Chapter II:

LITERATURE REVIEW

2.1 General

Conventional techniques employed for remediation of heavy metal(loid)s often suffer from demerits of excessive operation and maintenance costs, sludge generation and sometimes production of secondary pollutants (Leong and Chang, 2020). Also, at low concentrations of heavy metal(loid)s, most of these techniques are not very effective. To counter these limitations, microorganisms and plants, either separately or in combination, have been used for treatment of heavy metal(loid)s. Microorganisms such as algae, bacteria and fungi have developed means for biosorption as an effective method for detoxification of heavy metal(loid)s (Vaid et al., 2022). Charged functional groups at the surface of microbial cell walls bind with the metal(loid)s and play an important role in biosorption. Apart from biosorption, bioaccumulation, biotransformation and biovolatilization are other mechanisms of detoxification in microbes.

Plants use capping, degradation, extraction, filtration, stabilization, and volatilization to remove, degrade or immobilize heavy metal(loid)s from contaminated soil, water or sediments. The mobility and phytoavailability of heavy metal(loid)s are influenced by extractable forms rather than to the total metal contents (Singh and Kalamdhad, 2013). Based on the capacity of plant, it can accumulate various heavy metal(loid)s in different parts (flower, fruit, leaves, roots, stem) (Gavrilescu, 2022). Plants that grow on contaminated soils already have tolerance to heavy metal(loid)s. Some higher plants capable of accumulating 10-500 times more heavy metal(loid)s in their above-ground parts, compared to regular plants, are called hyperaccumulators (Skuza et al., 2022). *Brassicaceae*, *Euphorbiaceae*, *Asteraceae*, *Fabaceae*, *Lamiaceae* and *Scrophulariaceae* are some of the plant families that are recognized as good hyperaccumulators (Gavrilescu, 2022).

2.2 Bioremediation of arsenic

Bioremediation of arsenic is well documented (Irshad et al., 2021); however, arsenic remediation using microbes has not been fully recognized and the molecular mechanisms involved are still quite fragmented and need to be properly organized (Yin et al., 2022). Over the decades, diverse microorganisms—ranging from bacteria and fungi to archaea—have been reported to facilitate the bioremediation of arsenic-contaminated environments.

2.2.1 Algae

The use of algae for bioremediation of arsenic and other heavy metal(loid)s has been very well-understood and documented. Adsorption, oxidation-reduction and thiol complexation are some of the detoxification mechanisms of arsenic carried out by algae (Hussain et al.,

2021). Different algal species have varying arsenic removal capacities depending on arsenic species, pH, temperature and exposure duration. Algal species such as *Chlamydomonas reinhardtii* (Miyashita et al., 2011), *Closterium aciculare* (Hasegawa et al., 2001), *Chattonella antiqua* (Yamaoka et al., 1996), among others, have been known to remove various arsenic species. As(V) enters algal cells through phosphate transporters whereas adsorption of As(III) is carried out through the plasma membrane (Mitra et al., 2017). However, increased concentrations of PO_4^{3-} have been reported to inhibit the As(V) intake in algae (Guo et al., 2011). Table 2.1 lists various algal species with the potential for arsenic bioremediation.

Table 2.1: Arsenic remediation ability of some algae

Algal species	Mechanism	Salient features	Reference(s)
Lessonia nigrescens	Biosorption	Maximum As(V) biosorption of 45.2 mg/g	Hansen et al. (2006)
Ulothrix cylindricum	Biosorption	Maximum As(III) biosorption of 67.2 mg/g	Tuzen et al. (2009)
Maugeotia genuflexa	Biosorption	Maximum As(III) biosorption of 57.48 mg/g	Sari et al. (2011)
Synechocysis sp.	Bioaccumulation Biotransformation	Accumulated 1 mg/g As(V) and 0.9 mg/g As(III)	Yin et al. (2012)
Scenedesmus sp.	Bioaccumulation Biotransformation	Could oxidize As(III) to As(V); First report of biotransformation of arsenic by a soil alga; Accumulated 606 µg/g As(III) and 761 µg/g As(V)	Bahar et al. (2013b)
Biochar made from *Gracilaria* waste and *Oedogonium*	Biosorption	Arsenic biosorption ranged between 62.5–80.7 mg/g; Could also remove selenium and molybdenum	Johansson et al. (2016)
Algal (*Laminaria digitata*)/ polyethyleneimine composite beads	Biosorption	Maximum As(V) biosorption of 100 mg/g	Hamza et al. (2020)
Leptolyngbya boryana	Bioaccumulation	Accumulated 570 mg/kg As(III) and 268.5 mg/kg As(V)	Zhu et al. (2020)
Chlorella vulgaris	Bioaccumulation	Maximum adsorptive rate of arsenic (III) was 55 mg/gm	Alharbi et al. (2023)
Spirogyra sp.	Biosorption	Could remove 14605 µg/g/h As(III) and 6511 µg/g/h As(V)	Liu et al. (2024)

2.2.2 Bacteria

Bacteria are amongst the most effective microorganisms employed for arsenic bioremediation (William and Magpantay, 2024). Various mechanisms adopted for arsenic removal by bacteria involve biotransformation, bioaccumulation, biomethylation, biosorption, etc. (Irshad et al., 2021). Bacteria such as *Bascillus* sp. Bas1 has been known to oxidize As(III) to As(V) and can resist up to 1000 mM of As(V) (Biswas and Sarkar, 2018). Species such as *Bascillus*, *Achromobacter*, *Brevundimonas* and *Microbacterium* could oxidize arsenic and also exhibit plant growth-promoting traits (Cavalca et al., 2010). However, species like *Bacillus* strain As-12 can reduce As(V) to As(III) (Jebelli et al., 2018). As(III) is more toxic and difficult to remove as compared to As(V) and this antagonistic action prohibits bacteria from being used as a universal solution to arsenic bioremediation. Although there is a continuous increase in knowledge of bacteria used for arsenic remediation, the processes involved in arsenic oxidation need to be studied for their practical applications.

2.2.3 Fungi and yeast

Many studies have reported the potential use of fungi for arsenic bioremediation. Mechanisms involved in arsenic remediation by fungal species include biovolatilization, biotransformation and biosorption. Fungal biomass of *Aspergillus niger* coated with iron oxide has been found to remove nearly 95% of As(V) and 75% of As(III) from aqueous solutions (Pokhrel and Viraraghavan, 2006). Vala (2010) reported the facultative marine fungus *Aspergillus candidus* as a potential candidate for arsenic removal and found that removal for As(V) was higher than for As(III). Arsenic resistance in yeasts is found due to the presence of a set of genes namely, ACR1 (putative transcription factor), ACR2 (arsenate reductase) and ACR3 (plasma membrane As(III)-efflux transporter) (Kumar et al., 2020). The *WarsM* genes present in yeasts have been found to be responsible for better arsenic resistance through biovolatalization (Verma et al., 2019). Table 2.2 lists some of the yeast and fungal species used for bioremediation of arsenic.

2.2.4 Phytoremediation

Phytoremediation is the use of green plants to make pollutants harmless to the environment (Hansda et al., 2014). Different ways in which phytoremediation is carried out are phytodegradation (removal by degradation of pollutants), phytoextraction (removal of pollutants by accumulation), phytostabilization (reducing the bioavailability of pollutants), phytovolatilization (volatilization of pollutants), rizhofiltration (absorption and adsorption of pollutants by plant roots) (Kumar et al., 2022). Plants generally do not take trace

elements in quantities more than what is required for their metabolism, which is around 10-15 ppm for most of the trace elements with the exceptions being the hyperaccumulators (plants that can take up to 1000 ppm) (Tangahu et al., 2011; Mateo et al., 2019).

Table 2.2: Yeasts and arsenic resistant fungi reported from arsenic-contaminated areas

Yeast/Fungal Species	Mechanism	Salient features	Reference(s)
Tea fungus (symbiont of two yeasts *Pichia* and *Zygosaccharomyces* sp. and a bacterium *Acetobacter* sp)	Biosorption	Removed 100% of As(III) and Fe(II) after 30 min and 77% of As(V) after 90 min	Murugesan et al. (2006)
Fungi belonging to genera *Aspergillus; Trichoderma; Penicillium; Rhizopus; Sordaria; Neocosmospora*	Biovolatalization	Five fungal strains were resistant at 10,000 mg/L of As(V); Flux of biovolatilized arsenic ranged from 3.71 to 29.86%	Srivastava et al. (2011)
Aspergillus oryzae; *Fusarium* sp; *Aspergillus nidulans*; *Rhizomucor variabilis*; *Emericella* sp	Biovolatilization	Bioaccumulated 0·023 to 0·259 g/kg arsenic and biovolatalized 0·23 to 6·4 mg/kg	Singh et al. (2015)
Westerdykella sp. (FNBR_3); *Trichoderma* sp. (FNBR_6); *Lasiodiplodia* sp. (FNBR_13); *Rhizopus* sp. (FNBR_19)	Biosorption	Maximum adsorption of 70 mg/g for FNBR_3, 68 mg/g for FNBR_6, 113 mg/g for FNBR_13 and 90 mg/g for FNBR_19	Jaiswal et al. (2018)
Westerdykella aurantiaca, Saccharomyces cerevisiae	Biomethylation Biovolatalization	Yeasts significantly increased plant growth and arsenic accumulation	Verma et al. (2019)
Humicola sp.	Biovolatilization	Arsenic accumulation ranged between 0.146 to 11.36 g/kg biomass and volatilization between 0.05 to 53.39 mg/kg	Tripathi et al. (2020)
Rhizopus microsporus Os4	Biosorption	Resistant up to 15,000 mg/L of As(V) Could reduce 90% As(V) with initial concentration of 7000 mg/L	Flores-Amaro et al. (2024)
Pleurotus pulmonarius MT	Bioaccumulation	Maximum tolerance of 640 mg/L for Cd and 1280 mg/L for As(III)	Zhang et al. (2023)

Hyperaccumulation of arsenic was first reported in a fern, *Pteris vittata,* which could resist as much as 1500 μg/g of arsenic in soil (Ma et al., 2001). Plants act both as accumulators (accumulate the contaminant and then biodegrade/biotransform them) and excluders (restrict the uptake of contaminants) (Tangahu et al., 2011). Table 2.3 lists some of the advancements in the field of phytobial remediation of arsenic. There are some shortcomings of phytoremediation which makes its use limited to some extent. Treatment zone depth, in many cases, is shallow (FRTR, 2020). The method is seasonal, slow and also not applicable to all compounds (Kamusoko and Jingura, 2017). The complexity of the interrelated factors makes the optimization of phytoremediation a difficult process.

It is evident that arsenic is removed mainly through sorption, co-precipitation and precipitation. Direct uptake of arsenic by plants plays a very minor role in CWs. Zhang et al. (2017) established that most of the arsenic was retained in sediments or media, and more than 80% was retained in a solid medium, which suggested that arsenic uptake by plants was almost negligible. Removal efficacy and translocation of arsenic also depend on the type of plants present therein. Bedding material also plays an important part in arsenic removal in constructed wetlands. Buddhawong et al. (2005) found that arsenic was completely removed with gravel as bedding material, with the rate of removal being faster in subsurface flow wetlands than in surface flow wetlands. Manganese sand has been found to have a higher As(V) adsorption rate as bedding material compared to gravel, ceramsite and zeolite (Wu et al., 2014). Arsenic retention in peatland has also been found to be good (as high as 95%) but can vary sometimes (Palmer et al., 2015).

2.3 Arsenic removal using bacteria

2.3.1 Arsenic uptake and efflux pathways

The absence of an uptake system specifically for arsenic in any organism is due to the fact that it is not required for the sustenance of life. However, all organisms, ranging from bacteria to humans, have an arsenic detoxification pathway and extrusion from cells or sequestration is the major pathway (Fekih et al. 2018). Arsenic uptake is facilitated due to its molecular similarity with other substrates such as glucose, glycerol [similar to As(III)] and phosphate [similar to As(V)] (Satyapal et al. 2016; Urrialde et al. 2017). As(III) transport across membranes is facilitated by glycerol channel GlpF (glycerol uptake facilitator), first identified in 1917 in *Escherichia coli* (Garbinski et al., 2019). But in some microorganisms such as *Thiomonas* sp. 3As and *Herminiimonas arsenicoxydans*, no GlpF or homologous genes have been identified through which As(III) uptake is carried out (Kruger et al., 2013).

Table 2.3: Recent advances in phytoremediation of arsenic

Plant Species	Mechanism	Salient features	Reference(s)
Pteris vittata	Bioaccumulation	Accumulated 15861 µg/g of arsenic in 2 weeks and 22630 µg/g in 6 weeks Accumulated up to 6000 µg/g of arsenic Accumulated 149–694 mg/kg of arsenic	Ma et al. (2001) Visoottiviseth et al. (2002); Wei and Chen (2006)
Asparagus setaceous; Lolium temulentum	Bioaccumulation	Accumulated more than 1100 ppm of arsenic, but1400 ppm when assisted with trans-1, 2-cyclohexylenedinitrilotetraacetic acid	Bagga and Peterson (2001)
Pityrogramma calomelanos	Bioaccumulation	Accumulated up to 8000 µg/g of arsenic	Visoottiviseth et al. (2002)
	Phytostabilization	Phosphorous fertilizer and rhizosphere bacteria enhanced arsenic phytoextraction	Jankong et al. (2007)
Pteris cretica	Bioaccumulation	Arsenic accumulation ranged between 3–704 mg/kg	Wei and Chen (2006)
Arundo donax	Phytoextraction	Can treat contaminated waters containing arsenic up to 600 µg/L	Mirza et al. (2010)
Melastoma malabathricum L	Phytoextraction	Accumulated 2800 mg/kg of arsenic in roots and 570 mg/kg in stems and leaves	Selamat et al. (2014)
Brassica napus	Phytoextraction	Accumulated 35–45 and 81–132 mg/kg of arsenic in shoot and root	Niazi et al. (2017)
Brassica juncea		Accumulated 43–60 and 90–129 mg/kg of arsenic in shoot and root	
Eichhornia crassipes	Bioaccumulation	Accumulated up to 498.4 mg/kg of arsenic	de Souza et al. (2018)
Lemna Valdiviana	Bioaccumulation	Accumulated 1190 mg/kg arsenic under optimized conditions	de Souza et al. (2019)
Pteridium Aquilinum; Nymphaea Maculata	Bioaccumulation and translocation	Accumulated arsenic in root (910 mg/kg), shoot (798.2 mg/kg) and soil (960 mg/kg)	Onyia et al. (2020)
Acacia mangium	Bioconcentration and translocation	Could tolerate up to 790 mg/kg of arsenic	Rosli et al. (2021)
Arundo donax L.	Bioaccumulation	Maximum accumulation of 246 mg/kg without inducing any toxicity	Zeng et al. (2023)

As(V) uptake, as first observed in *E. coli*, is carried out through two systems, *Pst* (the high-affinity system) and *Pit* (the low-affinity system) (Yang et al., 2012). While *Pit* is the primary pathway, at higher concentrations, arsenic also enters through *Pst*. ArsB is the first identified As(III) efflux system in bacteria and is most widely employed, followed by Acr3 (Garbinski et al., 2019). Other mechanisms identified are through the synergistic effect of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ArsJ genes for As(V); ArsP for transportation of organic As(V), first identified in *Campylobacter jejuni*; and ArsK, identified in the chromosomes of *Agrobacterium tumefaciens* GW4 and a *Bacillus* sp. strain (Garbinski et al., 2019). Figure 2.1 shows the arsenic uptake and efflux pathways in bacteria. As(V) is reduced to As(III) through ArsC or is extruded through the synergistic effect of GAPDH and ArsJ. As(III) is either methylated through ArsM and then extruded by ArsP or is directly extruded by the action of genes like ArsB, Acr3 and ArsK.

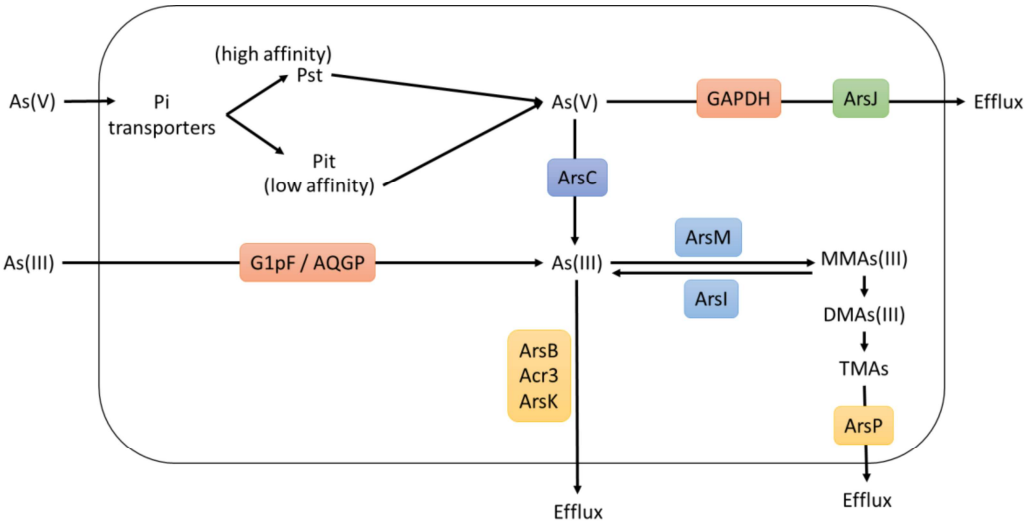


Figure 2.1: Mechanism of arsenic transport in bacteria [adopted and modified from: Garbinski et al. (2019); Hayat et al. (2017); Kruger et al. (2013)]

2.3.2 Genes involved in arsenic metabolism

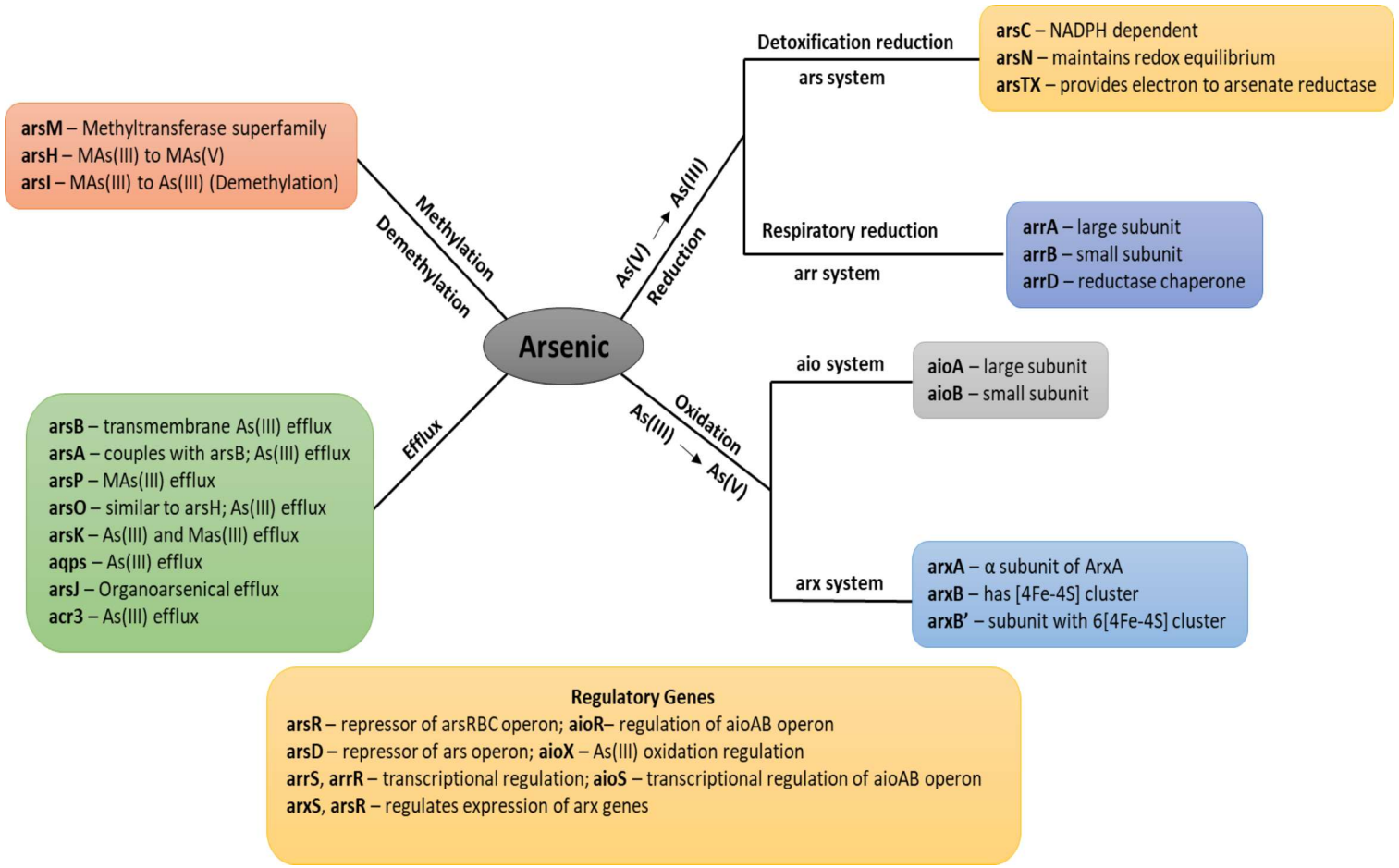
Arsenic resistance in bacteria is attributed to the gene products of *ars* operons. The *ars* operons are located either on plasmids or in chromosomes (Fekih et al. 2018). The three-gene operon arsRBC are the most common in the *E. coli* genome and extended five-gene operon arsRDABC (speculated to be formed by the insertion of *arsDA* in *arsRBC*) in *E. coli* plasmid R773 (Rosen, 2002). As(III) oxidation to As(V) is carried out by *aio/arx* systems, whereas As(V) reduction to As(III) is attributed to *arr* systems (Yan et al., 2019). As(III) oxidase, *aioAB* operon was first discovered in *Alcaligenes faecalis* and is widely distributed in bacteria (Silver and Phung, 2005).

AioS in *Agrobacterium tumefaciens* (Kashyap et al., 2006) and AioR in *Herminiimonas arsenicoxydans* (Koechler et al., 2010) were found to be vital for As(III) oxidation. ArxA has the ability to both reduce As(V) and oxidize As(III) (Yan et al., 2019). ArxB, ArxC and ArxD belong to the same Arx system. The detoxification of As(III) is achieved by its extrusion from the cell by ArsB enzymes encoded by the *arsB* gene. ArsA, encoded by *arsA* gene, provides increased resistance to the As(III) transport system by forming a tight membrane-bound complex (Yan et al., 2019). ArsD has been revealed to act as an arsenic chaperon that transfers As(III) to ArsA binding sites (Satyapal et al., 2016).

ArsR, encoded by *arsR* gene, acts as a transcriptional repressor responsible for the negative regulation of transcription of other genes of *ars* operons (Silver and Phung, 2005). Cytoplasmic As(V) is reduced by ArsC and is effluxed through ArsB, Acr3, or ArsAB complex (Rosen, 2002). The respiratory reductase of As(V) consists of a larger subunit, ArrA, and a smaller subunit ArrB (Afkar et al., 2003). Another As(III) oxidase family of genes is *aso* and AsoA, AsoB, AsoAB complexes belong to this family of genes. *asoA* is homologous to *aoxB* in *Alcaligenes faecalis* and *asoB* is identical to *aoxA* (Satyapal et al., 2016). Figure 2.2 illustrates various genes that are involved in arsenic metabolism by bacteria.

2.3.3 Arsenic metabolism

Due to continuous exposure over a period of time, bacteria have developed several metabolic pathways to counter the toxic effect of arsenic. Despite its toxicity, various bacteria use arsenic either as an electron donor or an acceptor to fuel their metabolism (Kruger et al., 2013). The arsenic reductase enzyme was absent during the formation of the earth but developed over time when the environment became reducing (Kabiraj et al., 2022). Arsenic metabolism mainly involves four mechanisms, namely oxidation, reduction, methylation and demethylation (Kabiraj et al., 2022). Various bacteria involved in oxidation, reduction, or methylation of arsenic have been well documented (Crognale et al. 2017; Kalimuthu et al. 2014; Ye et al. 2012). These mechanisms are also decontamination pathways for arsenic and have been discussed in detail below.



[*NADPH - Nicotinamide Adenine Dinucleotide Phosphate; MAs – Methylated Arsenics]

Figure 2.2: Genes involved in arsenic metabolism in bacteria [adopted and modified from Singh et al. (2021)]

2.3.3.1 Oxidation

Arsenic removal through oxidation of As(III) to As(V) is much easier as As(V) is more easily adsorbed on adsorbents as compared to As(III) (Ike et al., 2008). The first As(III) oxidizing bacterial strain was identified in 1918, but the finding finally came into the mainstream in 1949 when 15 strains of As(III) oxidizing bacteria were isolated by Turner (Yamamura and Amachi, 2014). Currently, many different strains have been identified which include both autotrophs and heterotrophs. Autotrophs like *Thiomonas arsenivorans* (Kruger et al., 2013), *Rhizobium* sp. NT-26 (Shi et al., 2020) have the ability to use As(III) as their only energy source, whereas heterotrophs can use As(III) as an auxiliary source as well and some heterotrophs simply oxidize As(III) to As(V) for detoxification (Ehrlich, 2002). As(III) oxidizing *Agrobacterium*/*Rhizobium* grow chemoautotrophically by using As(III) as an electron donor, whereas *Alcaligenes* sp. and *Agrobacterium albertimagni* detoxify As(III) heterotrophically (Macur et al., 2004). Aox operons are responsible for As(III) oxidizing ability of bacteria, whose expression is, in turn, controlled by AoxR. The *aioAB* operon, first discovered in *Alcaligenes faecalis* helps in catalyzing the oxidation of As(III) to As(V) (Yan et al., 2019).

It was then later discovered in β-proteobacterial strain ULPAs1, which shares similar strain sequences to *Alcaligenes facealis* (Muller et al., 2003). Genes similar to *aioA* have been identified from soils and sediments containing arsenic and other arsenic-rich environments such as mines, smelters and geothermal sites (Yamamura and Amachi, 2014). In both autotrophs and heterotrophs, the As(III) oxidase enzyme has been found to contain two subunits, one containing a molybdopterin center and a [3Fee4S] cluster, the larger subunit, and the other, a Rieske [2Fee2S] cluster, the smaller subunit (Ellis et al., 2001). These subunits were earlier known by different names (*aoxB-aoxA/aroA-aroB/asoA-asoB*) but have now been unified under the same name aio, *aioA* for larger subunits and *aioB* for smaller subunits (Lett et al., 2012).

Apart from these genes, in some bacteria, a Na^+/H^+ antiporter, a molybdate transporter, and the heat shock protein DnaJ have been reported to be responsible for As(III) oxidation (Kruger et al., 2013). As(III) oxidation can be carried out through both aerobic and anaerobic oxidizers but some cases of facultative oxidizers have also been reported. *Ancylobacter* strain OL-1, *Thiobacillus* strain S-1, *Hydrogenophaga* strain CL-3 (Garcia-Dominguez et al., 2008) oxidize As(III) aerobically, *Alkalilimnicola ehrlichii* sp. is a heterotrophic oxidizer (Yamamura and Amachi, 2014) whereas *Paracoccus niistensis* strain SY is a facultative oxidizer (Zhang et al., 2015) which uses O_2 or NO_3^- as electron acceptors under aerobic and anaerobic conditions, respectively. *Sinorhizobium* sp. KGO-5

strain was found to possess *aioA* gene similar to AioA of *Ensifer adhaerens* suggesting that KGO-5 is a facultative As(III) oxidizer (Dong et al., 2014). Many plant growth-promoting bacteria have been found to be capable of oxidizing As(III) to As(V), but the underlying genetic mechanism has not been fully understood yet and requires further investigation (Mondal et al., 2021). The class of the oxidizing bacteria is very diverse which includes both chemolithoautotrophs (CLA) and heterotrophs. Heterotrophs adopt a detoxification mechanism, whereas CLA uses As(III) as an electron donor, oxygen as an electron acceptor and CO_2 as a carbon source (Bahar et al., 2013a). This redox behavior (acceptance and donation of electrons) results in generation of bio-energy when metabolized (Rhine et al., 2007), which can be used in construction of microbial fuel cells in heavily contaminated areas (Irshad et al., 2021).

2.3.3.2 Reduction

As(V) is reduced by a variety of bacteria through detoxification mechanisms. The reduction of arsenic from a less toxic state [As(V)] to a more toxic state [As(III)] seems to be counterintuitive. But, since, As(V) oxyanions are very closely similar to phosphate oxyanions, the bacterial cells may fail to distinguish between them and absorb As(V) (Silver et al., 2001). As(V) enters bacterial cells through *Pit* and *Pst* phosphate transporters (Figure 2.1). Once inside the bacterial cell, As(V) is reduced to As(III) either through ArsC, which leads to extrusion of As(III) through ArsB, Arsk, Acr3 (detoxification) (Figure 2.1) or through arsenate reductase, ArrA (dissimilatory reduction) (Kruger et al., 2013). MIT13 was the first arsenic-respiring strain reported, which was later named as *Geospirillum arsenophilus* (Páez-Espino et al., 2009). As(V) respiring bacteria have been isolated from different phylogenetic groups belonging to gram-positive, β-, γ- and ε-proteobacteria and *chrysiogenes arsenatis* suggesting their widespread presence in the bacterial domain (Kruger et al., 2013).

Sulfurospirillum barnesii (Luijten et al., 2003), *Bacillus beveridgei* (Baesman et al., 2009), *Desulfitobacterium hafniense* DCB-2 (Niggemyer et al., 2001), *Shewanella* sp. ANA-3 (Saltikov et al., 2003), *Geobacter* sp. (Lovley et al., 2011), *Anaeromyxobacter dehalogenans* (Sanford et al., 2002) are some of the species of dissimilatory As(V) reducing prokaryotes. *Bacillus selenatarsenatis* strain SF-1 of the *Bacillus* sp. can release arsenic from iron and aluminium (hydro)oxides co-precipitated with As(V), whereas *Shewanella* sp. ANA-3 can use NO_3^-, Fe(III), Mn(IV), fumarate, thiosulfate, and oxygen as electron acceptors (Yamamura and Amachi, 2014). *Desulfovibrio* strain Ben-RA and *Desulfomicrobium* strain Ben-RB, can reduce both As(V) and sulfate (Macy et al., 2000). The strain Ben-RA reduces As(V) using a reductase similar to ArsC, but the nature of the

electron donor involved in the process is not known (Macy et al., 2000). Strain Ben-RB uses lactate as an electron acceptor, while the reduction of As(V) occurs through a membrane-bound enzyme (Macy and Joanne, 2002).

2.3.3.3 Methylation and demethylation

Methylation of arsenic is a prevalent phenomenon, reported in bacteria as well as in other higher organisms. Arsenic methylation was first demonstrated by bacteria in 1971 (Bentley and Chasteen, 2002). Microorganisms (bacteria, yeast and fungi) form both volatile (such as methylarsines) and non-volatile (such as methylarsonic acid and dimethylarsinic acid) compounds on arsenic methylation (Bentley and Chasteen, 2002). Methylation of arsenic results in the formation of methylarsenite [MAs(III)], arsenosugars, arsenobetaine, arsinothricin, and methylarsenical antibiotics (Chen and Rosen, 2020). In prokaryotes, the methylation pathways are the same as those described for fungus *Scopulariopsis brevicaulis* (Páez-Espino et al., 2009).

Huang et al. (2016) showed that *Cytophagaceae* strain SM-1 transformed all of As(III) to dimethyl arsenate and TMAO along with the production of trimethylarsine (TMA) gas, possibly attributing to an efficient ArArsM enzyme coupled with low As(III) efflux. The presence of TMA as nearly half of the total arsenic suggests that the increased toxicity of some intermediates can be outweighed by the increased volatility of methylated arsenicals. Methylation and subsequent formation of MMA(V), DMA(V), TMA(V) and MMA(III), DMA(III), TMA(III) involve alternative reduction and oxidation and S-adenosylmethionine (SAM) serves as a donor of methyl group and SAM gets converted to S-adenosylhomocysteine (SAH) (Dombrowski et al., 2005; Páez-Espino et al., 2009). The ArsM gene is widespread in microbes, while in humans, the AS3MT enzyme is responsible for arsenic methylation (Palmgren et al., 2017).

DMA(V) and TMAO have been detected in higher plants (Meharg and Hartley-Whitaker, 2002), but no ArsM orthologs have been found in plants (Heitkemper et al., 2001). To complete the geochemical cycle of arsenic, demethylation of methylarsenicals is necessary. Demethylation is faster under oxic conditions but can occur under both oxic and anoxic conditions (Huang, 2014). Methylated arsine in the atmosphere undergoes rapid photooxidative degradation, but the demethylation of arsenic present in the air through bacteria still needs to be investigated (Huang, 2014). Some bacterial species, such as *Burkholderia* and *Streptomyces* (Yoshinaga et al., 2011), have the ability for demethylation. Demethylation is also possibly responsible for the reduced efficiency of volatilization. However, the complete pathway and molecules involved in the pathway have not been fully understood.

2.3.4 Genetically modified bacteria

Genetic modifications in the field of biotechnology have been used for arsenic remediation. It has been used to enhance arsenic volatilization in the case of bacteria and fungi (Irshad et al., 2021). Since the first As(III) oxidizing bacterium genome of *Herminiimonas arsenicoxydans* (Muller et al., 2007), many more genomes have been sequenced. Because of the efficient *ars* efflux system, bacteria accumulate significantly less arsenic (Páez-Espino et al., 2009); however, genomics present an appealing possibility to enhance the arsenic remediation ability of bacteria. Arsenic accumulation in bacteria is achieved by three strategies: through the expression of arsenic-chelating proteins, by expressing membrane channels or porins and through the removal of genes responsible for As(III) efflux (Villadangos et al., 2014). A combination of these strategies can result in higher arsenic accumulation in bacterial cells. Phytochelatin (PC) synthase of *A. thaliana* has been expressed in *E. coli* successfully, which resulted in a 50-fold increase in arsenic accumulation (Sauge-Merle et al., 2003). PC synthase overexpression in plants also optimizes their metal-binding ability and helps in phytoremediation.

Genetically modified strains of *Corynebacterium glutamicum* showed 30 and 15-fold increases in As(V) and As(III) accumulation, respectively, but also resulted in lower arsenic resistance (Villadangos et al., 2014). Overexpression of As(III) uptake porins enhanced arsenic accumulation, not chelators. Expression of metallothionein with As(III) specific transporter GlpF in *E. coli* cells from marine alga *Fucus vesiculosus* has shown 30 fold increase for As(III) and 26 fold increase for As(V) accumulation (Singh et al., 2008). Genetically enhanced bacteria also have the possibility of arsenic removal through phytoremediation. Sizova et al. (2002) found that inoculation of sorghum with genetically modified *Pseudomonas* sp., including strains with arsenic-resistant plasmid, pBS3031, enhanced the arsenic extraction by the plants but impaired the plant growth. While *Pseudomonas aureofaciens* BS1393 from the rhizosphere has been developed to resist high arsenic content and dissolve arsenic and increase the arsenic accumulation ability of *Sorghum saccharatum* L. and also helps in its survival (Sizova et al., 2004). Expression of *arsM* gene in genetically engineered bacteria has resulted in volatilization of arsenic and a many-fold increase in methylation of arsenic (Singh et al., 2011).

ArsR, because of its high affinity and selectivity towards As(III), has been found to increase arsenic accumulation (13-60 fold) when overexpressed in *E. coli* (Singh et al., 2011), but *arsR* also competes with the extrusion system for available As(III). Generally, the genomics approach may help to improve the understanding of microbial metabolism. It may also give insight into the evolution and diversity of the enzymes and may result in the

development of an arsenic-specific removal mechanism. However, as a result of the horizontal transfer of recombinants, the genetically modified bacteria used for the treatment of polluted sites can have adverse effects on the environment and also face the challenge of long-term stability (Perpetuo et al., 2011). Figure 2.3 represents a simple schematic diagram of different mechanisms involved in bioremediation of arsenic through bacteria. Table 2.4 lists various bacterial species responsible for bioremediation of arsenic and mechanisms involved in the process.

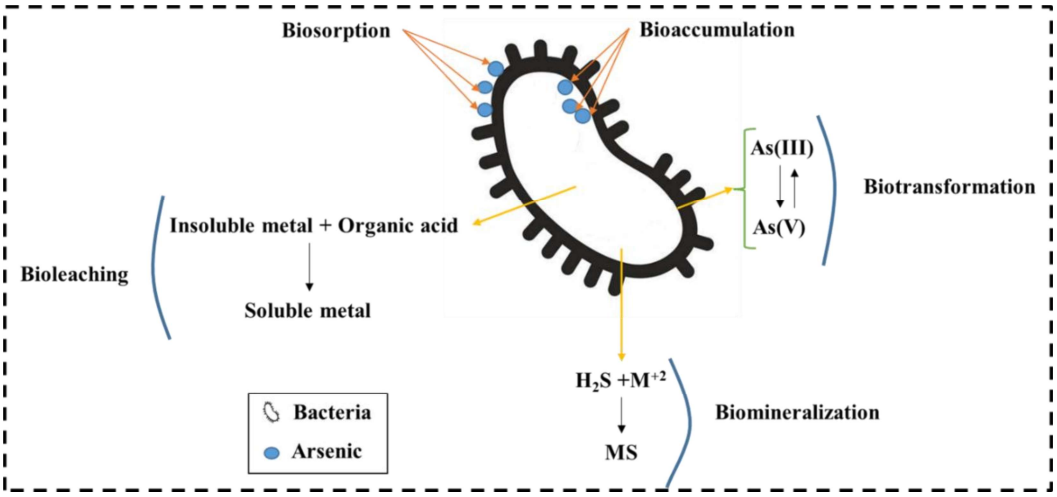


Figure 2.3: Simplified schematic diagram of different mechanisms involved in bacterial remediation of arsenic

2.4 Use of immobilized bacteria for arsenic removal

Bioremediation can be carried out either through a free cell (suspension) or through an immobilized cell (attached) system (Swain et al., 2020). Various studies by previous researchers have found that immobilized cells perform better than free cells in removing heavy metal(loid)s and several other pollutants (Al-Hasawi et al., 2020; Liu et al., 2018). Unlike free cells, immobilized cells offer greater advantages by providing low hydraulic retention time and higher biomass concentration availability per unit carrier volume (Hsu et al., 2010). Immobilization of bacteria also prevents decrease in catalytic activity, stability, and selectivity (Vikrant et al., 2018). Immobilization can be carried out either on surface or within a carrier matrix (Girijan and Kumar, 2019; Kiran et al., 2007). Biochar is one of the most common materials used for immobilization of bacteria. It has also been one of the most preferred adsorbents due to its high sorption capacity and surface functional groups (Pan et al., 2021; Zhang et al., 2013). Modification of biochar can result in increased specific surface area and increased functional groups, resulting in enhanced removal efficiency by multiple folds (Sun et al., 2022).

Table 2.4: Different bacteria used for bioremediation of arsenic

Species	Mechanism	Salient features	Location	Reference(s)
Sulfolobus BC	Bioleaching	Resistant up to 600 mg/L As(III) and 1000 mg/L As(V)	-	Escobar et al. (2000)
Sporosarcina ginsengisoli CR5	Biomineralization	High As(III) tolerance of up to 50 mM	Arsenic contaminated site near Urumqi, China	Achal et al. (2012)
Rhizobium; Ochrobactrum; Achromobacter	Biotransformation	Highly resistant to As(III), As(V), Cr(VI), Cu(II), Co(II), Ni(II), Zn(II), Cd(II), Hg(II)	Kolsure village, North 24 Parganas, West Bengal, India	Sarkar et al. (2013)
Leptospirillum sp.; *Sulfobacillus* sp.	Bioleaching	Resulted in 92.6% extraction of arsenic after 12 d	Dexing copper mine, Jiangxi Province, China	Xie et al. (2013)
Acidithiobacillus thiooxidans; Acidithiobacillus ferrooxidans	Bioleaching	Mixed culture showed higher efficiency on bioleaching of copper and arsenic from mine tailings after 600 h of incubation	Dizon Mine located in San Marcelino, Luzon, Philippine.	Nguyen et al. (2015)
Exiguobacterium (As-9)	Biotransformation	Resistant to high levels of As(V) (700 mM) and As(III) (180 mM) and removed 99% arsenic in 10 d	Rajnandgaon district, Chhattisgarh, India	Pandey and Bhatt (2015)
Bacillus megaterium Strain SS9; *Lysinibacillus* sp. Strain SS11	Bioaccumulation	Bioaccumulation capacity of 23.43 mg/L for As(V) and 5.65 mg/L for As(III)	Bengal Delta Sediments	Singh et al. (2015)
Bacillus arsenicus MTCC 4380	Bioaccumulation	Could accumulate up to 79.4% As(III) and 85.03% As(V)	Purchased from Microbial Type Culture Collection and Gene Bank (Chandigarh, India)	Podder and Majumder (2016)

Burkholderia sp. Z-90	Bioleaching	Can remove multiple heavy metals including Mn, Zn, Cd, Cu, As, Pb	Soil in the vicinity of a smelter in southern China	Yang et al. (2016)
Acidithiobacillus ferrooxidans BY3	Biotransformation	10.07 fold increase in As(III) oxidation in presence of Fe^{2+} ions	Baiyin of Gansu, China	Gao et al. (2018)
Klebsiella pneumoniae strain SSSW7	Bioaccumulation; Biotransformation	Could oxidize 10 mM of As(III) to As(V) within 24 h	Shipyard waste, from Bicholim, Goa, India	Mujawar et al. (2019)
Azospirillum brasilense Cd	Biotransformation	Arsenic resistance genes mediate the redox As transformation	-	Vezza et al. (2020)
Shewanella sp. O23S	Biomineralization	Reduction yields: SeO_3^{2-} (90%), AsO_4^{3-} (60%), and SeO_4^{2-} (<10%)	Former gold mine in Złoty Stok (SW Poland)	Staicu et al. (2022)
Enterobacter Cloacae RSC3	Biotransformation	Tolerated arsenic up to 6000 ppm and could transform 50% of As(III) to As(V) in 12 h	Industrial area near Delhi NCR	Bhati et al. (2022)
Lactobacillus reuteri HS12	Biosorption	Could remove Cd (98%), Pb (97%) and Cu (56%)	Human and albino mice fecal samples	Bhakta et al. (2022)
Lysinibacillus sp.*; Bacillus safensis*	Bioaccumulation	Arsenic removal between 30-40% with an initial concentration of 50 ppm	Asanpara village, Murshidabad, West Bengal, India	Sun et al. (2022)
Chlamydomonas sp.	Biosorption	Maximum removal of 95.2% and biosorption capacity 53.8 mg/g of arsenic	Phycological Laboratory, Faculty of Science, Beni-Suef University	Mohamed et al. (2022)
Bacillus pacificus strain AKS1a	Biosorption	Resistant up to 20 mM A(V) and 10 mM As(III) and also exhibited plant growth-promoting characteristics	West Bengal, India	Kim et al. (2022)
Enterobacter sichuanensis WCHECL 1597*; Enterobacter sp.* NA11309	Biotransformation	Resistant up to 16 mM of As(III), 241 mM of As(V) and 20 mM of Fe(II)	Chakdaha block, Nadia district, West Bengal	Barman et al. (2024)

Several researches have reported the removal of arsenic from water with the help of modified biochar (Srivastav et al., 2022). Municipal solid waste, rice husk, oak bark, pine cone, oil palm, sewage sludge, pine wood, mango bark, tree/flower leaves, etc. (Srivastav et al., 2022) have been used as a feedstock for biochar preparation. Peanut shell (PS) (*Arachis hypogaea*) biochar has also been used as an adsorbent for arsenic removal. With a total worldwide production of 43.9 million tons, China being the leader, followed by India (Sharma et al., 2012), PS is a cost-effective and easily available waste. Peanut Shell Biochar (PSB) has been used for the removal of heavy metals such as lead, but not much has been reported for arsenic removal (Sattar et al., 2019). Also, the potential of using modified peanut shell biochar (MPSB) for removal of arsenic has so far not been fully investigated. However, in both systems, microorganisms are directly exposed to adverse environmental conditions, which results in their decreased metabolic activity (Jaiswal et al., 2023a). Providing a protective cover to the microorganisms (already immobilized on biochar) can help solve this problem and encapsulation inside beads is an effective way to do this.

PVA and SA beads have been widely used for encapsulation of bacteria for treatment of pollutants. PVA is a biodegradable and biocompatible polymer and reacts strongly with SA. Due to excellent chemical resistance, it does not cause any environmental pollution when used as a coating material (Amiri et al., 2023). SA can be extracted from brown algae and is a biodegradable copolymer that is non-toxic to bacteria (Baigorria et al., 2020). PVA-SA beads provide the advantage of easy separation and enhanced mechanical strength (Wen et al., 2018).

While single PVA or SA hydrogels are weak and prone to swelling, their cross-linking with Ca^{2+} enhances the stability of the structure significantly (Zhang et al., 2022). Alginate beads have previously been employed for removal of arsenic. They have been used in combination with zirconium oxides for the removal of copper and arsenic (Kwon et al., 2016), ferric oxides for the removal of As(III) and As(V) (Sarkar et al., 2010), water treatment residuals for the removal of As(III) and As(V) (Ociński et al., 2016). Banerjee et al. (2016) reported that *Pseudomonas alcaligenes* (RJB-B) and *Pseudomonas resinovorans* (RJB-3) immobilized on Ca-alginate could uptake 465 µg/L and 425 µg/L of arsenic, respectively. Arsenic removal using bacteria immobilized on biochar or biochar immobilized in PVA-SA beads has been reported by various researchers. However, arsenic removal by bacteria immobilized on PSB and further encapsulation in a PVA-SA bead is very limited.

2.5 Bacterial removal of arsenic from soil

Removal of heavy metal(loid)s such as arsenic and chromium from soil is very challenging as they are bound to the soil matrix and, hence, are difficult to mineralize. Several methods, such as physical, chemical and biological, have been employed for the removal of heavy metal(loid)s from soil (Gholizadeh and Hu, 2021). However, most of these methods are highly cost-intensive (Satapute et al., 2019). Heavy metal(loid)s, due to their non-biodegradability, persist in soil for a long time. They are permanently fixed by soil and interact with soil through ion exchange, precipitation, adsorption and complexion (Dhaliwal et al., 2020).

Recently, bioremediation through use of bacteria has emerged as a cost-effective method for removal of heavy metal(loid)s from soil. *Pseudomonas aeruginosa* could remove 89.67% of chromium (initial concentration of 20 ppm) after 20 days from nutrient broth media (Oyewole et al., 2019). Cell walls of *Pseudomonas aeruginosa* contain lipopolysaccharides, protein and phospholipids. The lipopolysaccharides contain phosphate and carboxyl groups and phospholides which serve as principal sites for metal binding and thus, facilitating biosorption of metal(loid)s (Oyewole et al., 2019). Vaxevanidou et al. (2008) reported that removal of arsenic from soil increased from 35% to 90% as bacterial activity of *Desulfuromonas palmitatis* increased. Shafique et al. (2017) reported that *Pseudomonas* sp. MX6 was resistant to multi metals which included As(III), As(V), Cd, Co, Cr, Cu, Hg, Ni, Pb, Se and Zn.

He et al. (2014) reported that *Pseudochrobactrum saccharolyticum* W1 and *Sporosarcina saromensis* W5 showed high chromium removal ability. On the other hand, remediation of heavy metal(loid)s by *Porteus* species is very limited. Adebo et al. (2023) reported that *Proteus mirabilis* was highly resistant to chromium. Plants also play an important role in the treatment of soils contaminated with heavy metal(loid)s. Several researchers have reported the use of microorganisms in combination with plants to remove contaminants at a higher rate and in greater amounts (Mushtaq et al., 2022; Yang et al., 2020). Several studies have reported the effect of arsenic or chromium individually on plant growth and physiology. In spite of arsenic and chromium cooccurring in soil, their combined effect on plants is very little, and studies on crop plants are even scarce (Islam et al., 2015).

2.6 Research gaps

A lot of strides have been made in the field of bioremediation of arsenic using bacteria. However, there are still a lot of areas that have remained underexplored. Some of the research gaps identified have been listed below:

- Studies employing polyurethane foam (PUF) for the immobilization of bacteria aimed at arsenic removal are still lacking.
- PSB has been used for removal of some heavy metals such as cadmium, copper, lead etc., but very little has been reported for arsenic removal.
- Work on bacterial immobilization on biochar and subsequent encapsulation in hydrogels has remained an extremely underexplored area.
- The combined effects of arsenic along with chromium on crop plants has not been thoroughly investigated.

2.7 Aims and objectives

The overarching objective of this Ph.D. research was to employ bioremediation strategy for the effective removal of arsenic and chromium contamination under different conditions, using isolated bacterial species and immobilization techniques to enhance remediation efficiency. Based on the research gaps identified in Section 2.6, and to achieve aim of this work, the following objectives were identified and have been listed below:

- Isolation and identification of potential bacterial species capable of resisting and remediating arsenic and optimization of various parameters for maximum removal of arsenic
- Evaluation of performance of different kinds of bioreactors for arsenic removal and toxicity assessment of samples before and after treatment using the isolated bacteria
- Bioremediation of arsenic and chromium from contaminated soil and evaluation of phytotoxicity and chlorophyll content in plants
- Immobilization of bacteria on biochar and encapsulate in PVA-SA beads for arsenic removal