Chapter IV:

ISOLATION AND IDENTIFICATION OF ARSENIC RESISTANT BACTERIA

4.1 General

Due to the geomorphological factors (such as meandering) of the Ganga River, arsenic contamination in the Gangetic plain is not uniform (Olea et al., 2018). A higher level of arsenic contamination is expected to be on the convex side (depositional part) of the meander. Since the majority of Varanasi city lies on the concave side (erosional part) of the Ganga River and thus, high arsenic concentration is found in the villages of Bahadurpur, Bhakhara, Bhojpur, Jalilpur, Kateswar, Kodupur, Madhiya, Ratanpur, and Semra (Raju, 2012) which are situated within the newer alluvium deposit. Ratanpur has been reported to have an arsenic concentration of 23 µg/L in the groundwater, and sediments have an average arsenic concentration of 5.3 mg/kg (Olea et al., 2018). The present work aimed to isolate arsenic-resistant and remediating bacteria from the Ratanpur Village, Varanasi, Uttar Pradesh (India) region. The extent of arsenic resistance, biotransformation ability, arsenic removal efficiency and resistance of the indigenous bacteria towards other heavy metals and phytotoxicity assessment on chickpea and *Vigna radiata* (mung bean) seeds were also evaluated. The genes involved in the biotransformation of arsenic were also determined.

4.2 16S rDNA amplification and phylogenetic analysis

Soil can be home to various microorganisms depending on the soil characteristics of a particular area. Arsenic concentration in the soil was determined by digesting the soil. The soil sample had a concentration of 0.118±0.01 mg/kg arsenic. The presence of arsenic in the soil can serve as a potential source for the isolation of arsenic-resistant bacteria, as exposure to arsenic over time can result in the adaptation of bacteria against arsenic (Majumder et al., 2013). The genomic DNA analysis of the isolates was done by the cetyltrimethylammonium bromide (CTAB) method and was visualized on an agarose gel. The DNA concentration for both isolates was 180 ng/µL. The electropherogram data revealed the sequences for both isolates. Phylogenetic analysis revealed that the bacteria belonged to the genus *Proteus* and *Pseudomonas*, respectively (Figure 4.1). The accession number for the isolated bacteria obtained from NCBI Genebank were MK332344.1 and KY419152.1 for *Proteus alimentorum* strain TY6 and *Pseudomonas aeruginosa* strain K7Pb, respectively (*It may please be noted that Pb in K7Pb does not refer to the element, lead*). *Pseudomonas aeruginosa* has previously been reported to be arsenic-resistant (Abdelbary et al., 2019; Fekih et al., 2018; Takeuchi et al., 2007). Arsenic-resistant and remediating bacteria from various different genera, such as *Bacillus* (Dey et al., 2016; Sher et al., 2021; Tiwari et al., 2016), *Pseudomonas* (Satyapal et al., 2018; Sher et al., 2021), *Planococcus* (Chowdhury et al., 2009), etc. have also been reported.

4.3 Bacterial resistance against arsenic

The arsenic resistance property of the isolated bacterial strains was determined by the MIC test by varying As(III) and As(V) concentrations. The bacteria did not show any growth for As(III) above a concentration of 10 mM and 610 mM for As(V). The isolated bacteria were further incubated by varying the As(III) concentration between 5 and 10 mM (5, 6, 7, 8, 9 mM). The strain, TY6, showed visible growth up to 8 mM of As(III) and 350 mM of As(V). The strain K7Pb showed a MIC of 10 mM for As(III) and 610 mM for As(V). The growth pattern for the isolates was also studied with respect to time in the presence of arsenic (refer to Appendix A). It was observed that initially, there was a lag phase due to the acclimatization of bacteria to the arsenic environment. After some duration, the exponential growth phase was observed and it reached a maximum in about 32 h. The growth reached a stationary phase after 35 h and then it started to decline.

Bacteria showed greater resistance towards As(V) as compared to As(III) during enrichment for selective isolation. Both isolates, TY6 and K7Pb, showed very high resistance towards As(V). Takeuchi et al. (2007) have reported As(V) resistance of 100 mg/L for *Pseudomonas aeruginosa,* whereas Sathe et al. (2021) have reported a tolerance of 1500 mg/L. Several other bacterial species have also been known to show resistance to considerably high concentrations of As(V). As-9 and As-14 belonging to *Exiguobacterium* sp. and *Bacillus hornekiae,* have shown increased resistance to As(V) at 700 and 900 mM, respectively (Pandey and Bhatt, 2015). Some bacterial species have shown still higher resistance to As(V) (up to 1000 mM), such as *Delftia* sp. strain BAs29 (Biswas et al., 2019) and *Bacillus* sp. strain BAs1 (Biswas and Sarkar, 2018). Such high resistance to arsenic might result from the development of an additional resistance system during enrichment for isolation (Pandey and Bhatt, 2015).

Various species of *Pseudomonas* have also been reported to show good tolerance towards As(III) such as *Pseudomonas tiwanensis* showed As(III) tolerance up to 10 mg/L (Satapute et al., 2019), *Pseudomonas* AK1 (13 mM) and *Pseudomonas* AK9 (15 mM) (Satyapal et al., 2018), *Pseudomonas monteilii* (IT6) (26.5 mM) (Sher et al., 2021). *Pseudomonas citronellolis* have been reported to exhibit a MIC for As(V) and As(III) from 300 – 500 mM and 250 mg/L, respectively (Adhikary et al., 2019). Lower resistance to As(III) can be attributed to the higher toxicity of As(III) as compared to As(V), as As(III) is 25 to 60 times more toxic than As(V) (Shukla et al., 2020).

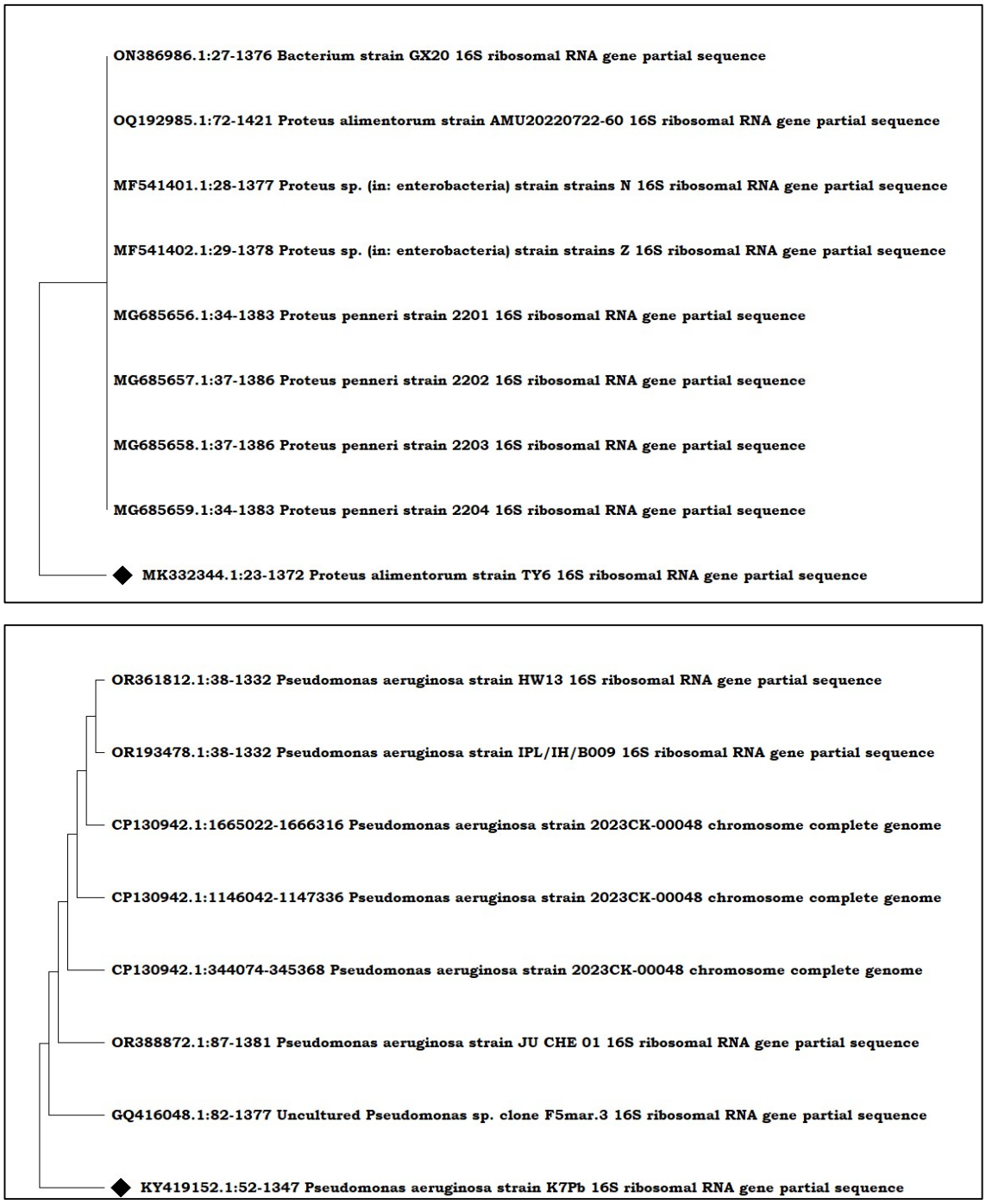

Figure 4.1: Phylogenetic tree based on 16S rRNA gene sequence for isolates *Proteus alimentorum* strain TY6 and *Pseudomonas aeruginosa* strain K7Pb

4.4 Effect of initial arsenic concentration and inoculum dose on bacterial growth

TY6 showed maximum growth at a lower concentration (1 mM) in presence of both As(III) and As(V) (Figure 4.2). However, growth in presence of As(V) was more than that of As(III) at concentrations greater than 5 mM. For K7Pb, maximum growth was observed at a concentration of 2 mM for As(V) and at 1 mM for As(III). Bacterial growth decreased with an increase in arsenic concentration, but decrease in presence of As(III) was more as compared to As(V). For inoculum dose, maximum growth for TY6 was obtained at a dose of 1.0 mL in the presence of both As(III) and As(V), whereas it was 1.5 mL for K7Pb (refer to Appendix A).

4.5 Effect of pH and temperature on bacterial growth

TY6 showed optimum growth in the alkaline medium (at pH 8). At lower pH (≤4) and higher pH (≥10), very little or no bacterial growth was observed after 24 h. Samples at lower and higher pH were further incubated for 48 h and 72 h, but no bacterial growth was observed. In acidic range, best bacterial growth was observed at pH 5 when maintained using HCl as compared to H_2SO_4 and HNO_3. In alkaline range, effect of KOH and NaOH was comparable as the bacterial growth observed (when pH was maintained using KOH and NaOH) which was nearly the same (Figure 4.2). For K7Pb, maximum growth was observed at pH 5 when H_2SO_4 was used to maintain pH in the acidic range (Figure 4.3).

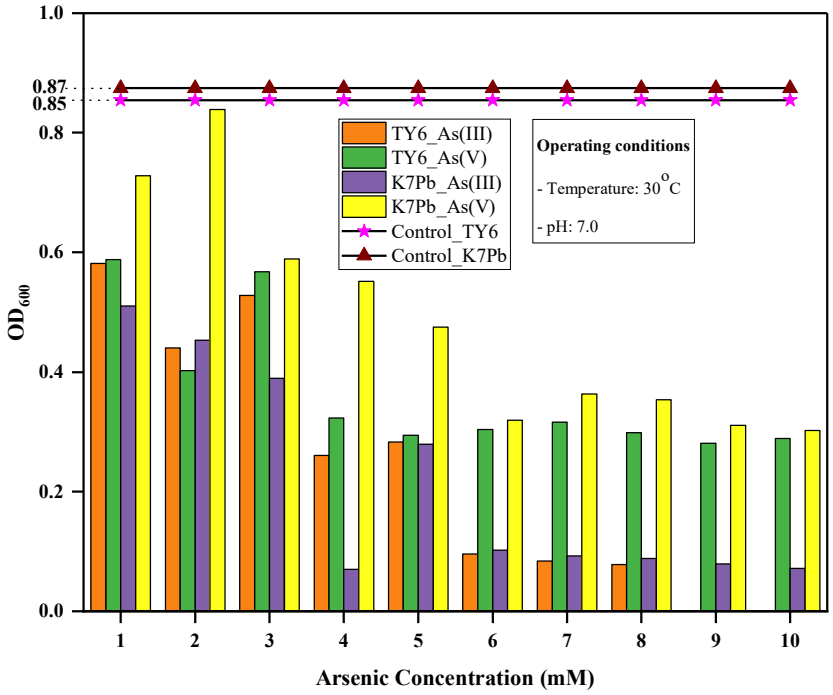

Figure 4.2: Effect of arsenic concentration on bacterial growth

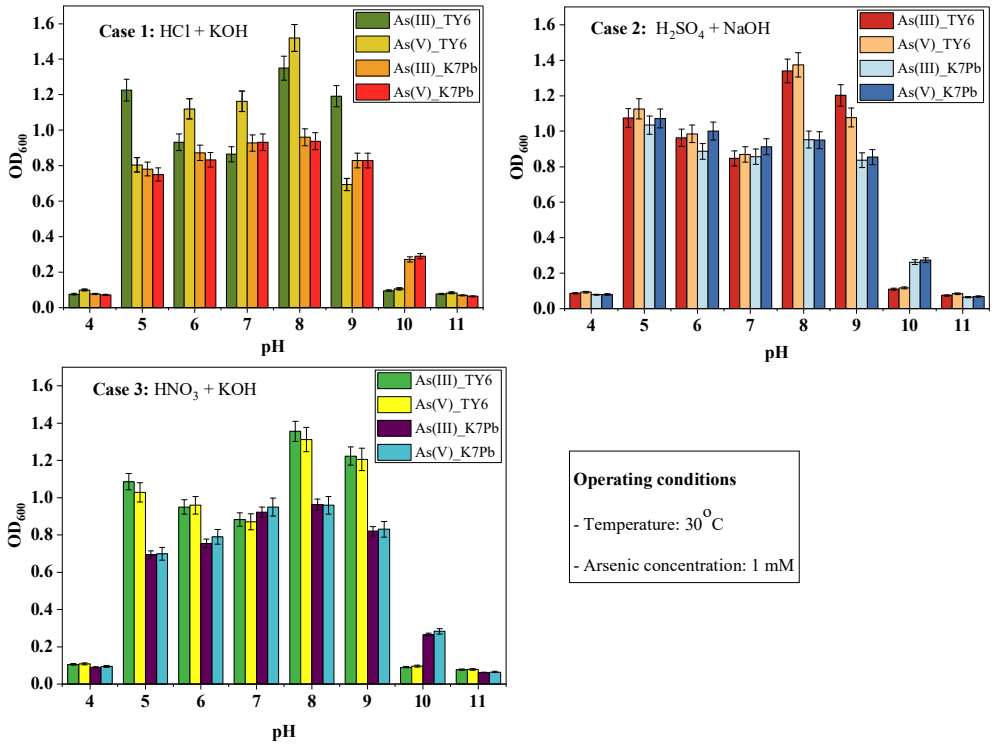

Figure 4.3: Effect of pH on the growth of TY6 and K7Pb for various combinations of acids and alkalis for case 1, case 2 and case 3

The optimum temperature for TY6 was observed to be 30°C. At lower temperatures (≤20°C) and higher temperatures (≥50°C), very little or no bacterial growth was observed. Optical density increased up to 30°C in presence of As(III) and 35°C in presence of As(V), then decreased with an increase in temperature (Figure 4.3). K7Pb showed maximum growth at 30°C in presence of As(V) and 35°C in presence of As(III). Optical density increased up to 30°C in presence of As(V) and 35°C in presence of As(III), then decreased with an increase in temperature (Figure 4.4).

Arsenic-resistant bacteria have been found to grow over a wide range of pH and temperature (Rahman et al., 2020; Taran et al., 2019). *Proteus alimentorum* has shown good enzymatic activity and growth in the pH range of 4 – 10 and temperatures between 35°C and 55°C (Ullah et al., 2021). In this study, optimum growth was obtained at pH 8 and temperature 30°C [for As(III)] and 35°C [for As(V)], which is in the range of pH and temperature reported for the growth of *Proteus alimentorum* in previous studies. *Pseudomonas aeruginosa* can survive over a wide temperature range (4°C to 42°C) and is grows well at 37°C (LaBauve and Wargo, 2012) and has been known to show good enzymatic activity in the pH range of 5 – 9 (Gupta et al., 2005; Silva et al., 2009; Thompson et al., 2001). In this study, K7Pb showed excellent growth between 30°C and 35°C and pH 5 and 7.

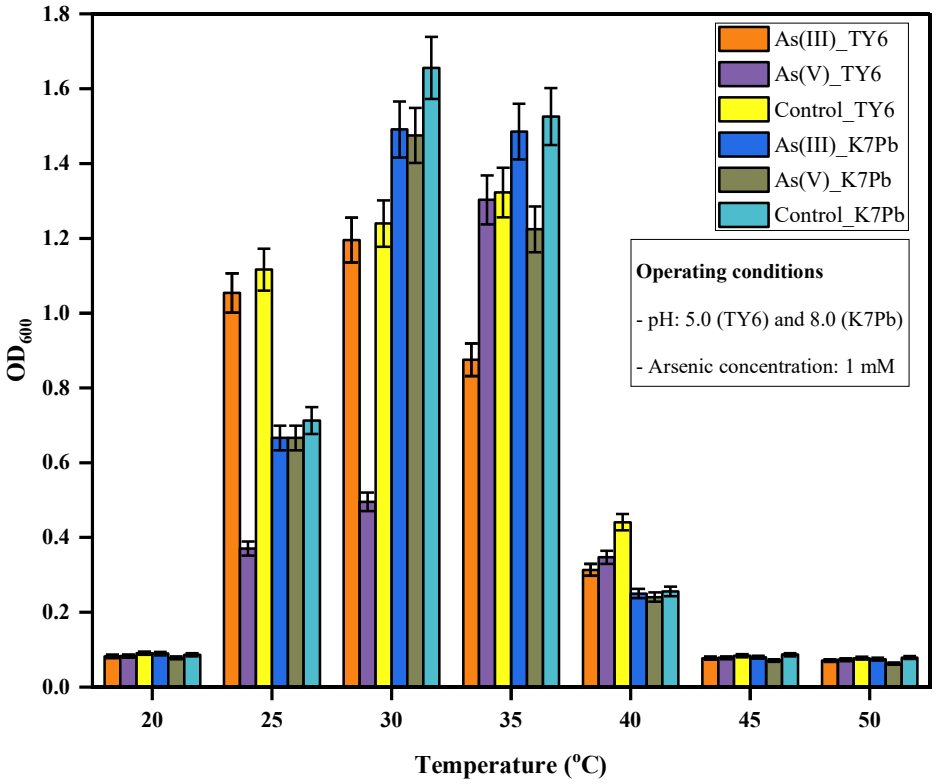

Figure 4.4: Effect of temperature on growth of TY6 and K7Pb

4.6 Biotransformation and bioaccumulation ability of bacteria and genes involved in biotransformation of arsenic

$AgNO_3$ and $KMnO_4$ tests affirmed the biotransformation ability of the bacterial isolates (Figure 4.5). The formation of yellow precipitate confirmed the ability of K7Pb to reduce As(V) to As(III), and the formation of brown precipitate confirmed the ability of TY6 to oxidize As(III) to As(V). Further, it was observed that with time, the color of the precipitates changed to purple and then to dark brown when $AgNO_3$ was used (refer to Appendix A). The PCR amplification profiles of K7Pb and TY6 were evaluated under As(V) and As(III) stress, respectively. The results showed the presence of *arrA*, *arsC*, *arxA* and *aioA* genes in both isolates (refer to Appendix A). The expected size of the amplified products was 160-500 bp for *arrA*, 200-400 bp for *arsC*, 400-800 bp for *arxA* and ~500 bp for *aioA*.

The arsenic accumulation test showed that both isolates had different arsenic accumulating capacities for As(III) and As(V). TY6 could accumulate up to 96±0.82% and 66±0.47% As(V) and As(III), respectively, with an initial concentration of 500 µg/L (Figure 4.6) after an incubation period of 6 d. K7Pb had an accumulation capacity of 98±0.94% and 78±1.25% for As(V) and As(III), respectively. However, both isolates had arsenic accumulation capacity and As(V) accumulation capacity was higher in both isolates.

Figure 4.5: Biotransformation ability of K7Pb and TY6 using $AgNO_3$ and $KMnO_4$ test. **(A)** K7Pb_As(V) – Formation of yellow precipitate (reduction of As(V) to As(III)) **(B)** TY6_As(III) – Formation of brown precipitate (oxidation of As(III) to As(V)) **(C)** Biotransformation ability of K7Pb using $KMnO_4$ test

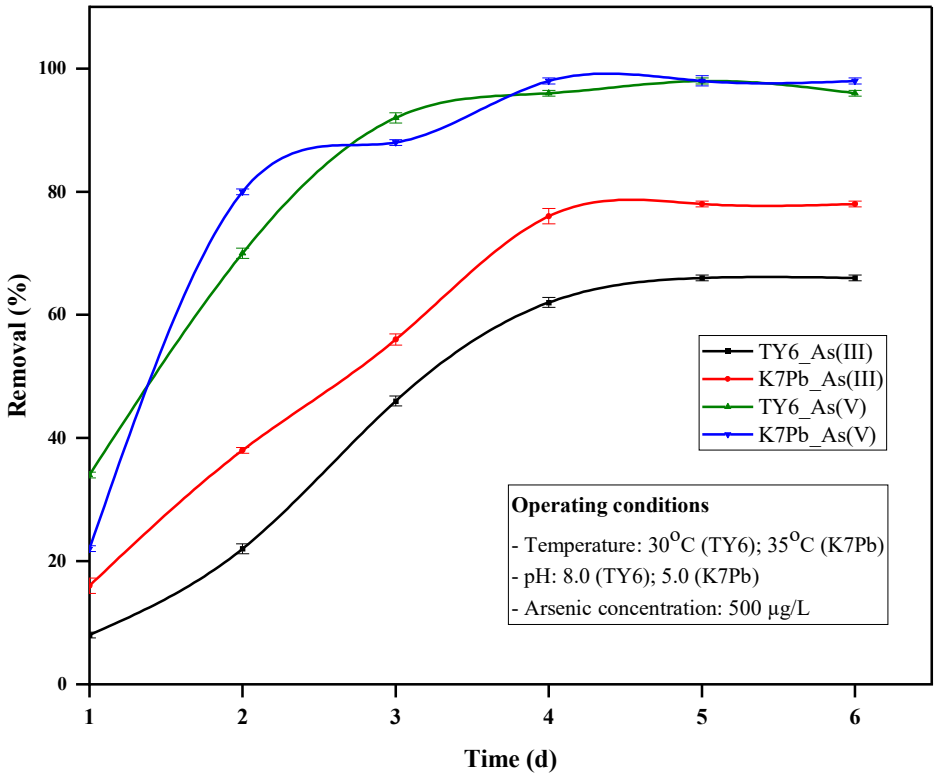

Figure 4.6: Removal efficiency of As(III) and As(V) by TY6 and K7Pb

Various studies have reported the presence of genes responsible for the biotransformation of arsenic (Escudero et al., 2013; Zargar et al., 2012). *arrA* and *arsC* are responsible for reduction of As(V) to As(III) whereas *arxA* and *aioA* take part in oxidation

of As(III) to As(V) (Yan et al., 2019). The presence of all four genes in both isolates implied that they are capable of both oxidation and reduction of arsenic. However, the oxidation ability of K7Pb and the reduction ability of TY6 were not observed in $AgNO_3$ and $KMnO_4$ tests during this study. One of the possible reasons for this observation may be that the expression of these genes was not strong enough to perform the designated function. Another possibility can be that these bacteria obtained the genes from other organisms through horizontal gene transfer but, do not possess the required mechanism to carry out the biotransformation (Neethu et al., 2022).

Pseudomonas aeruginosa has been known to reduce As(V) to As(III) (Sathe et al., 2021), but the effect of arsenic on TY6 has not been reported yet. Several bacterial species have been known to oxidize (Herrera et al., 2021; Sher et al., 2021) and reduce (Laha et al., 2021; Satyapal et al., 2018) arsenic. However, the change in color of the precipitates formed with time can be a result of the formation of different species of arsenic or an entirely different action by the bacteria, which still warrants further exploration.

Bioaccumulation study revealed that both the isolates were significantly efficient in removing both forms of arsenic [As(III) and As(V)] from the growth medium. As(V) accumulated in the cells ranged from 96 – 98%, and As(III) ranged from 66 – 78% (Figure 4.6). The results are in good agreement with the previous studies reported on the bioaccumulation of arsenic by various bacteria (Aguilar et al., 2020; Anjanapriya et al., 2022; Dey et al., 2016; Majumder et al., 2013). The removal in case of K7Pb was higher (98±0.94%) as compared to TY6 (96±0.82%) for As(V). Bioaccumulation results from higher uptake and lower efflux of arsenic by the bacteria (Rahman et al., 2012). It was observed that bioaccumulation affinity was higher towards As(V) as compared to As(III) for both bacteria.

4.7 Resistance of bacteria against other heavy metal(loid)s

The ability of isolates TY6 and K7Pb to resist heavy metal(loid)s other than arsenic, such as Cd, Co, Cr, Hg, Ni, and Pb was individually determined at different concentrations by following the same procedure as adopted for finding out the MIC for arsenic. MIC of elements has been presented in Table 4.1.

The isolates showed maximum resistance towards Pb (250 mg/L for TY6 and 300 mg/L for K7Pb) and minimum for Hg (2 mg/L for TY6 and 0.6 mg/L for K7Pb). Various strains of *Pseudomonas aeruginosa,* such as *Pseudomonas aeruginosa* DSM 1117 DSM (SN36) (Pb and Cd – 2mM) (Desoky et al., 2020), *Pseudomonas aeruginosa* ASU 6a (Cd, Co, Cr, Ni, Pb – 4.4, 5.9, 5.8, 6.8, 3.1 mM) (Hassan et al., 2008), *Pseudomonas aeruginosa*

AT18 (Silva et al., 2009) have been reported to show resistance towards different heavy elements. Several other bacterial species have also been known to grow in the presence of heavy metal(loid)s, such as *Pseudomonas fluorescens* (Cr – 250 mg/L) (Kalaimurugan et al., 2020), *Providencia rettgeri* AVRB20 (Anjanapriya et al., 2022) and *Paenibacillus jamilae* DSM 13815 T DSM (LA22) (Desoky et al., 2020), *Bacillus safensis* (Kalaimurugan et al., 2020). Raja et al. (2006) have also reported that *Pseudomonas aeruginosa* BC15 had very high resistance towards Cd (500 mg/L), Cr (400 mg/L) and Pb (800 mg/L). *Pseudomonas aerugeinosa* has also been reported to grow in very high concentrations of heavy metal(loid)s such as Co (590 mg/L), Cd (843 mg/L), Cu (222 mg/L), Cr (26 mg/L), Pb (570 mg/L) and Zn (1310 mg/L) (Anjanapriya et al., 2022; Hafeez et al., 2018). The resistance towards several heavy metal(loid)s by the two isolates indicates that they both may have the potential to be used for the remediation of other metal(loid)s also from water or the environment.

Table 4.1: MIC of isolated bacteria for different heavy metal(loid)s

Metal(loid)s	TY6	K7Pb
Cd	120±4.08 mg/L	100±4.71 mg/L
Co	40±2.05 mg/L	20±2.36 mg/L
Cr	90±6.24 mg/L	100±4.08 mg/L
Hg	2±0.41 mg/L	0.6±0.12 mg/L
Ni	110±4.71 mg/L	150±6.24 mg/L
Pb	250±4.08 mg/L	300±16.33 mg/L

4.8 Phytotoxicity assessment

The phytotoxicity test revealed that the bacteria treatment affected the seed germination and growth capability. The germination of seeds was highest for control as compared to treated and untreated samples. The test results have been shown in Table 4.2 and Table 4.3 for chickpea and *Vigna radiata*, respectively. Treated samples had better germination than untreated samples. Consortium (TY6+K7Pb) showed much better growth than samples treated with single strains (TY6 or K7Pb).

Several strains of *Pseudomonas aeruginosa* have been known to promote plant growth (Ahemad and Khan, 2010; Rizvi et al., 2020). TY6 and K7Pb both promoted the germination of seeds. However, the germination of chickpeas was more as compared to *Vigna radiata*. Also, seeds irrigated with solutions treated with the consortium (TY6+K7Pb) showed better germination than individual solutions but germination was lesser than that with control. Since seeds do not have defense mechanisms, seed germination is a very sensitive process for metal(loid)s accumulation (Shri et al., 2009).

Previous studies have also reported a decrease in seed germination due to exposure to arsenic (Liu et al., 2005; Shri et al., 2009). However, the germination of *Vigna radiata* was much lower than chickpea for all cases, which may be due to the effect of LB media used to irrigate the seeds.

Table 4.2: Seed germination parameters for chickpea

Parameters	Control	Untreated		Treated		
		As(III)	As(V)	As(III)_TY6	As(V)_K7Pb	As(V)+As(III)_TY6+K7Pb
RL (cm)	3.5±0.09	1.2±0.08	1.4±0.05	1.9±0.08	2.0±0.12	2.3±0.08
%RRG	100	34.28	40	54.28	57.14	65.71
%RSG	100	80	80	80	50	90
%GI	100	27.42	32	43.42	28.57	59.14
PI	0	0.66	0.60	0.46	0.43	0.34

Table 4.3: Seed germination parameters for *Vigna radiata*

Parameters	Control	Untreated		Treated		
		As(III)	As(V)	As(III)_TY6	As(V)_K7Pb	As(V)+As(III)_TY6+K7Pb
RL (cm)	3.3±0.12	0.6±0.09	0.7±0.12	0.9±0.12	1.1±0.12	1.3±0.05
%RRG	100	18.18	21.21	27.27	33.33	39.39
%RSG	100	10	10	20	30	50
%GI	100	1.82	2.12	5.45	9.99	19.69
PI	0	0.82	0.79	0.73	0.67	0.61

4.9 Summary

Arsenic-resistant bacteria were isolated from Ratanpur Village of Varanasi District, Uttar Pradesh, by enrichment technique. The isolates, *Proteus alimentorum* strain TY6 and *Pseudomonas aeruginosa* strain K7Pb showed high resistance to As(V) with a minimum inhibitory concentration of 610 mM and 350 mM, respectively, and 10 mM and 8 mM for As(III), respectively. TY6 could remove 96±0.82% As(V) and 66±0.47% As(III), whereas K7Pb could remove 98±0.94% As(V) and 78±1.25% As(III). Biotransformation ability was determined using $AgNO_3$ and $KMnO_4$ tests. PCR amplification showed the presence of *aio*, *arr*, *arx* and *ars* genes. Strains also showed resistance against other heavy metal(loid)s such as Cd, Cr, Co, Pb, Hg, and Ni. Phytotoxicity assessment on chickpea and *Vigna radiata* (mung bean) revealed that the arsenic-contaminated water treated with the isolates can be used for irrigation purposes.