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Transformation of arsenic by indigenous soil microbes as affected by phosphorus and arsenic

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Highly arsenic-polluted soil (16.5 mg kg^{-1}) of West Bengal, India, was used for isolation, screening and identification of indigenous soil microbes. *Citrobacter koseri* significantly removed (7.6) and bioaccumulated (4.95) highest arsenic in $P_{15}As_{15}$ treatment, while loss (2.9) was higher in $P_{10}As_{15}$. Similarly, *Pseudomonas putida* significantly removed (7.4) and bioaccumulate (4.8) highest As in $P_{15}As_{15}$ and while loss (2.8) was higher in $P_{10}As_{15}$. Percentage removal of As was 47–59, bioaccumulation was 29–38 and loss 17–23 with *Citrobacter* sp., while it was 47–58% (removal), 29–39% (bioaccumulation) and 17–21% (loss) with *Pseudomonas putida*. Maximum removal and bioaccumulation of phosphorus was 37.8% and 32.1% respectively, for $P_{10}As_{15}$ in *Citrobacter* sp. In *P. putida* it was 33.1% and 27.2% respectively, for $P_{10}As_{15}$. At the same level of arsenic, increase in phosphorus significantly increased its removal and bioaccumulation, but the opposite was true during calculation in terms of percentage removal and percentage bioaccumulation.

Keywords: Arsenic, bioaccumulation, *Citrobacter*, phosphorus, *Pseudomonas*.

ARSENIC (As) pollution in the environment has been detected on a large scale in many districts of West Bengal,

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India and huge populations are suffering from skin diseases and carcinogenesis, leading to death[1]. Arsenic has the ability to exist in oxidation states like +5, +3, –1 in a sedimentary or aquatic system which depends on redox conditions[2]. Transformation of arsenic through biochemical process plays an important role in determining the fate in arsenic-rich treatment waste[3]. Diverse bacterial communities can undergo different microbial transformation reactions like oxidation, methylation or reduction[4]. *Pseudomonas fluorescens* reduces iAs(V) to iAs(III) microbially, whereas *Bacillus arsenoxydans* microbially oxidize iAs(III) to iAs(V)[5]. In the soil system, PO_4^{3-} competes with arsenate As(V) for sorption sites and affects As availability in the soil[6]. In the plant system, As(V) is taken up via PO_4^{3-} transporters. PO_4^{3-} is able to reduce plant uptake of As(V) which depends on the resistance characteristics of plants as well as the amount of soluble P and As in the rhizosphere. Phosphates become more competitive over time since they are capable of slow sorption, but As(V) is adsorbed more strongly than phosphate[7]. As arsenic can transfer from soil to animals or humans via plants, we should look into the process for remediation of arsenic toxicity through different biotic and abiotic technologies[8]. There are a group of soil microorganisms which are capable of transforming arsenic into different forms[8]. As(+III) is more toxic than As(+V) on bacteria and actinomycetes. Besides, tolerance to arsenic (both As(+III) and As(+V)) is more in fungi than bacteria and actinomycetes[9]. For remediation of As-contaminated soils, there is increasing interest in the application of bioremediation technology[10]. *Pseudomonas* sp. is a Gram-negative bacterium, which has the prospect of rhizoremediation of organic compounds. This species has not been used for arsenic removal till now. *Citrobacter* sp. is a Gram-negative facultative anaerobic bacterium that can be used to reduce arsenic by its reductase activities. Till date, few studies have been carried out to investigate the effect of phosphorus and arsenic on transformation of arsenic by indigenous soil microbes. In order to fulfil the objective of the present study on bacterial transformation of arsenic as influenced by phosphorus using *C. koseri* and *P. putida* bacterial strains, an experiment was conducted under controlled conditions in broth to explore the possibilities of reducing arsenic contamination.

Highly arsenic-polluted (16.5 mg kg^{-1}) composite soil sample (0–15 cm) of Gotera village under Chakdah block, Nadia district, West Bengal, India (23°00′44.9″N and 88°34′59.6″E) was used for the experiment (Figure 1). For isolation and purification of As-resistant bacterial strains, diluted soil samples were prepared in sterile saline solution and plated on solidified Luria Bertani (LB) medium (an enriched medium for the growth of isolates under stressed conditions) with 500 mg l^{-1} As(III) and incubated at 30°C for 48 h. Arsenic volatilization capacities of purified bacterial isolates were analysed by a modified trapping method. Bacterial isolates showing considerable As(III)-volatilizing ability and higher As tolerance capacity were selected for molecular identification by 16S rDNA sequence analysis. The partial 16S rDNA sequences of the isolated strains were compared with those available in the GenBank database by BLASTN algorithm to identify sequences with a high degree (≥98%) of similarity. Among the various isolates, CTN-7 (NCBI accession number FJ605176) showed the highest sequence similarity with *Pseudomonas* sp. and thus we assumed it as *Pseudomonas putida* in this experiment. The isolated ATCC (NCBI accession number NR_075520) showed the highest sequence similarity with *Citrobacter koseri* ATCC BAA-895, and thus we assumed it as *Citrobacter koseri* in this experiment. The two selected bacterial isolates for the study were maintained as slants in nutrient agar medium at 4°C. Total bacteria, fungi, actinomycetes and cyanobacteria (colony forming unit (CFU)) in the soil were studied by serial dilution pour plate technique. Physico-chemical parameters of the soil were analysed using different standard methods[11]. Soil pH (1 : 2.5 H_2O, $CaCl_2$), Olsen P (0.5 M sodium bicarbonate extraction), cation exchange capacity (CEC) (1 M ammonium acetate (pH 7.0) extraction) and SO_4^{2-} (0.04 M $Ca(H_2PO_4)_2$ extraction) were measured by the method of Black *et al.*[12]. Available N of soils was determined using the method of Subbiah and Asija[13]. Available K was determined by the Brown and Warncke[14] method. Maximum water-holding capacity of the soil was determined by Keen Rackjowskii method and mechanical analysis was carried out following the international pipette method[15]. Total and extractable As concentrations were estimated according to the method of Sparks *et al.*[16]. The significance of field soil characteristics was to estimate the initial soil total arsenic and Olsen extractable arsenic. Before the isolation of microbes from any soil system, it is essential to analyse the soil properties to gather basic data and to establish a correlation between the isolated microbes and soil properties (suitable soil environment for the isolated microbes). Different levels of phosphorus were 0, 10 and 15 mg l^{-1} and arsenic 10 and 15 mg l^{-1}. Composition of the nutrient broth and nutrient agar medium for enumeration of bacteria was 5 g peptone, 3 g beef extract, 5 g sodium chloride, 15 g agar; 1000 ml distilled water (pH 7.0), agar 20 g (ref. 17). Two arsenic-transforming bacteria *C. koseri* and *P. putida* were grown in the broth with different concentrations of phosphorus and arsenic along with control replicated thrice. Anaerobic conditions were maintained by circulating 80% N_2, 10% H_2, 10% CO_2 gas mixture through alumina pellets coated with palladium. The arsenic extracting solution used in this treatment was 0.5 M $NaHCO_3$ (sodium bicarbonate). Stock solution of arsenic was prepared by dissolving Na_2HAsO_4, $7H_2O$ in 1 litre volumetric flask. Then 50 ml nutrient broth was taken in 100 ml capacity conical flasks made up of Borosil. A

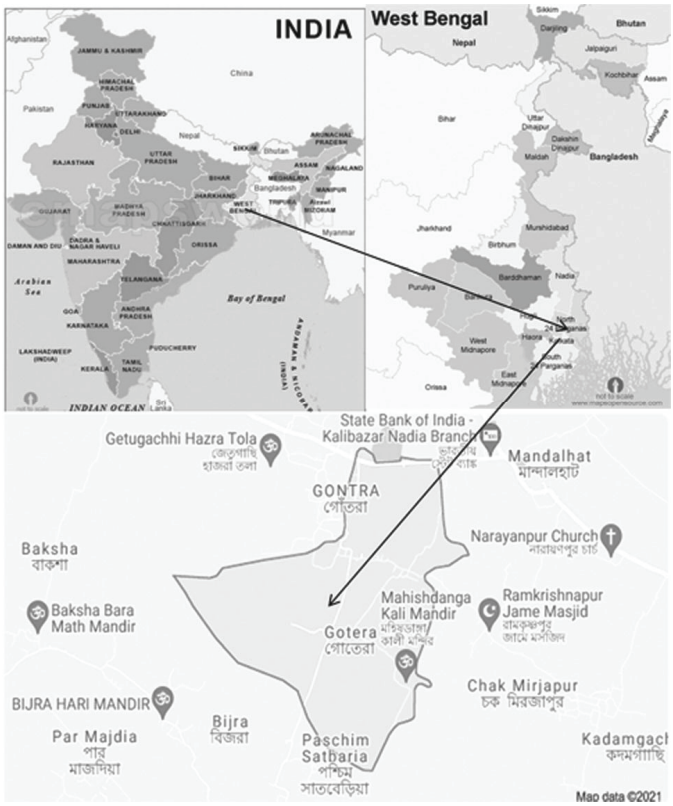

Figure 1. Site for soil sample collection.

Table 1. Physico-chemical properties of the experimental soil

Parameters	Value
Soil pH (1 : 2.5 :: soil : water suspension)	7.5
Electrical conductivity (dsm^{-1}; 1 : 2.5 :: soil : water suspension)	0.37
Oxidizable organic carbon (g/kg)	4.7
Available nitrogen (kg/ha)	174.0
Cation exchange capacity (cmol (p^+) kg^{-1} of soil)	22.9
Sand (%)	6.1
Silt (%)	70.7
Clay (%)	23.2
Textural class	Silty loam
Soil taxonomy	Typic Haplustepts
Exchangeable cation (cmol (p^+) kg^{-1} of soil)	8.37
Exchangeable Ca^{++} Mg^{++} (cmol (p^+) kg^{-1} of soil)	3.28
Exchangeable K^+ (cmol (p^+) kg^{-1} of soil)	0.22
Exchangeable Na^+ (cmol (p^+) kg^{-1} of soil)	0.10
Amorphous Fe (%)	0.34
Total As (mg/kg)	16.5
Olsen extractable arsenic (mg/kg)	4.29
Available P (mg/kg; Olsen extractable)	24.9
Water-holding capacity (%)	34.0

requisite volume of solution containing 10 and 15 mg l^{-1} arsenic was added to the treatments. A requisite volume of the phosphorus solution containing 10 and 15 mg l^{-1} phosphorus was also added to each treatment. Bacterial inoculation was made with 1 ml cell suspensions having density of ~10^8 CFU ml^{-1}. All these treatment combinations were incubated in the laboratory for 14 days at 30° ± 1°C on a rotary set at 150 rpm. After incubation, arsenic and phosphorus accumulation by cell biomass, and arsenic and phosphorus in nutrient broth upon removal of bacterial cell biomass were estimated. For estimation of bioaccumulation of arsenic and phosphorus, the harvested cell pellets obtained by centrifugation were resuspended and washed first in 0.02 M $MgCl_2$ and thereafter in deionized water before drying at 65°C for 24 h. The dried cell pellets were digested with HNO_3 (ref. 18). The total arsenic content was measured using atomic absorption spectroscopy (model: PerkinElmer PinAAcle900F with FIAS400) @λ_{max} ~193.7 nm) and total phosphorus using UV–visible double-beam spectrophotometer[17]. Loss of arsenic from the broth was calculated by subtracting arsenic removal in broth from bioaccumulation of arsenic in bacterial cells. Using SPSS Statistics 17, all statistical analyses were carried out along with analysis of variance (ANOVA) to examine statistical significance. Statistical analysis of data was subjected to Fisher's least significant difference (LSD) test at the significance level $P < 0.05$.

Physico-chemical properties and microbial compositions of the arsenic-affected soils were studied for a better understanding of their interactions (Tables 1 and 2). It was observed that the soil was neutral (pH 7.5) in reaction and non-saline. It showed medium range of available phosphorus (24.9 mg/kg), low in organic carbon (4.7 g/kg), low in available potassium (0.22 mg/kg) and low in available nitrogen (174 kg/ha). CEC of the soil was high (22.9 c mol (p+) kg^{-1} of soil). Soil textural class

Table 2. Microbial population of experimental soil

Soil sample	Bacterial CFU* $\times 10^5$ g^{-1}	Cyanobacteria CFU $\times 10^2$ g^{-1}	Actinomycetes CFU $\times 10^5$ g^{-1}	Fungal CFU $\times 10^3$ g^{-1}
Gotera soil	41	40	25	28

*CFU, Colony forming unit.

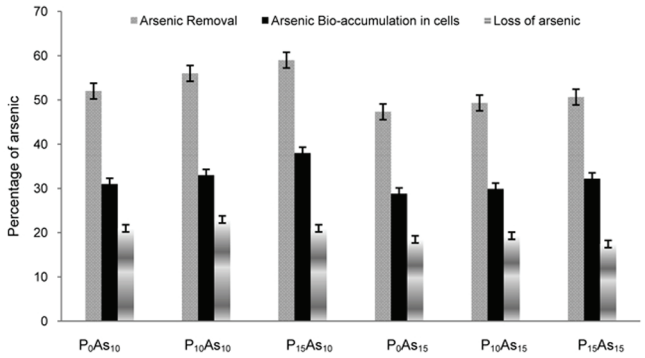

Figure 2. Arsenic removal, bioaccumulation in cells and loss (%) in As(V)-enriched broth after 14 days of incubation by *Citrobacter koseri.*

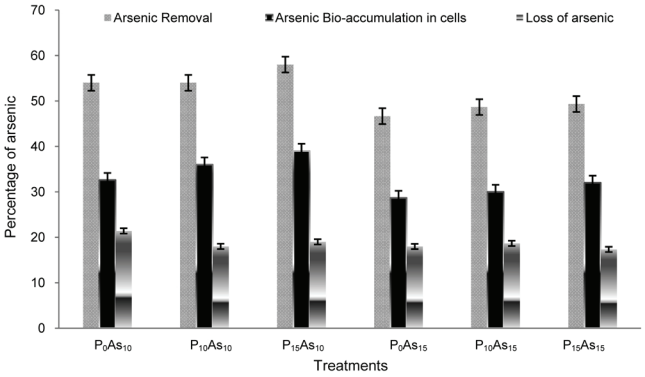

Figure 3. Arsenic removal, bioaccumulation in cells and loss (%) in As(V) enriched broth after 14 days of incubation by *Pseudomonas putida.*

was recorded as silty loam and soil was Typic Haplustepts. The total arsenic loading was 16.5 mg kg^{-1} and Olsen extractable arsenic was 4.29 mg kg^{-1}. The magnitude of arsenic contamination in the experimental soil was in the higher range. The mean natural content of arsenic in the soils of about 5–6 mg kg^{-1} with a typical range of 1–40 mg kg^{-1} is in a higher range as reported by Das *et al.*[19]. Das and Mondal[20] observed higher range of arsenic in soils of Nonaghata village (22°57′29.1″N and 88°34′22.4″E) of Haringhata block, Nadia district, from 8.4 to 24.3 mg kg^{-1} of total arsenic and 2.9 to 15.8 mg kg^{-1} of Olsen extractable arsenic. The bacterial population was 41×10^5 g^{-1} CFU and medium in range. The cyanobacterial, actinomycetes and fungal populations were in medium range. Das and Mondal[20] observed that the total population of bacteria and cyanobacteria was significantly lower in arsenic-polluted soils than the non-polluted soils of West Bengal and with increase in soil arsenic loading, a decreasing trend in the population was reported.

Two bacterial strains *C. koseri* and *P. putida* having greater tolerance of arsenic were selected for observing the effect of different levels of phosphorus and arsenic on the arsenic-transforming ability after 14 days of incubation. Table 3 shows removal of arsenic, bioaccumulation in the cells and loss after 14 days from As(V)-enriched broth. Results revealed that arsenic removal (7.6 mg l^{-1}) and bioaccumulation (4.98 mg l^{-1}) by *Citrobacter* sp. was highest in $P_{15}AS_{15}$ and loss of arsenic (2.9 mg l^{-1}) was higher in $P_{10}AS_{15}$. Table 3 also shows that arsenic removal (7.4 mg l^{-1}) and bioaccumulation (4.8 mg l^{-1}) by *P. putida* was highest in $P_{15}AS_{15}$ and loss of arsenic (2.8 mg l^{-1}) was higher in $P_{10}AS_{15}$. The lowest removal was seen when phosphorus at 0 mg l^{-1} and arsenic at 10 mg l^{-1} (P_0A_{10}) were applied, and the highest removal was observed when phosphorus at 15 mg l^{-1} and arsenic at 15 mg l^{-1} ($P_{15}A_{15}$) were applied. The trend was applicable to both the bacterial strains. Similar trend was observed in case of bioaccumulation of arsenic in both the bacterial strains. No significant difference ($P = 0.95$) was observed between *C. koseri* and *P. putida* in terms of removal, bioaccumulation in cells and loss of arsenic. This showed that both the organisms performed in a similar manner in terms of transformation of arsenic at different levels of phosphorus and arsenic. It can be recommended that both the organisms were similarly inefficient for transformation of arsenic. A gradual increase in arsenic removal and bioaccumulation after 14 days of incubation was observed when the phosphorus content of nutrient broth increased, but this increase was non-significant ($P = 0.95$). Therefore, increase in the phosphorus level or addition of phosphorus non-significantly affected the increase in arsenic removal or bioaccumulation. The same trend mentioned earlier was observed for both the bacteria. Almost in all the treatments arsenic removal or bioaccumulation was significantly higher in broth containing 15 mg l^{-1} than 10 mg l^{-1} of arsenic at the same level of phosphorus. In terms of percentage removal of arsenic, the opposite was true, i.e. it was higher at 10 mg l^{-1} than 15 mg l^{-1} of arsenic broth at the same level of phosphorus. No significant difference ($P = 0.95$) was observed in percentage arsenic loss, but it was slightly higher in 10 mg l^{-1} than 15 mg l^{-1} of arsenic broth. Figures 2 and 3 show that removal of arsenic ranges from 47% to 59%, bioaccumulation from 29% to 38% and loss from 17% to 23% with *Citrobacter* sp. It is 47–58% (arsenic removal),

Table 3. Arsenic removal from broth, bioaccumulation in cells and loss of arsenic by selected bacterial strains from As(V)-enriched broth after 14 days of incubation

Bacterial strain	Treatment	Arsenic removal from broth (mg l^{-1})	Arsenic bioaccumulation cells (μg g^{-1})	Loss of arsenic (mg l^{-1})
Citrobacter sp. *koseri*	P_0As_{10}	5.2	3.1	2.1
	$P_{10}As_{10}$	5.6	3.3	2.3
	$P_{15}As_{10}$	5.9	3.8	2.1
	P_0As_{15}	7.1	4.3	2.7
	$P_{10}As_{15}$	7.4	4.5	2.9
	$P_{15}As_{15}$	7.6	4.9	2.6
	Mean	6.5	4.0	2.5
Pseudomonas putida	P_0As_{10}	5.4	3.2	2.1
	$P_{10}As_{10}$	5.4	3.6	1.8
	$P_{15}As_{10}$	5.8	3.9	1.9
	P_0As_{15}	7.0	4.3	2.7
	$P_{10}As_{15}$	7.3	4.5	2.8
	$P_{15}As_{15}$	7.4	4.8	2.6
	Mean	6.4	4.0	2.3
Bacteria (*B*)	SEm (±)	0.175	0.146	0.212
	CD ($P = 0.05$)	NS	NS	NS
Treatment (*T*)	SEm (±)	0.304	0.253	0.367
	CD ($P = 0.05$)	1.330	1.058	NS
($B \times T$)	SEm (±)	0.329	0.258	0.418
	CD ($P = 0.05$)	NS	NS	NS

NS, Non-significant.

29–39% (bioaccumulation), and 17–21% (loss) with *P. putida*. Das and Mondal[19] observed that *Citrobacter* sp. caused 17% removal, 15% bioaccumulation and 2% loss of arsenic, while *P. putida* caused 21% removal, 18% bioaccumulation and 3% loss of arsenic in 50 μg l^{-1} of broth. The higher efficiency in both the organisms in terms of percentage removal, bioaccumulation and loss in the present experiment compared to earlier findings of Das and Mondal[20] with the same organisms might be due to the lower levels of arsenic (10 and 15 mg l^{-1}) in the present experiment. Although it was statistically at par, percentage removal or bioaccumulation or loss of arsenic in 10 mg l^{-1} broth was higher than 15 mg l^{-1} broth in the present experiment. The efficiency of the bacterial strains might be better at lower levels of arsenic. *Citrobacter* sp. strain NC-1 reduced arsenate within 24 h and exhibited arsenate-reducing activity[21]. The strain NC-1 was able to extract arsenic from contaminated soils (arsenate to arsenite) through solid-phase reduction[20]. *Citrobacter* sp. strain RPT can survive under As stress and has been identified for application in bioremediation of As (ref. 22).

After 14 days of incubation, removal of phosphorus from broth and bioaccumulation of phosphorus within bacterial cells were observed. Table 4 presents the data. In terms of removal and bioaccumulation of phosphorus, the two bacterial isolates were similar. Both the bacterial strains increased phosphorus removal and bioaccumulation from the broth. There was no significant difference between the two bacterial isolates. There was significant difference ($P = 0.05$) between all the treatments. Phosphorus removal by *Citrobacter* sp. from the broth was higher in $P_{15}As_{15}$ and for *P. putida* it was $P_{15}As_{10}$. Similarly, phosphorus accumulation in the cells was higher in $P_{15}As_{15}$ by *Citrobacter* sp. and in $P_{15}As_{10}$ by *P. putida*. In case of *P. putida*, both phosphorus removal and bioaccumulation were higher in $P_{15}As_{10}$ than $P_{15}As_{15}$. Thus, at higher concentration of arsenic, the bacterial activity of *P. putida* was hampered. *C. koseri* was efficient in phosphorus removal and bioaccumulation at slightly higher concentration of arsenic ($P_{15}As_{15}$). Phosphorus removal and bioaccumulation were negligible for both the bacterial strains at P_0 level of phosphorus at both As_{10} and As_{15}, which indicated that phosphorus nutrient is essential for both the microbes for removing phosphorus in highly arsenic-contaminated soil. From Figures 4 and 5, in *C. koseri* the maximum percentage removal of phosphorus and percentage bioaccumulation was 37.8% and 32.1% respectively, for $P_{10}As_{15}$. In *P. putida*, the maximum percentage removal of phosphorus and percentage bioaccumulation was 33.1 and 27.2 respectively, for $P_{10}As_{15}$. At the same level of arsenic, increase in phosphorus significantly increased its removal and bioaccumulation, but the opposite was true during calculation in terms of percentage removal and percentage bioaccumulation. This shows that percentage removal or bioaccumulation of phosphorus is higher at lower concentration of arsenic. This pattern was similar to that observed in case of arsenic. There was no significant difference between treatments and bacterial strains ($P = 0.95$).

Arsenic immobilization by *Pseudomonas* sp. strain GE-1-induced ferrihydrite which can be applied as an alternative remediation strategy[23]. *Pseudomonas* sp. exhibited maximum accumulation of 4 mg As g^{-1} (dry wt.)[24]. Xiu *et al.*[25] obtained 24 bacterial isolates of which 5 had higher

Table 4. Phosphorus removal from broth and bioaccumulation in cells by selected bacterial strains from As(V)-enriched broth after 14 days of incubation

Bacterial strain	Treatment	Phosphorus removal from broth (mg l^{-1})	Phosphorus bioaccumulation in cells (μg g^{-1})
C. koseri	P_0As_{10}	–	Negligible
	$P_{10}As_{10}$	3.5	3.1
	$P_{15}As_{10}$	4.9	4.3
	P_0As_{15}	–	Negligible
	$P_{10}As_{15}$	3.7	3.1
	$P_{15}As_{15}$	5.0	4.5
	Mean	4.3	3.8
P. putida	P_0As_{10}	–	Negligible
	$P_{10}As_{10}$	3.1	2.8
	$P_{15}As_{10}$	4.3	4.0
	P_0As_{15}	–	Negligible
	$P_{10}As_{15}$	3.2	2.8
	$P_{15}As_{15}$	4.3	3.9
	Mean	3.7	3.4
Bacteria (*B*)	SEm (±)	0.055	0.046
	CD ($P = 0.05$)	NS	NS
Treatment (*T*)	SEm (±)	0.077	0.065
	CD ($P = 0.05$)	1.406	1.31
($B \times T$)	SEm (±)	0.110	0.092
	CD ($P = 0.05$)	NS	NS

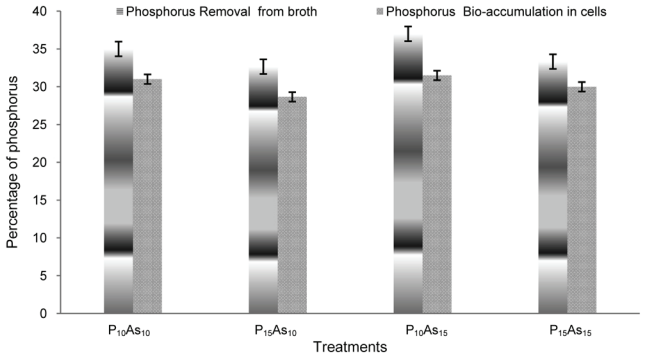

Figure 4. Phosphorus removal and bio-accumulation in cells (%) in As(V)-enriched broth after 14 days of incubation by *C. koseri.*

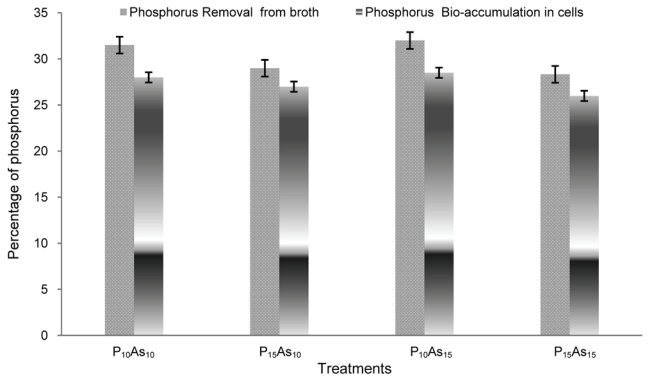

Figure 5. Phosphorus removal and bioaccumulation in cells (%) in As(V)-enriched broth after 14 days of incubation by *P. putida.*

arsenic-adsorbing capacities ranging from 80.9% to 96.9%. The above findings were comparatively in a higher range than those of the present study. In this study, there was loss of 17–23% with *C. koseri* and 17–21% with *P. putida.* The reason for the loss or removal of arsenic from the broth may possibly be due to volatilization as well as microbial cell accumulation. Again, volatilization of arsenic depends upon several factors, viz. initial concentration of arsenic, substrate time and amount. Transformation of arsenite and arsenate into mono, di or trimethyl arsenic through a number of microorganisms has been reported[26]. In the present study, loss (17–23%) of arsenic from the broth by *C. koseri* and *P. putida* was due to methylation. The results of this study using different arsenic concentrations, revealed that the highest recorded resistance was found in both the strains. Simeonova *et al.*[27] found that approximately 37% of As(III) (aerobic) and 30% As(V) (anaerobic) were volatilized by bacterial isolates within three days. Tables 2 and 3 show that the bacterial strains are resistant and effectively remove arsenic in arsenic-polluted soil of West Bengal. This may be because the microbes develop various intrinsic arsenic tolerance mechanisms to sustain under adverse environmental conditions. Few bacterial microbes have some genes which are located on plasmids and the genetic system called as ARSOPERON may be the main functional unit which is responsible for arsenic resistance[28]. *Synechocystis* sp. strain PCC6803 has strong ability for arsenic accumulation and tolerance, which may be applied in the phytoremediation of aquatic arsenic[29]. In the present study, at the same level of phosphorus (P_{10} or P_{15}) with increase in arsenic content, phosphorus removal and bioaccumulation also increased non-significantly ($P = 0.95$).

The bacterial inoculants *C. koseri* and *P. putida* efficiently removed, bioaccumulated and lost arsenic from As(V)-enriched broth after 14 days of incubation. The

two microbes also efficiently removed and bioaccumulated phosphorus from As(V)-enriched broth. Therefore, these two promising bacterial strains can be used to ameliorate soil arsenic. The successful exploitation of these isolates may deliver an eco-friendly and cost-effective tool for As mitigation compared to genetically engineered alternatives.

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