at a wavelength of 600 nm
A450	Absorbance at a wavelength of 450 nm
A530	Absorbance at a wavelength of 530 nm
Rpm	Revolutions per minute
QC	Quality control
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
CDS	Coding sequence
rRNA	Ribosomal ribonucleic acid
mRNA	Messenger ribonucleic acid
tRNA	Transfer ribonucleic acid
Bp	Base pair
CFU	Colony-forming unit
GC	Gas chromatography
HCN	Hydrogen cyanide

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POPULASI BAKTERIA PADA *Pteris vittata* DENGAN POTENSI SEBAGAI BIOPEMULIH TANAH YANG KAYA DENGAN ARSENIK DAN MENGGALAKAN PERTUMBUHAN TUMBUHAN

ABSTRAK

Dalam persekitaran tanah, bakteria tertentu boleh menahan tekanan yang disebabkan oleh arsenik dan memainkan peranan penting dalam pertanian sebagai rizobakteria yang menggalakan pertumbuhan tumbuhan (PGPR). Bakteria ini meningkatkan pertumbuhan tumbuhan dan menyumbang kepada biopemulihan tanah daripada kesan toksik arsenik. Proses ini juga melibatkan kumpulan tumbuhan terpilih yang dikenali sebagai hiperakumulator, seperti *Pteris vittata*, yang meningkatkan kecekapan penyingkiran arsenik daripada tanah. Pelbagai kaedah digunakan untuk membersihkan alam sekitar daripada kesan buruk pencemaran arsenik, tetapi kebanyakan kaedah ini tidak menjimatkan kos dan mempunyai prestasi yang sangat buruk tidak memuaskan. Biopemulihan digunakan sebagai pendekatan praktikal untuk menangani pencemaran yang disebabkan oleh arsenic. Kajian ini bertujuan untuk menentukan populasi bakteria tumbuhan *P. vittata* dengan potensi biopemulihan tanah yang tercemar arsenik dan menggalakan pertumbuhan tumbuhan. Analisis metagenomik amplikon 16S digunakan untuk menentukan komposisi, fungsi, dan diversiti bakteria daripada tanah yang kaya dengan arsenik dan mineral semula jadi. Kaedah konvensional telah digunakan untuk mengasing, menyaring, dan mengenal pasti potensi bakteria untuk menggalakan pertumbuhan tumbuhan, toleransi ke atas arsenik dan logam-logam lain, detoksifikasi, dan kebolehan biopemulihan. Isolat terpilih dinilai untuk kecekapan mereka dalam mengurangkan kesan toksik arsenik, menggalakan pertumbuhan tumbuhan dan

meningkatkan keupayaan hiperakumulasi *P. vittata* di bawah tekanan arsenik yang berbeza. Jujukan amplikon 16S sampel tanah mendedahkan bahawa rizosfera tanah *P. vittata* daripada tanah yang kaya dengan arsenik mempamerkan kepelbagaian spesies yang lebih tinggi tetapi mempunyai diversiti keseluruhan yang lebih rendah berbanding tanah mineral semula jadi. Tanah yang kaya dengan arsenik didominasi oleh filum Actinobacteria (48%), Proteobacteria (19%), Chloroflexi (12%), Acidobacteria (6%), Planctomycetes (3%), Gammatimonadetes (3%), Firmicutes (2%) dan Myxococcota (2%). Tanah mineral semula jadi didominasi oleh filum yang sama, tetapi dengan tahap kelimpahan yang berbeza-beza. Filum yang paling banyak dalam tanah mineral semula jadi ialah Actinobacteria (41%), Proteobacteria (21%), Acidobacteria (9%), Chloroflexi (8%), Verrumicrobia (6%), Planctomycetes (5%), Gammatimonadetes, dan Bacteriodetes. (2%). Gen untuk toleransi arsenik dan logam lain dan penggalakkan pertumbuhan tumbuhan telah berjaya diramal dalam metagenom. Sebanyak enam (6) isolat bakteria telah dipilih berdasarkan ciri-ciri PGP dan toleransi arsenik dan telah dikenal pasti oleh penjujukan 16S rDNA sebagai *Enterobacter* sp. 2P5 (CCB-MBL 5011), *Enterobacter* sp. 3P19 (CCB-MBL 5012), *Bacillus* sp. 3P20 (CCB-MBL 5013), *Enterobacter* sp. 5P7 (CCB-MBL 5015) daripada tanah kaya dengan arsenik dan *Enterobacter* sp. 3U4 (CCB-MBL 5014) dan *Enterobacter* sp. 6U3 (CCB-MBL 5016) daripada tanah mineral semula jadi. *Bacillus* sp. 3P20 dan *Enterobacter* sp. 3U4 telah dipilih untuk menentukan kesannya terhadap detoksifikasi arsenik dan hiperakumulasi oleh *P. vittata*. Hasilnya mendedahkan bahawa inokulasi bakteria menggalakan pertumbuhan tumbuhan dan secara ketara meningkatkan kecekapan detoksifikasi arsenik dan hiperakumulasi oleh *P. vittata*. Jujukan lengkap genom *Bacillus* sp. 3P20 dan *Enterobacter* sp. 3U4 mendedahkan kehadiran jangkaan gen untuk ciri-ciri PGP, arsenik, dan ketahanan logam berat lain dalam genom yang telah diisolasi. Berdasarkan keputusan, isolat tersebut mempunyai tahap toleransi terhadap logam arsenik yang tinggi, menggalakan pertumbuhan tumbuhan, dan memainkan peranan penting dalam biotransformasi arsenik, hiperakumulasi oleh *P. vittata*, dalam tanah yang tercemar. Oleh itu, isolat tersebut boleh digunakan sebagai inokulum untuk meningkatkan hiperakumulasi arsenik dan bioremediasi apabila ditambahkan ke dalam tanah yang tercemar.

BACTERIAL POPULATION OF *Pteris vittata* WITH POTENTIALS FOR BIOREMEDIATION OF ARSENIC RICH SOILS AND PLANT GROWTH PROMOTION

ABSTRACT

In soil environments, certain bacteria can withstand arsenic-induced stress and play vital roles in agriculture as plant growth-promoting rhizobacteria (PGPR). The bacteria enhance plant growth and contribute to the bioremediation of toxic arsenic in soils. This process also involves a selected group of plants known as hyperaccumulators, such as *Pteris vittata*, which enhance the efficiency of arsenic removal from the soil. Various methods are being used to cleanse the environment from the adverse effects of arsenic pollutant, but most of these methods are not cost-effective and have very poor performance. Bioremediation is used as a practical approach to address pollution caused by arsenic. This study aims to determine the bacterial population of *P. vittata* with the potential for bioremediation of arsenic-rich soils and plant growth promotion. The 16S amplicon metagenomics analysis was employed to determine the composition, functions, and bacterial diversity of soils from arsenic-rich and natural mineral sites. Conventional methods were employed to isolate, screen, and identify bacteria potentials for plant growth promotion, arsenic and other metals tolerance, detoxification, and bioremediation abilities. The selected isolates were evaluated for their efficiency in mitigating the toxic effects of arsenic, plant growth promotion and enhancement of the hyperaccumulation ability of *P. vittata* under different arsenic stress condition. The 16S amplicon sequencing of the soil samples revealed that the rhizosphere soil of *P. vittata* from arsenic-rich soil exhibited higher species richness but lower overall diversity than the natural mineral

soils. The arsenic-rich soils were dominated by the phyla Actinobacteria (48%), Proteobacteria (19%), Chloroflexi (12%), Acidobacteria (6%), Planctomycetes (3%), Gammatimonadetes (3%), Firmicutes (2%), and Myxococcota (2%). The natural mineral soils were dominated by the same phyla, but with varying levels of abundance. The most abundant phyla in the natural mineral soils were Actinobacteria (41%), Proteobacteria (21%), Acidobacteria (9%), Chloroflexi (8%), Verrumicrobia (6%), Planctomycetes (5%), Gammatimonadetes, and Bacteriodetes (2%). Genes for arsenic and other metal tolerance and plant growth promotion were successfully predicted in the metagenome. A total of six (6) bacterial isolates were selected based on their PGP traits and arsenic tolerance and were identified by 16S rDNA sequencing as *Enterobacter* sp. 2P5 (CCB-MBL 5011), *Enterobacter* sp. 3P19 (CCB-MBL5012), *Bacillus* sp. 3P20 (CCB-MBL 5013), *Enterobacter* sp. 5P7 (CCB-MBL 5015) from arsenic-rich soils and *Enterobacter* sp. 3U4 (CCB-MBL 5014) and *Enterobacter* sp. 6U3 (CCB-MBL 5016) from natural mineral soils. *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 were selected to determine their effects on arsenic detoxification and hyperaccumulation by *P. vittata*. The result revealed that bacterial inoculation promotes the plant's growth and significantly improves arsenic detoxification efficiency and its hyperaccumulation by *P. vittata*. The complete genome sequence of *Bacillus* sp. 3P20 and *Enterobacter* sp. 3U4 revealed the presence of predicted genes for PGP traits, arsenic, and other heavy metal resistance in the genomes of the isolates. Based on the results, the isolates tolerated high levels of arsenic metal, promoted plant growth, and played a significant role in arsenic biotransformation, its hyperaccumulation by *P. vittata*, in the contaminated soils. Hence, the isolates can be used as inoculum to enhance arsenic hyperaccumulation and its bioremediation when introduced into contaminated soils.

CHAPTER 1

INTRODUCTION

1.1 Background

Environmental contamination has become a serious public health concern because it is a major source of health risks and causes several diseases worldwide (Sumiahadi & Acar, 2018; Sun et al., 2020). The pollution of heavy metals is a major concern in terms of environmental degradation (Sher & Rehman, 2019). Despite the well-known dangers of heavy metal exposure, there has been an increase in the occurrence of arsenic and other heavy metals in some parts of the globe (Ali et al., 2019). This exposure can result in severe health issues, and extreme cases can even be fatal. Controlling and minimising exposure to heavy metals is crucial to prevent serious health consequences (Sumiahadi & Acar, 2018).

Heavy metals are found naturally in the environment and can enter the body through ingestion, inhalation, or skin contact (Jiang et al., 2020a; Priyadarshanee & Das, 2021). Metals are naturally present in soil and have a high adsorption capacity (Timková et al., 2018). Toxic metals, such as arsenic (As), lead (Pb), zinc (Zn), chromium (Cr), cadmium (Cd), copper (Cu), manganese (Mn), iron (Fe), and nickel (Ni), are emitted from industrial production and catalytic procedures through the discharge of water and waste materials, contaminating the surroundings (Priyadarshanee & Das, 2021; Timková et al., 2018). These metals are persistent environmental contaminants because they cannot be degraded or destroyed, leading to their continuous accumulation in the food chain and posing a serious environmental threat (Priyadarshanee & Das, 2021). Although a few heavy metals, such as Cu^{2+}, Zn^{2+}, Fe^{2+}/Fe^{3+} Mn^{2+}, Co^{2+}, are required for vital biological functions, excessive exposure to these elements can lead to toxicity (Priyadarshanee & Das, 2021; Timková et al., 2018). The impact of metal toxicity varies based on the dose, mode of exposure, the form of metal, and the characteristics of the exposed individual, including age, gender, and nutritional status (Ali et al., 2019; Priyadarshanee & Das, 2021; Singh et al., 2019).

Arsenic is a ubiquitous element found everywhere on Earth and is the 20th most abundant element in the Earth's crust (Sher & Rehman, 2019). It is widely distributed in the environment, primarily in rocks and minerals, and it is found in various natural reservoirs such as oceans, rocks, atmosphere, biota, and soil (Hussain et al., 2021). However, over 99% of it is present in rocks and minerals. Arsenic was first identified by Albertus Magnus in 1250 and has held the reputation of being the supreme poison since ancient times (Sher & Rehman, 2019). Arsenic has been present in the environment for a long time, but the recent increase in its exposure is causing a significant rise in cancer cases which has serious consequences for human health (Jing et al., 2022; Pandey et al., 2018; WHO, 2019). It is mainly found in two forms, arsenite As(III) and arsenate As(V), the As(III) is more toxic than As(V) (Sher & Rehman, 2019). Arsenic is a highly dangerous and carcinogenic substance that can accumulate in the food chain, leading to serious health risks for humans and wildlife (Jia et al., 2022).

Microbes in arsenic-rich environments have evolved strategies to either utilize or detoxify arsenic within the cell, leading to their involvement in the biochemical cycle of arsenic. This transformation produces species with differing solubility, mobility, bioavailability, and toxicity properties. Heavy metals are crucial in small amounts but can be harmful in contaminated environments. Certain bacteria use reduction, oxidation, and methylation processes to transform arsenic, which helps them tolerate its toxicity.

Phytoremediation is a low-cost technology which uses hyperaccumulator plants to remove metal pollutants from soils (Ashraf et al., 2019; Kafle et al., 2022). By accumulating arsenic, the hyperaccumulator plants developed arsenic tolerance due to acclimatization to contaminated soils (Kafle et al., 2022; Mairaj et al., 2022; Tiwari & Lata, 2018). *Pteris vittata* (Chinese brake fern) was the first known arsenic hyperaccumulator, capable of accumulating up to 23 g kg^{-1} arsenic in fronds with high arsenic translocation (Jia et al., 2022; Wei et al., 2021). Microorganisms that can promote plant growth and tolerate arsenic stress could be beneficial for *P. vittata* in a-stressed conditions and may also aid in arsenic remediation by enhancing the plant's phytoremediation capabilities. When inoculated with arsenic resistance bacteria such as *Bacillus* sp. and *Variovorax* sp., *P. vittata* can improve arsenic stress tolerance and enhance phytoremediation abilities (Ghosh et al., 2015). The use of various bacterial genera, including *Alcaligenes, Pseudomonas, Bacillus, Acidovorax, Paenibacillus, Rhodococcus,* and *Mycobacterium*, has been extensively documented in the phytoremediation process (Sharma, 2021).

The rhizosphere, the portion of soil directly influenced by plant roots, is home to various microbial populations that can have either neutral, beneficial, or detrimental effects on the plants (Lopez et al., 2020). Bacteria that help the host plant to grow through direct or indirect means are termed plant growth-promoting rhizobacteria (PGPR) (Alves et al., 2022; Gouda et al., 2018). Plant growth-promoting rhizobacteria (PGPR) is a group of bacteria found in the rhizosphere in association with the roots of plants (Gouda et al., 2018; Khan et al., 2021; Manoj et al., 2020). The PGPR, including *Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Pseudomonas, Enterobacter, Burkholderia*, *Klebsiella Rhizobium,* and *Serratia,* have been reported to improve the health and development of the plants (Gouda et al., 2018; Khan et al., 2021; Rahman et al., 2020). The PGPR have been shown to help plants grow in many ways, such as dissolving minerals like phosphorus and potassium, fixing atmospheric nitrogen, producing siderophores, and synthesizing plant growth hormones such as gibberellic acid, Indole-3-acetic acid (IAA), ethylene, and cytokinins (Etesami & Maheshwari, 2018; Ghosh et al., 2018; Rahman et al., 2017).

PGPR can assist in the growth and development of plants in both natural mineral soils and arsenic-contaminated soils (Kong & Glick, 2017). PGPR can boost plant growth in natural mineral soils by increasing nutrient accessibility and facilitating more efficient nutrient absorption. Moreover, they can enhance plant growth by generating growth hormones and promoting stress tolerance (Gao et al., 2022; Kumar & Verma, 2018; Mondal et al., 2021). In arsenic-rich soils, PGPR can play a crucial role in reducing the harmful effects of arsenic on plants. They can facilitate the absorption and tolerance of arsenic by modifying its chemical composition or immobilizing it, which lowers its toxicity to plants (Alka et al., 2020; Xiao et al., 2020). In addition, some PGPR can promote plant growth in arsenic-contaminated soils by producing enzymes that break down organic matter and release nutrients trapped with arsenic (Kumar et al., 2019; Manzoor et al., 2019).

Microorganisms present in arsenic-contaminated and natural mineral soils have developed various strategies to endure and thrive in these environments (Sun et al., 2020). Certain bacteria, including *Pseudomonas, Bacillus,* and *Arthrobacter*, are resistant to arsenic and can withstand high levels of the toxic element. Other bacteria, such as *Shewanella, Desulfovibrio,* and *Geobacter,* can transform arsenic from one form to another, reducing its toxicity or increasing its mobility. Arsenic-reducing bacteria, including *Alcaligenes* and *Rhizobium*, can convert arsenic into a less harmful form (Paul et al., 2018). On the other hand, some bacteria, like *Acidithiobacillus* and *Leptospirillum*, can oxidize arsenic, making it more toxic and mobile.

Selected microbes have developed a range of mechanisms to survive in soils contaminated with arsenic (Osagie et al., 2021). For instance, some bacteria utilize efflux pumps with specialized transporters to remove arsenic from their cells (Mondal et al., 2021; Saeed et al., 2021). Others produce enzymes that detoxify arsenic by breaking down or modifying arsenic compounds, which reduces their toxicity (Dubey et al., 2018; Suhadolnik et al., 2017). Certain bacteria form biofilms to protect themselves from high levels of arsenic. Finally, some bacteria adapt to the high arsenic levels by altering their genetic makeup, which enables them to survive in contaminated soil. The ability of these microbes to survive in arsenic-contaminated soils depends on various factors, such as their metabolic pathways, genetic makeup, and environmental conditions, such as acidity or alkalinity and temperature. These microbes have a significant role in the natural cycling of arsenic in the environment, and their ability to adapt and thrive in arsenic-contaminated soils is essential for bioremediation strategies (Mirza et al., 2014; Saeed et al., 2021).

1.2 Statement of the research problem

Arsenic pollution has become a serious concern worldwide due to its adverse effects on flora, fauna, and human health (Alka et al., 2020). Different methods are being used to cleanse the environment from the adverse effects of these pollutant, but most of these methods are not cost-effective and have very poor performance. In response to this pervasive challenge, bioremediation is increasingly considered a practical approach to address the environmental pollution caused by arsenic. Bioremediation utilizes microorganisms, plants, or their derivatives to restore polluted environments to their original state by reducing or removing toxic pollutants. This approach is cost-effective, environmentally friendly, and causes minimal harm to the environment. However, there are limitations to this method. Hence there is a need for a better, more affordable, and quicker approach that can be achieved by using both plants and microorganisms together. Recent research has introduced a new method that involves using PGPR-enhanced phytoremediation to address plant stress in soils contaminated with arsenic (Alka et al., 2020). This new method has proven to be more effective in phytoremediation, causing shifts in how arsenic exists in the soil, improving its mobility within plants, and decreasing the harmful effects of arsenic on plants. Addressing soil detoxification and promoting healthy plant growth is crucial in arsenic-contaminated soil. Exploring the potentials of PGPR linked to *P. vittata* offers a promising solution for bioremediation and plant growth enhancement. Although there are some initial indications of the abilities of bacteria in enhancing bioremediation and plant growth, there's a significant gap in understanding specific bacterial types, their functions, genes responsible for arsenic tolerance, plant growth promotion, and effectiveness in arsenic bioremediation. This research aims to fill this gap by investigating the bacterial population from the rhizosphere of *P. vittata*. The focus is on isolating, screening, and identifying bacterial strains and genes with impressive arsenic tolerance, detoxification abilities, and potential for stimulating plant growth. By studying interactions between *P. vittata*-related PGPR, in arsenic-rich and natural mineral soils and plant development, this research provides insights into sustainable bioremediation strategies.

1.3 Research questions

i. What PGPR are associated with *P. vittata* grown on arsenic-rich and natural mineral soils?

ii. Do the PGPR of *P. vittata* promote plant growth and the plant's arsenic accumulation /extraction abilities for bioremediation?

iii. What are the genes that enhance plant growth, arsenic tolerance, and accumulation of the PGPR?

iv. What are the mechanisms involved in arsenic decontamination, and how is the PGPR isolated involved in one of those mechanisms?

1.4 Research hypothesis

i. Selected PGPR are associated with hyperaccumulator plants (rhizosphere soils of *P. vittata*) growing on arsenic- rich ex- tin mining soils.

ii. The PGPR can promote growth and arsenic accumulation and extraction ability of the selected hyperaccumulator plants for bioremediation.

iii. Genes of selected PGPR enhance plant growth and arsenic tolerance, accumulation, and decontamination ability.

1.5 Aim and objectives

1.5.1 Aim

This research aimed to determine the bacterial population of the rhizosphere of *P. vittata* from arsenic-rich (ex-tin mining) and natural mineral (Universiti Sains Malaysia Campus) soils for bioremediation of arsenic and plant growth-promoting activities.

1.5.2 Objectives

1. To compare the population of arsenic-tolerant PGPR of *P. vittata* collected from arsenic-rich (RHT) and natural mineral (USM) rhizosphere soils of *P. vittata*.

2. To isolate and screen different arsenic-tolerant PGPR from the rhizosphere soils of *P. vittata* collected from both sampling sites.

3. To determine the oxidation and reduction potential of arsenic tolerant PGPR in the decontamination of arsenic.

4. To determine the efficiency of the best PGPR in enhancing hyperaccumulation and plant growth promotion abilities of *P. vittata* in arsenic-spiked soil.

5. To carry out complete genome sequencing of the best performing PGPR associated with *P. vittata* and investigate the genetic basis for bacterial arsenic metal tolerance, detoxification, and plant growth promotion.

CHAPTER 2

LITERATURE REVIEW

2.1 Arsenic (As) and the environment

Arsenic, a metalloid that exists in small amounts throughout the Earth's crust, is known to be highly toxic and carcinogenic (Abou-Shanab et al., 2020; Cai et al., 2019; Marzi et al., 2021). It has been listed among the World Health Organization's top 10 chemicals of significant health concern due to its detrimental impact on human health (Suhadolnik et al., 2021). It is common and widely distributed in different forms in soil, water, and air environments (Xiong et al., 2010). Ecosystems contaminated with arsenic seriously threaten food safety and human well-being because of its persistence, toxicity, and widespread presence in the environment (Ghosh et al., 2021). It is widely recognized as a carcinogen, even at low levels of exposure. Arsenic contamination in soil and water has been observed in many regions globally.

Recently, in certain areas of Asia, such as China, the long-term consumption of arsenic-tainted groundwater has resulted in a condition known as endemic arsenicosis, which poses a significant risk to public health (Xiong et al., 2010). Globally, the average level of arsenic in soil is typically less than 10 mg/kg. However, in contaminated soils, the levels can be much higher, often ranging from 10^2-10^3 mg/kg, and sometimes as high as 10^4 mg/kg have been reported. The form in which arsenic is present in the soil, whether inorganic or organic, can influence its solubility, bioavailability, and toxicity (Matzen et al., 2020a). Despite its toxic nature, it has been found that certain microorganisms, known as "arsenotrophic" microbes, can use arsenic for respiratory metabolism. With a rapidly growing population, food production needs to be significantly increased across the globe (Xiong et al., 2010).

2.1.1 Sources of arsenic (As) in the environment

Arsenic pollution is increasingly recognized as a major global environmental concern (Kumar et al., 2021). Arsenic is ubiquitous, and it is the 20th most common element in the Earth's crust (Reid et al., 2020; Sher & Rehman, 2019) and it is a naturally occurring element found in soils, rocks, atmosphere, organisms, and natural waters (Ullah et al., 2021). Arsenic, a toxic element, has been getting more attention lately because of its presence and spread in the environment (Sher & Rehman, 2019). It can come from two main sources: natural and human-caused (anthropogenic). Arsenic occurs naturally in various forms and concentrations, with trace amounts present in the soil, the Earth's crust, and ocean water. However, there is a growing concern about the transfer of arsenic from these sources to the air and drinking water (Sher & Rehman, 2019). Natural processes such as leaching, erosion, weathering of rocks, forest fires, and volcanic activity can increase the concentration of arsenic in the environment (Chandrakar et al., 2018; Ullah et al., 2021).

Additionally, geothermal water and biological activities may contribute to the rise in arsenic concentration in the intricate soil-water interface. Recent observations indicate that the oxidation of minerals containing arsenic in groundwater has caused an increase in arsenic concentration in valuable groundwater systems (Sher & Rehman, 2019). The levels of arsenic present in both soil, ground, and surface waters come from both natural and artificial sources, and its potential to cause harm and its movement through the environment is closely tied to its chemical form (Kumar et al., 2022).

Anthropogenic activities such as wood preservation, smelting, manufacturing, gold mining, glass, chemical, weapons production, printing, pigment production, tanning, and the use of rodent poisons, pesticides, insecticides, herbicides, and fungicides and soil sterilant can also contribute to arsenic contamination in the environment (Baloch et al., 2020). These man-made sources are a significant concern because they can increase the amount of arsenic in the environment and make it available for human and animal exposure. Human actions are responsible for discharging substantial amounts of arsenic into the environment and can lead to soil, air, and water contamination (Sher & Rehman, 2019). Mining activities and industrial processing are major sources of environmental arsenic contamination, posing potential dangers to human health. The handling and disposal of mine tailings, which contain high concentrations of arsenic, is a major problem in the mining industry (Tang & Zhao, 2020).

Gold extraction activities produce substantial amounts of waste materials that negatively impact the nearby surroundings, especially the residue from the mining process known as tailings, which includes elevated levels of heavy metals like arsenic, lead, and copper. These pollutants can seep into groundwater, rivers, and soil, leading to contamination, threatening human health, and impacting microorganisms' variety, numbers, and behaviour (Sonthiphand et al., 2021). The pollution caused by the waste generated from gold mining tailings has demonstrated an impact on the microbial ecosystem in sediment and water samples, resulting in increased growth of specific microorganisms and decreased total productivity, size, and diversity of the microbial population (Sonthiphand et al., 2021).

Arsenic can also be found in sedimentary rocks and certain minerals such as pyrite and other sulphide minerals (Baloch et al., 2020). High concentrations of arsenic can also be found in many hydrous metal oxides and oxide minerals (Chandrakar et al., 2018; Ullah et al., 2021). This can lead to potential risks to human health. Mines produce large volumes of waste materials, including slag, rock, topsoil, tailing, and other associated materials that must be removed to access the mineral resource during extraction (Masuda, 2018). Geogenic and anthropogenic activities such as precipitation, weathering, volcanic actions, and human activities such as coal mining can lead to the mobilization of arsenic and contamination of groundwater, which is a major concern in semi-arid and arid areas where groundwater is used for irrigation (Ullah et al., 2021). Certain human activities can increase arsenic levels in the environment, such as mining, pesticide and insecticide use, coal burning, and petroleum refining (Baloch et al., 2020). These activities can lead to the mobilization of arsenic and its presence in groundwater, which can be used for irrigation in semi-arid and arid areas. This can result in high levels of arsenic in crops and poses a risk to human health (Masuda, 2018). A summary of the sources of arsenic in the environment is presented below.

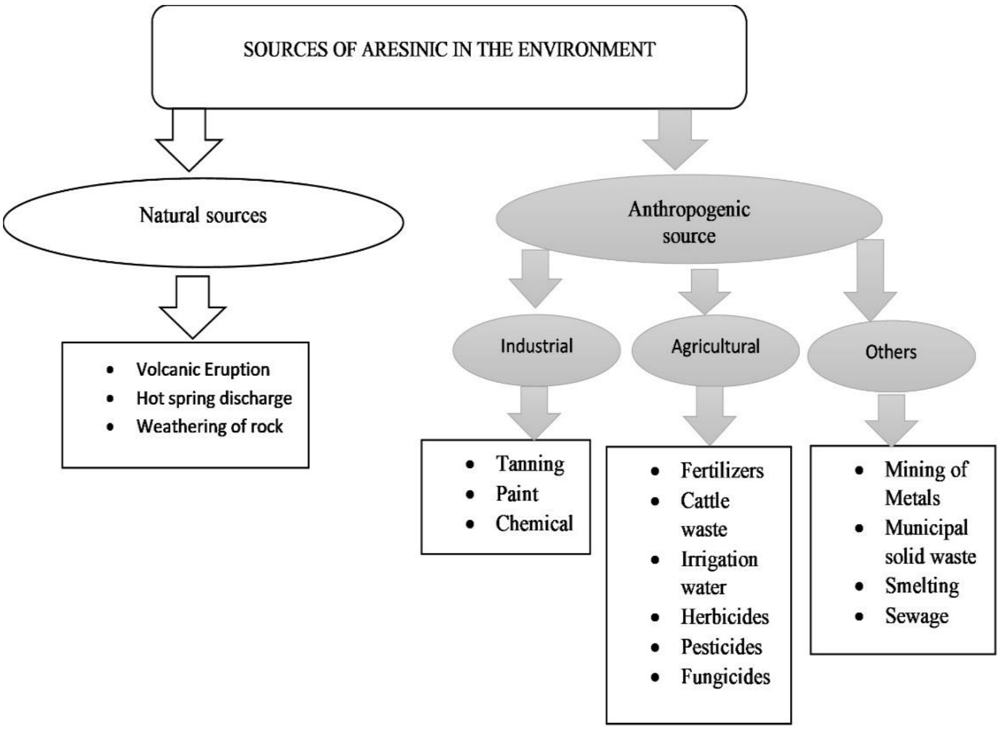

Figure 2.1 Sources of arsenic in the environment

Arsenic can leach into natural water sources through the erosion of minerals and soils that contain it (Reid et al., 2020; Sher & Rehman, 2019). The concentration of arsenic in soils can vary depending on the source of the soil. Typically, the amount of arsenic in the soil varies between 5 to 10 mg/kg. However, in European topsoil, the average concentration is reported to be 7 mg/kg (Abbas et al., 2018). Soils rich in peat and bog are typically more enriched with arsenic, with an average concentration of 13 mg/kg. Some soils, such as acid sulphate soils, have even higher concentrations of arsenic. The recommended permissible concentration of arsenic in soil set by the United States Environmental Protection Agency (USEPA) is 24 mg/kg, but many countries have surpassed this limit due to human activities (Abbas et al., 2018; Alka et al., 2020).

2.2 Arsenic speciation in the soil

Speciation" refers to the different chemical forms an element can take, including its oxidation levels and mineral phases (Abbas et al., 2018). It can indicate how easily the element is absorbed and its potential for toxicity in the soil. Arsenic can exist in various species or chemical forms in soil, such as organic or inorganic components, precipitated solids, free ionic forms, exchangeable states, and primary secondary minerals' structural elements (Abbas et al., 2018). The most common method for quantifying individual arsenic species to understand their distribution, toxicity, metabolism, and health effects in biological systems better is high-performance liquid chromatography (HPLC) separation coupled with inductively coupled plasma mass spectrometry (ICP-MS) detection (Reid et al., 2020; Sher & Rehman, 2019). HPLC separates individual arsenic species, allowing them to be quantified with the appropriate detector (ICP-MS). The ICP-MS is commonly used due to its high ionization efficiency, low matrix interference, high selectivity and wide dynamic range for sensitive detection (Reid et al., 2020). However, this method gives no molecular information, and the identification of arsenic species relies on matching retention times with available standards. ICP-MS is a sensitive and selective detector that does not provide molecular information. Therefore, HPLC-ICP-MS can only determine the identities of compounds when reference standards are used (Reid et al., 2020). These methods are powerful but can be expensive and time-consuming. The Safranin O dye spectrophotometry technique, can be used for arsenic removal from biological samples. However, if the goal is to specifically identify arsenic species such as arsenite and arsenate, the micro-plate $AgNO_3$ can be used (Sher & Rehman, 2019).

Arsenic infrequently occurs in a free state and is mostly found in combination with oxygen, sulphur and iron. It can be found in a variety of forms, including sulfides, oxides, and salts of sodium, calcium, iron, and copper. It is a widespread environmental pollutant that is found in many regions of the world (Kumar et al., 2022). In groundwater, arsenic combines with oxygen (O_2) to form inorganic pentavalent arsenate and trivalent arsenite. Contrary to other heavy metalloids and oxyanion-forming elements, arsenic can be mobilized at the pH 6.5 to 8.5, normally found in the surface and groundwaters and under oxidizing and reducing conditions (Kumar et al., 2022). Arsenic can also be found within the mg/L range, while all other oxyanion-forming elements are within the μg/L range. Arsenic occurs in the environment in different oxidation states (−3, 0, +3 and +5), often as metal arsenides, arsenates, or sulfides. Arsenic exists in two main forms in the environment, arsenite As(III) and arsenate As(V) (Sher & Rehman, 2019). The forms of arsenic can be grouped into three categories: inorganic, which includes arsenate [As(V)] and arsenite [As(III)] and are the most dangerous and toxic; methylated forms such as methylarsonate and dimethylarsenate, which are moderately toxic; and nontoxic forms such as arsenobetaine (AsB), arsenocholine (AsC) and other arsenosugars (AsS) (Stevens et al., 2018).

2.3 Hyperaccumulator plants

Plants have different mechanisms for arsenic toxicity, such as sequestration in vacuoles, detoxification by methylation, and exclusion at the root surface. Some plant species, known as hyperaccumulator plants, have developed mechanisms to tolerate high arsenic levels in their tissues (Souri et al., 2017). Hyperaccumulators are plants that can absorb and store high concentrations of metal ions, such as heavy metals, in their leaves, stem, and roots (Matzen et al., 2022a). They can grow in soil or water containing very high levels of metals and tolerate these conditions, while other plants would not survive (Vandana et al., 2020). They can absorb the metals through their roots and thus accumulate extremely high levels in their tissues (Silva et al., 2018; Matzen et al., 2022a). The plants can absorb and store high amounts of metal/metalloids from the soil in their aboveground biomass. When exposed to polluted soils or water, these plants can accumulate high concentrations of heavy metals in their fronds without phytotoxicity symptoms. Multiple research has been done on using these plants to clean up soil and water contaminated with metal(loids) through phytoextraction (Matzen et al., 2022b).

Hyperaccumulation is a plant's capacity to amass heavy metals in its above-ground parts, even at a concentration 100-1,000 times higher than non-hyperaccumulating species exposed to similar conditions, without exhibiting any symptoms of phytotoxicity (Suman et al., 2018). Hyperaccumulator plants have a unique ability to extract metals from the soil at a high rate and store large amounts in their leaves and roots (Das et al., 2017). This is due to the differential gene expression that regulates metal uptake and transport in these plants. The number of known hyperaccumulator species has been increasing, with over 450 identified in 45 different families of flowering plants as of 2015 (Suman et al., 2018). The Chinese brake fern (*P. vittata* L.) was the first known arsenic-hyperaccumulating plant.

Studies have identified other fern species, such as the Cretan brake fern (*Pteris cretica* L.), which can accumulate arsenic. However, not all fern species can hyper-accumulate arsenic. Ferns that cannot tolerate and accumulate high arsenic levels in their tissues are considered non-hyperaccumulators (Popov et al., 2021). *P. vittata* is among the best options used for arsenic hyperaccumulation. This plant tends to store a high arsenic concentration in its shoots but needs an appropriate water supply for growth. Arsenic toxicity in plants can produce Reactive Oxygen Species (ROS), resulting in damage to biomolecules and even cell death (Ali et al., 2021; Nahar et al., 2022). Therefore, in these cases, the plants must evolve a mechanism to protect the cell from ROS by synthesizing enzymatic and non-enzymatic antioxidants (Abd-Allah et al., 2018).

2.3.1 *Pteris vittata* and arsenic hyperaccumulation in soil

Pteris vittata, a fern species in the *Pteridaceae* family, is native to China, Taiwan, and the Philippines. It is a perennial plant that typically reaches 1-2 feet in height. The fronds are large, reaching up to 2-3 feet in length, and feature glossy, bright green leaflets that are organized in a ladder-like pattern, which gives the plant its common name (Figure 2.2). It is also called the Chinese brake fern, and it has been identified as a model plant for its ability to hyper-accumulate arsenic (Matzen et al., 2022a; Yan et al., 2019a). *P. vittata* has unique underground stems called rhizomes, which connect their roots and fronds. These rhizomes are different from those found in other plants. In studies, the rhizomes, and roots of *P. vittata* are often grouped together and referred to simply as "roots" (Figure 2.2). This fern is used in traditional medicine and grown as an ornamental plant. It is also known for its ability to remove arsenic and other pollutants from the environment through phytoremediation, making it valuable for environmental remediation (Yan et al., 2019b). The ferns (*Pteris vittata*) are diverse and survive in great numbers, and have adapted to both competitions from seed plants and changing environments (Gonzaga et al., 2007; Ma et al., 2001). *P. vittata* is a widespread species that can grow in tropical and temperate climates. The distribution of this plant is limited by its need for well-drained, alkaline soil and exposure to ample sunlight (Yan et al., 2019b).

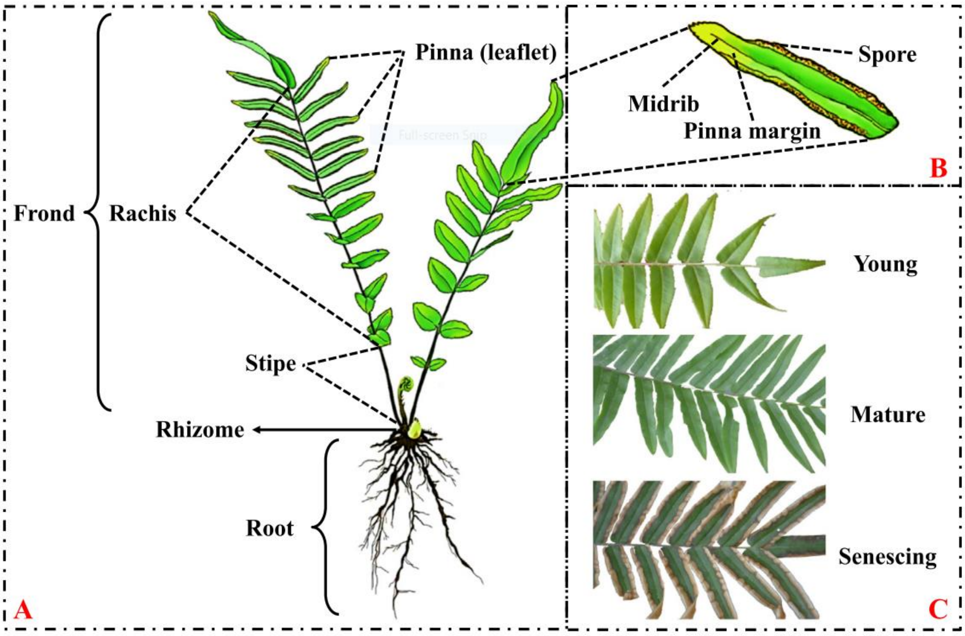

Figure 2.2 Components of *P. vittata* comprising (A); the root, rhizome, stipe (stem without pinna), pinna (leaflet), rachis (stem with pinna), and frond (aboveground biomass), (B); illustrates the inner structure of the pinna, encompassing the midrib, pinna margin, and spore, and (C); shows the fronds at various stages of maturity from a single *P. vittata* plant (Han et al., 2020)

P. vittata was the first plant known to hyperaccumulate arsenic. Other species belonging to the *Pteris* genus have also been found to hyper-accumulate arsenic, including *Pityrogramma calomelanos, P. cretica, P. longifolia, P. umbrosa, P. argyraea, P. quadriaurita, P. ryiunkensis, and P. biaurita* (Ma et al., 2001). *P. vittata* has the remarkable ability to absorb and tolerate large amounts of arsenic and transport it to its fronds in large quantities, with shoot concentrations reaching levels that are about 100 times higher than the soil concentrations (Wan et al., 2017; Yan et al., 2019b). In addition, *P. vittata* is easy to grow, has high biomass, is resistant to disease and pests, and is a hardy perennial with a high arsenic accumulation rate. While most plants naturally contain very little arsenic, with concentrations rarely exceeding 1 mg/kg, *P. vittata* was found to accumulate up to 438-755 mg/kg of arsenic when grown in uncontaminated soil, and 3,525 to 6,805 mg/kg of arsenic when grown in contaminated soil for 6 weeks (Abou-Shanab et al., 2020; Ma et al., 2001). *P. vittata* is an ideal plant for studying arsenic hyperaccumulation as it can effectively extract arsenic from soil and transport it to its shoots (Matzen et al., 2022a; Yan et al., 2019b).

Studies have shown that *P. vittata* can amass up to 27,000 mg kg^{-1} of arsenic (Wang et al., 2002), but signs of phytotoxicity start to manifest at about 10,000 mg kg^{-1} dry weight (Ma et al., 2001; Popov et al., 2021; Tang & Zhao, 2020; Vandana et al., 2020; Wang, 2002). In contrast, the threshold amount for non-accumulator plants is roughly 5-100 mg kg^{-1} dry weight (Wang et al., 2002). Researchers have paid significant attention to the underlying arsenic-resistance mechanism of *P. vittata* because of its ability to accumulate a high amount of arsenic in its above-ground biomass. Phosphate transporters help in the take up As(V) in the root of *P. vittata,* which is then transferred to rhizomes, where it is converted to As(III). Thus, little information on arsenic accumulation in *P. vittata* rhizomes is currently available (Kohda et al., 2021). Therefore, *P. vittata,* as a strong arsenic hyperaccumulator, can provide genetic components for engineering transgenic plants for cleaning up contaminated soils (Gu et al., 2018). The ability of the *P. vittata* to take up high concentrations of arsenic and then sequester into aboveground portions when grown on either arsenic-enriched or uncontaminated soils implies that the fern has highly effective arsenic scavenging mechanisms (Yan et al., 2019b). Gaining knowledge about the mechanisms by which brake fern tolerates and accumulates arsenic is important for improving the use of plants in remediating soils contaminated with arsenic {Formatting Citation}.

2.4 Mechanisms of arsenic tolerance and detoxification by microbes in plants

There are three ways of arsenic biotransformation, namely methylation, oxidation, and reduction. To regulate the levels of arsenic, microorganisms utilize different mechanisms like metal ion uptake and chelation, as well as extrusion of metal ions (Sher & Rehman, 2019). When exposed to arsenic, bacterial strains utilize various detoxification strategies. Some strains may adsorb the arsenic onto their cell wall surface, while others may allow it to enter their cell cytoplasm for detoxification (Elahi et al., 2019). They can change the chemical properties of these metals through oxidation and reduction processes, increase their solubility, and immobilize them through precipitation. Microorganisms can also remove toxic metals from contaminated sites through anaerobic digestion and bioleaching. The detoxification of arsenic in plant tissues involves the reduction of As(V) to As(III) as the initial step (Abbas et al., 2018; Mandal et al., 2022). The enzyme responsible for this reduction is called arsenate reductase (AR), which utilizes glutathione (GSH) as a cofactor. By reducing As(V) to As(III), it promotes the efflux of As(III) to the external environment, thus reducing the overall concentration of arsenic in the plant tissue

(Mandal et al., 2022). They can also detoxify heavy metals using specific enzymes such as arsenite oxidase.

There are various ways that plants can tolerate and detoxify arsenic, including expelling accumulated arsenic, limiting the uptake of As(V), converting inorganic arsenic to less toxic organic forms, binding it with other molecules, and storing it in non-reactive locations. Different plants may use varying combinations of these mechanisms, depending on their biology. A hyper-tolerant plant uses various techniques to lower the amount of arsenic present in its shoots, while a hyperaccumulator can efficiently take in and transport arsenic and minimally release it to increase the amount of arsenic in its shoots. For example, some plants, like *Holcus lanatus and Cytisus striatus* (Zhang et al., 2022), may have a high tolerance for arsenic by slowing down the rate of arsenic infiltration rather than decreasing total accumulation (Bertin et al., 2022).

Plants that grow in areas with high levels of arsenic contamination develops hyper-tolerance to arsenic by slowing down the rate at which arsenic enters the plant. This allows the plant more time to respond but does not decrease the overall amount of arsenic accumulated. Arsenate is like phosphate, and it enters the plant through phosphate transporters. Many different types of microorganisms that can metabolize arsenic, such as *Lactobacillus* sp., *Rhodococcus Bacillus* sp., *Stenotrophomonas, Alcaligenes, Lysinibacillus* sp., *Kocuria* sp., *Pseudomonas* sp., *Aliihoeflea* sp., *Herminiimonas* sp., *Achromobacter xylosoxidans,* etc. can tolerate high levels of arsenic and can be used in the bioremediation of arsenic from groundwater (Diba et al., 2021). These microorganisms can use both heterotrophic and autotrophic As (III) oxidation. Using indigenous arsenite-oxidizing bacteria with bioaccumulation capacity for arsenic biomining could be an alternative method (Diba et al., 2021). Several bacterial species, such as *Staphylococcus aureus, Escherichia coli, Bacillus* sp., *Staphylococcus xylosis, Pseudomonas* sp., and *Chromobacterium violaceum* have been found to have the ability to detoxify arsenic. They can select variants with genetic resistance determinants that allow them to tolerate higher levels of arsenic in their environment.

2.4.1 Arsenic absorption, transportation, and detoxification mechanisms in *P. vittata*

The mechanism by which *P. vittata*, an arsenic hyperaccumulator, takes up, detoxifies, and transports arsenic is characterized by three main features: the capacity to efficiently uptake metals from soil or water, rapid movement of heavy metals from roots to shoots, and a better capacity for detoxifying and storing heavy metals in the shoots (Abbas et al., 2018; Danh et al., 2014). The bioavailability of heavy metals in the rhizosphere, the area surrounding the plant roots, is also an important factor in metal uptake from soil (Vandana et al., 2020). Hyperaccumulator plants like *P. vittata* have been found to have a greater bioavailability of heavy metals compared to non-hyperaccumulator plants, possibly due to the presence of mechanisms that solubilize metals (Matzen et al., 2022a). These mechanisms include the release of proteins and root exudates containing organic acids and plant-based compounds called siderophores, which facilitate the mobilization of arsenic to the plant. Studies have also shown that certain components of root exudates, such as phytic acid and oxalic acid, enhance arsenic uptake in *P. vittata* (Etesami, 2018).

Additionally, it has been suggested that arsenic movement from soil to plant in the presence of root exudates is coupled with the translocation of arsenic to the leaves (Vandana et al., 2020). The ability of *P. vittata* to accumulate high arsenic in its above aground biomass imply that the plant possesses a remarkably effective mechanism for eliminating arsenic. The unique defence mechanism of this fern against arsenic can be understood by studying the genetic and biochemical differences between *P. vittata* and other non-hyperaccumulator plants, particularly the plants that are sensitive to arsenic (Danh et al., 2014; Souri et al., 2017). The accumulation of arsenic in plants, such as *P. vittata*, can result in the production of reactive oxygen species (ROS) like hydroxyl radicals (OH^-), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2), which can cause oxidative stress. This stress can harm important biomolecules such as proteins, lipids, and DNA, disrupt metabolic processes, enzyme activity, and photosynthesis, and ultimately result in leaf chlorosis and necrosis (Danh et al., 2014). Plants employ two approaches to mitigate the toxic effects of heavy metals, such as indirect and direct defence mechanisms. Indirect defence mechanisms involve using antioxidant molecules and enzymes to scavenge for ROS. In contrast, the direct defence mechanism involves binding heavy metals to cell walls, chelating them in the cytosol, or storing them in vacuoles to avoid cell damage. *P. vittata* may employ both strategies to detoxify arsenic at higher levels than non-accumulator plants (Danh et al., 2014; Matzen et al., 2022a).

2.5 Biotransformation of arsenic

Microorganisms play a significant role in the presence, toxicity and chemical makeup of the toxic element arsenic in the environments (Hasegawa et al., 2019). Various pathways for the biotransformation of arsenic have been suggested for different microorganisms, which typically involve oxidation or reduction reactions (Hasegawa et al., 2019; Hirano, 2020; Mujawar et al., 2019). Microbes conduct these redox reactions to either protect themselves from this metalloid's toxic effects or produce energy to promote cellular growth. Due to the similarities in chemical makeup between As(V) and phosphate, phytoplankton absorb As(V) through the phosphate uptake system and then transform it within their cells (Hasegawa et al., 2019). The high toxicity of As(V) is caused by its binding to phosphate receptors that have important functions within cells. Biotransformation is a change in the chemical properties of a substance due to chemical reactions (Hirano, 2020; Mujawar et al., 2019). The toxicity of metal can be altered by adding or removing electrons. For example, the toxicity of arsenic can be reduced through an oxidation process using arsenite-oxidizing genes that convert As(III) into As(V) (Hasegawa et al., 2019). In the case of reduction, As(V) can be converted into As(III) which is 100 times more toxic than As(V). The mechanism of biotransformation of metals through microorganisms is well-understood by scientists worldwide, and the current focus is on finding ways to apply this mechanism on a large scale for commercial use (Hirano, 2020; Mujawar et al., 2019).

Arsenic is a toxic metal that can be degraded by certain types of bacteria through various mechanisms, such as oxidation, reduction, and methylation (Mengmeng et al., 2018). These processes help reduce arsenic toxicity (Hasanuzzaman et al., 2015; Reid et al., 2020). Bacteria use the energy generated from degrading arsenic for their growth. However, the idea that certain strains of bacteria can incorporate arsenate into their DNA to support growth has been challenged by scientific research. Studies have also shown that certain bacteria can utilize arsenate as a source of phosphate for growth by degrading ribosomal RNA and allowing for phosphate incorporation in the cell (Mengmeng et al., 2018). A variety of bacterial species have been identified as capable of degrading arsenic, including *Herminiimonas arsenicoxydans*, and many other species, such as *Bacillus, Delftia, Firmicutes, Pseudomonas, Acinetobacter, Stenotrophomonas, Crenarchaea,* *Agrobacterium, Achromobacter, Citrobacter, Clostridium, Rhodobium, Cyanobacteria* and *Methanobacterium* (Mengmeng et al., 2018; Reid et al., 2020).

2.5.1 Reduction of Arsenate [As(V)]

To eliminate arsenate As(V) from their surroundings, arsenate-reducing bacteria employ various reduction systems, transforming it into arsenite and expelling it from the cell. One such mechanism is using a cytoplasmic arsenate reduction system (ArsC). When arsenate enters the bacterial cell through phosphate transporters (Pit or Pst), ArsC enzyme reduces arsenate to arsenite using ferredoxin or glutathione as an electron donor. This conversion of arsenate to arsenite is followed by the expulsion of As(III) from the cell through an ArsB efflux pump. Bacteria employ two different systems for reducing As(V): the cytoplasmic As(V) reduction system mediated by ArsC and the periplasmic respiratory As(V) reduction system mediated by Arr proteins (Mengmeng et al., 2018). The latter is also referred to as dissimilatory As(V) reduction and is utilized by dissimilatory As(V)-reducing prokaryotes (DARPs). These bacteria can use a variety of electron donors for As(V) reduction, including inorganic hydrogen, as well as sulfide (Mengmeng et al., 2018), organic acids, sugars, ethanol, and complex aromatic compounds (Brown et al., 2018). Examples of As(V) reducing strains *Alkaliphilus oremlandii, Chrysiogenes arsenates,* and *Bacillus selenitireducens* (Mengmeng et al., 2018; Mondal et al., 2021).

Certain bacteria may have an advantage over others when they possess an ATPase called ArsA that is bound to ArsB for the extrusion of arsenite (Escudero et al., 2013; Firrincieli et al., 2019). Another mechanism of arsenate reduction is when some bacteria use As(V) as a terminal electron acceptor during anaerobic respiration. These bacteria are called dissimilatory arsenate respiring prokaryotes (DARPS) and are phylogenetically diverse, including members such as *Chrysiogenes arsenatis*,