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pH modulates arsenic toxicity in *Bacillus licheniformis* DAS-2

K. Tripti, Shardendu *

Laboratory of Environment and Biotechnology, Department of Botany, Patna Science College, Patna University, Patna 800005, India

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ABSTRACT

The toxic characteristics of arsenic species, As(V) and As(III) result in ecological risks. Arsenic tolerant bacterium was isolated and identified as the *Bacillus licheniformis* DAS-2 through 16SrDNA sequencing. *B. licheniformis* DAS-2 was efficient to tolerate and remove both the As(V)[MIC 8 mM] and As(III)[MIC 6 mM] from the growth medium. The potential for the removal/uptake of arsenic from the 3, 5 and 7 mM As(V) enriched growth media was 100%, 60% and 35% respectively and from the 1, 3 and 5 mM As(III) enrichment it was 100%, 99% and 58% respectively at neutral pH. 80% of uptake As(V) was reduced to As (III) in 3 mM As(V) enrichment which was gradually decreased to only 17% at 7 mM As(V) enrichment at neutral pH. The arsenic toxicity in *B. licheniformis* DAS-2 was found modulated by pH and was examined through alteration in growth, uptake/removal, reduction and measurement of chemical toxicity.

1. Introduction

Bengal Delta has been reported as the most arsenic contaminated region on the Earth, due to the presence of toxic level of arsenic in ground water, where 36 million people are exposed to the risk of arsenic contamination (Nordstrom, 2002). Bihar state is a part of Bengal Delta located at 85° 32′E longitude and 25° 11′N latitude on the Earth. Majority of the population of Bihar depends upon agriculture for their livelihood. They are getting polluted with arsenic, due to bio-magnified level of arsenic in their food, via irrigation water. Arsenic has been reported as carcinogenic (Rosen, 1971) and has a wide range of adverse health effects including skin lesions (Chakraborti et al., 2004) and neurological disorders.

The sources of arsenic are both natural and anthropogenic. Inorganic arsenic occurs in various oxidation states including As (V) [arsenate], As(III) [arsenite], elemental arsenic (0) and As(-III) arsenide. Among them trivalent and pentavalent arsenic are most common forms but, the trivalent form is most toxic.

Soil with long term exposure of As(V) and As(III) may result in the evolution of highly diverse arsenic resistant bacteria. Relative abundance of As(V) and As(III) in the soil is influenced by the microbial transformations. The transformation of arsenic into different chemical species might be the best survival strategy of soil bacteria in arsenic-contaminated environment. Arsenic species exhibit variation in solubility, mobility, bio-availability and toxicity (Masscheleyn et al., 1991; Inskeep et al., 2002) in the environment. Thus bacteria utilizes the most available form of arsenic [As(V) and As(III)] during the transformation, as an alternative source of energy. The phenomenon of bacterial transformation occurs through the processes like extrusion after oxidation, reduction and methylation (Anderson and Bruland, 1991) or by intracellular chelation. The above phenomenon occurs in almost all bacteria, for which it needs arsenic uptake transporters. Transport of Arsenate [As(V)] into bacterial cell in the form of an oxyanion (AsO_4^{3-}) is carried out by the phosphate (PO_4^{3-}) transporters due to its chemical analogy with the phosphate (Sanders et al., 1997; Wolfe-Simon et al., 2011). As(III) is mostly found in unionized state as $As(OH)_3$ at neutral pH and it is transported into the cells by aqua glycophorins (glycerol transport proteins) (Sanders et al., 1997; Rosen and Liu, 2009).

pH of the growth medium effects the availability and transport of arsenic, whereas maximum uptake and transport for both As (V) and As(III) occur at neutral pH (Tu and Ma, 2003). Since availability and transport of arsenic species are highly controlled by the pH, it may be possible that, pH modulates the arsenic toxicity. This study explores some survival strategies of soil-native bacteria under arsenic stress by the assessment of growth, uptake/removal, transformation, biochemical analysis and influence of pH over them.

2. Materials and methods

Bacillus licheniformis DAS-2 was isolated by the spread plate technique (Prescott, 2002). 16sRNA gene was amplified by using universal primer (Grifoni et al., 1995). PCR product sequenced at Banaras Hindu University (B.H.U. Varanasi, India). The 16Sr DNA sequence was compared with the nucleotide sequences present in the NCBI database using the standard nucleotide BLAST search (Altschul

* Corresponding author.
E-mail address: shardendu77@rediffmail.com (Shardendu).

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et al., 1997). The nucleotide sequences of 16S rRNA of isolate have been deposited in GenBank under the accession number KF664028 with species name *B. licheniformis* DAS-2. Studies of growth and other physiological parameters were done by growing bacteria at 37° C and 150 rpm in TYEG broth (Oh et al., 1995) for all the given conditions (control, As(V), As(III), variable pH conditions of arsenic stress [As(V), As(III)]). Cell cultures were taken at different time intervals during lag, log and stationary phases (from 0 to 32 h). Optical density (OD) was measured at 600 nm. Quantification of As(V) and As(III) was done by digesting the residual media/cell biomass by nitric acid method (APHA, 2005; Tripti et al., 2014) and estimating the arsenic by azure B method (Cherian and Narayana, 2005) with some modifications (Tripti et al., 2014). Biochemical analysis for measuring chemical toxicity (Dehydrogenase activity) was done by Resazurin test (Liu, 1981).

2.1. Data analysis

All the data in the figures are the means ± standard errors of three replicates. The correlation statistics (r-value) were calculated for various parameters and effects at 0.05 levels by using software. Analysis of variance (ANOVA) were also performed by using software STATISTICA v5.52.164.0 for the statistical evaluation of the variations in growth, removal, and reduction due to change in pH with the differences considered significant at $P < 0.05$. The graphs were drawn using MICROSOFT EXCEL 2003, 2007.

3. Results

3.1. Growth profile of Bacillus licheniformis DAS-2 in presence of arsenate As(V) and arsenite As(III) stress

Bacillus licheniformis DAS-2 was isolated from rhizosphere of *A. viridis* and was selected for further studies; from the colonies grown on 1 mM As(V) amended TYEG plate. Isolated *B. licheniformis* was further allowed to grow in TYEG broth amended with 1, 3, 5, 7 and 8 mM of As(V) and 1, 3, 5 and 6 mM of As(III) at various time intervals. Growth comparison of cells grown in arsenic-free media and arsenic-containing media revealed the toxic effect of arsenic. It was observed that *B. licheniformis* was growing in control (no added arsenic) (Fig. 1) spent first 2 h in lag phase then 26 h in exponential phase and then moved to stationary phase with few deaths. In 1 mM of As(V) amendment, 5.5% higher cell growth was determined in comparison to control, showing As(V) as growth promoting factor. It might be due to the fact that, in presence of As (V) *B. licheniformis* might switch on its strategies by activating arsenic-utilizing system. After that, gradual reduction in cell growth was observed on increasing As(V) concentration in growth media. About 10% cell growth was reduced in 3 mM As(V), 19% in 5 mM As(V), 74% in 7 mM As(V) and 92% in 8 mM As(V). The duration of lag phase was also gradually increased on increasing As(V) concentration in medium, 10 h of long lag phase was observed in both 5 and 7 mM As(V) supplied medium. The Minimal Inhibitory Concentration (MIC) was determined as 8 mM As(V).

The growth pattern of *B. licheniformis* in As(III) enriched media revealed that the As(III) is more toxic than As(V), as reduction in cell growth were, by 13.5% in 1 mM, 45% in 3 mM, 66% in 5 mM and 85% in 6 mM (MIC). There was significant reciprocal correlation between the growth of bacterium and concentration of As (V) with ($\mathbf{r = -0.92523}$) and As(III) with ($\mathbf{r = -0.94060}$) enrichment in growth media.

3.2. Effects of pH on growth of Bacillus licheniformis DAS-2

Statistically significant ($\mathbf{p = 0.010}$) variation in growth due to change pH was observed. Maximum growth was observed at neutral pH, which was reduced on moving towards acidic as well as basic pH (Fig. 2). But the data supports an alkaline condition for better growth than an acidic condition. Thus, near neutral to slightly alkaline was the most suitable growth medium for *B. licheniformis* DAS-2.

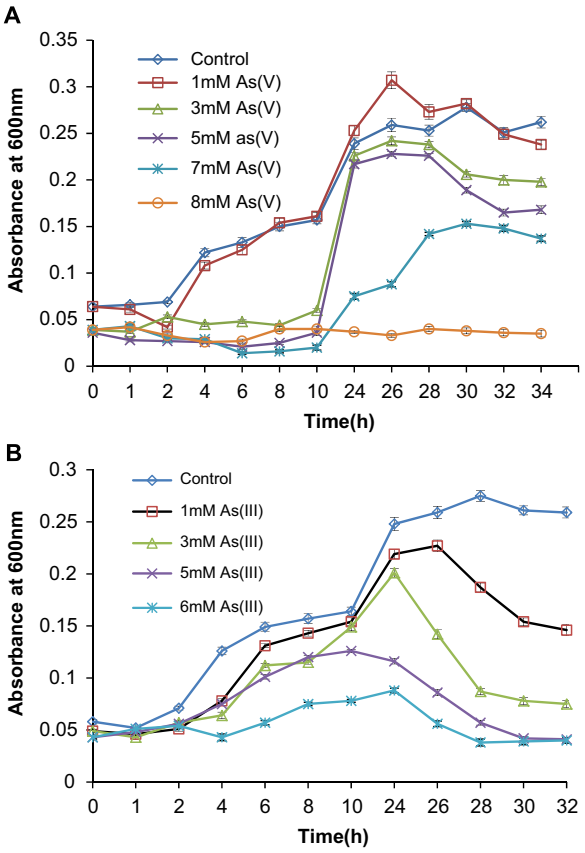

Fig. 1. Growth pattern of *Bacillus licheniformis* DAS-2 in arsenic containing TYEG broth, over the range of arsenic concentrations (A) Arsenate [As(V)] (1 mM, 3 mM, 5 mM 7 mM and 8 mM) (B) Arsenite [As(III)] (1 mM, 3 mM, 5 mM and 6 mM). Control cultures with no added arsenic [As(V) or As(III)] i.e (0 mM) are shown. Change in OD_{600} (Absorbance) of culture was measured over 32 h. Error bars indicate the standard error of the mean of three experiments.

On adding 3 mM and 7 mM of As(V) in normal TYEG broth, pH of broth had changed from 7 to 7.6 and 7.7 respectively. pH of As (V) enriched media was further increase only by 0.2 (7.8 and 7.9) till the end of experiment/growth. Hence there was no need of buffering the media. Further changes in pH to 5, 6, 8 and 9 were done by adding 10% HCl or 1 mM NaOH. Both acidic and basic pH (6, 8 and 9) had slightly favoured the growth but, at more acidic pH (5) growth was diminished in both the concentrations of As(V) (3 mM and 7 mM) stress. Significant variation ($\mathbf{p = 0.019}$) was found on comparison of the growth (absorbance/O.D) of the *B. licheniformis* in different pH conditions of the medium, without added arsenic, with growth in different pH of As(V) stress (added arsenic [As(V)] in medium). It was found that at both acidic and alkali condition, spiked As(V) was observed as a stimulant for the growth of *B. licheniformis* (Figs. 2 and 5. A) providing evidence for the presence of arsenate/As(V) utilizing system in bacteria, which also work in different pH stress. Addition of 3 mM and 5 mM of As (III) contributed to change the pH of TYEG broth from 7 to 7.9 and 8.1 respectively and were further decreased to 7.5 and 7.7 respectively at the end of experiment/growth. Further change in pH to 5, 6, 9 and 10 was done by adding acid or base in the As(III) amended TYEG broth. It was observed that at pH 9 growths were slightly favoured in As(III) stress otherwise growth was diminished in

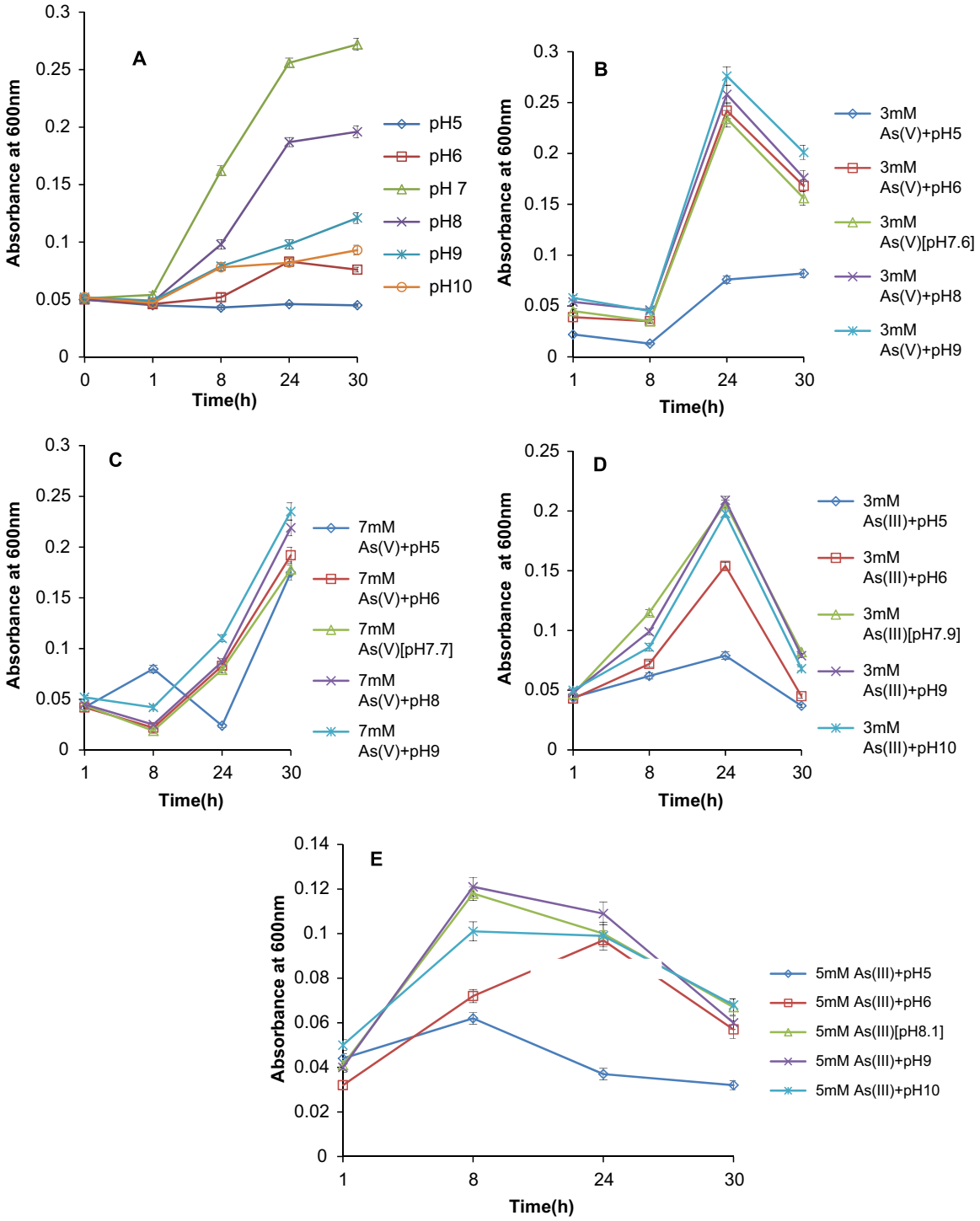

Fig. 2. Effects of pH on growth pattern of *Bacillus licheniformis* DAS-2 (A) Growth pattern in different pH of TYEG broth, (B and C) Growth pattern in different pH of As (V) stressed[3 mM As(V) and 7 mM As(V)] (D and E) As(III) stressed [3 mM As(III) and 5 mM As(III)] TYEG broth. Change in OD_{600} (Absorbance) of culture was measured over 32 h. Error bars indicate the standard error of the mean of three experiments.

acidic pH (5 and 6) as well as in basic pH (10). Statistically significant effect of pH on growth of bacterium in As(III) stress was found with $\mathbf{p=0.044}$.

3.3. As(V) removal potential of Bacillus licheniformis DAS-2

Removal of As(V) from the As(V) enriched growth media by *Bacillus licheniformis* DAS-2 depends upon both, the As (V) enrichment and also on pH of the growth medium. Significant reciprocal correlation $\mathbf{r=-0.90237}$ was found between concentration of As(V) enrichment and removal of As(V). There was gradual reduction in percentage removal of As(V) as the supplied amount of As(V) was increased in media (Fig. 3). Almost 100% removal of As(V) was measured in initial enrichment (3 mM) of the medium. As the concentrations were increased like 5 mM and 7 mM of As(V), the respective removal of As(V) was 60% and 35% only. pH was the second most important factor which affected the removal of As(V). Change in pH of As(V) (acidic or basic) enriched

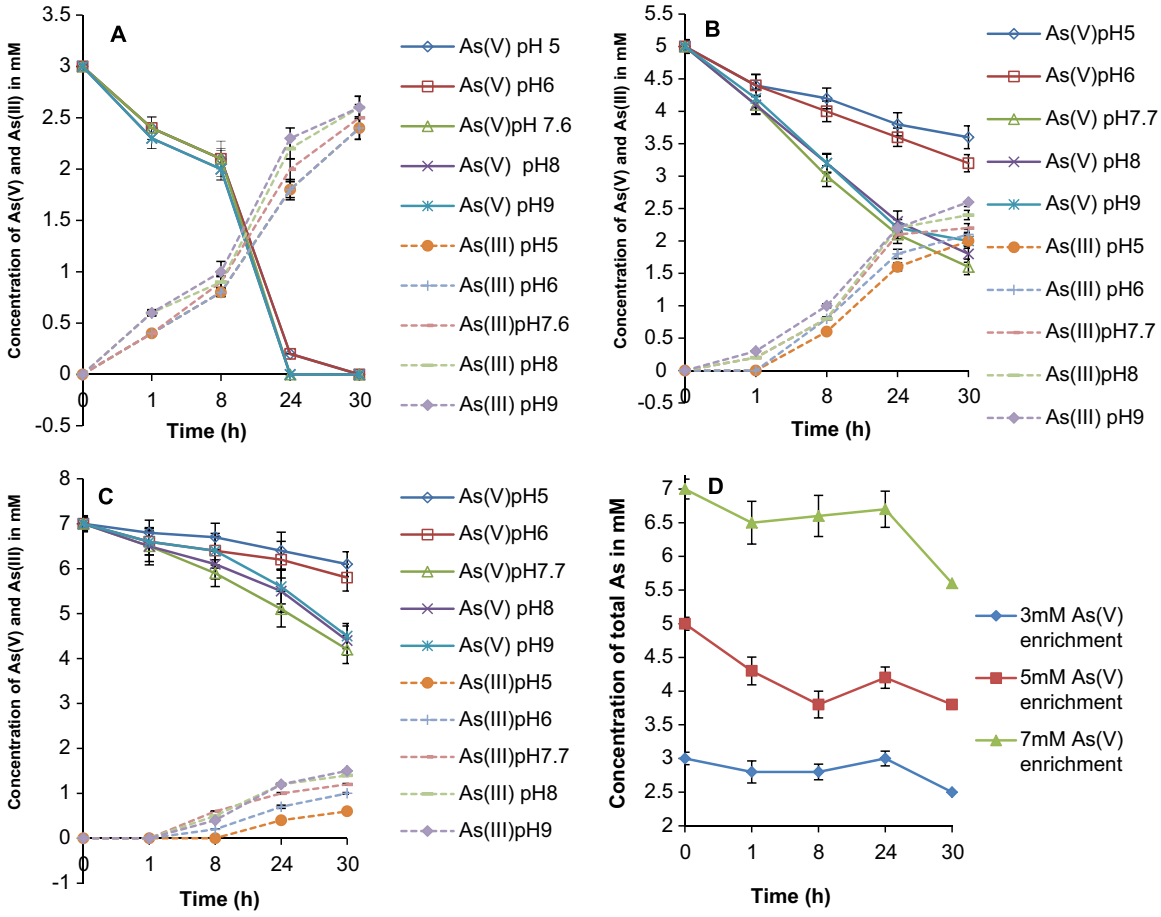

Fig. 3. Removal of As(V) from the growth media and reduction of As(V) to As(III) by *Bacillus licheniformis* DAS-2. Y-Axis represents the concentration of As(V) and As(III) in residual media at different time point (X-axis) of growth phase at different concentration of As(V) enrichment (A) 3 mM As(V), (B) 5 mM As(V), (C) 7 mM As(V) and (D) showing total arsenic in residual media at different time point of growth phase at different concentration of As(V) enrichment. All values are mean of three replicates and standard errors (SE) are presented as error bars (±).

growth medium, resulted in reduction of removal of As(V), mostly at all concentrations. Minimum removal of As(V) was observed as a result of combined toxicity of acidic (pH 5) and higher concentration of As(V) [7 mM]. Whereas, slight increment in removal of As(V) was observed in alkaline pH (8 and 9) at lower concentration of As(V) i.e 3 mM. Statistically significant value i.e **p=0.011** particularly during log phase, shows the pH dependent As(V) removal efficiency.

3.4. As(III) removal potential of Bacillus licheniformis DAS-2

It is significant to note that *Bacillus licheniformis* DAS-2 tolerated and removed both As(V) as well as As(III) at different concentrations. As(III) removal was also dependent on the supplied concentration of As(III) in the media with value of the correlation **r=−0.49485** between As(III) enrichment and As(III) removal. Initially, concentration of both As(V) and As(III) (1 mM and 3 mM), the removal efficiency was almost 100% (Fig. 4). As(III) was found more toxic than As(V) because *B. licheniformis* DAS-2 has tolerated the As(III) maximum to only 6 mM with 8% removal efficiency, whereas for As(V), the maximum tolerance concentration was 7 mM with 35% removal efficiency. At 5 mM enrichment [As(III) and As(V)] the removal potential of *B. licheniformis* DAS-2 was almost the same i.e 58% and 60%. The effect of change in pH on As (III) removal was almost similar to As(V) i.e. both acidic and basic pH change had caused decrement in removal efficiency and minimum removal was observed in more acidic pH (5). Variation **p=0.0061** in As(III) removal due to the effect of pH particularly during log phase was found.

3.5. Reduction of As(V) to As(III) by Bacillus licheniformis DAS-2

Potential of reduction of As(V) to As(III) in different [As(V)] stress with variable range of pH has been shown in Fig. 3. Potential of reduction of As(V) into As(III) was also dependent on the concentration of supplied As(V) in the media. There was a positive and significant correlation between As(V) uptake and As(III) formation at different pH that are **r=0.895991** at pH 5**, r=0.920861** at pH 6**, r=0.94601**at neutral pH, **r=0.948379** at pH 8 and **r=0.970795.**

80% As(V) was reduced to As(III) with 0.12 mM per h reduction rate in 3 mM As(V) enriched media, whereas 44% of As(V) was reduced to As(III) with a reduction rate of 0.036 mM per h, in 5 mM As(V) enrichment and 17% of reduction with 0.012 mM per h reduction rate was estimated in 7 mM As(V) enriched media. Change in efficiency of reduction, due to change in pH during the log phase was evaluated with **p=0.067**. Acidic pH (5 and 6) slightly diminished the reduction and basic pH (8 and 9) slightly enhanced the reduction of As(V) to As(III) in all concentration of As (V) stress (Fig. 3). Maximum reduction (86%) was observed at pH 9 in 3 mM As(V) supplied media.

3.6. Chemical toxicity

Toxicity effect at cellular (enzyme) level have the advantage of being more sensitive than investigation at population level (Liu, 1981). Percentage inhibition of microbial dehydrogenase in

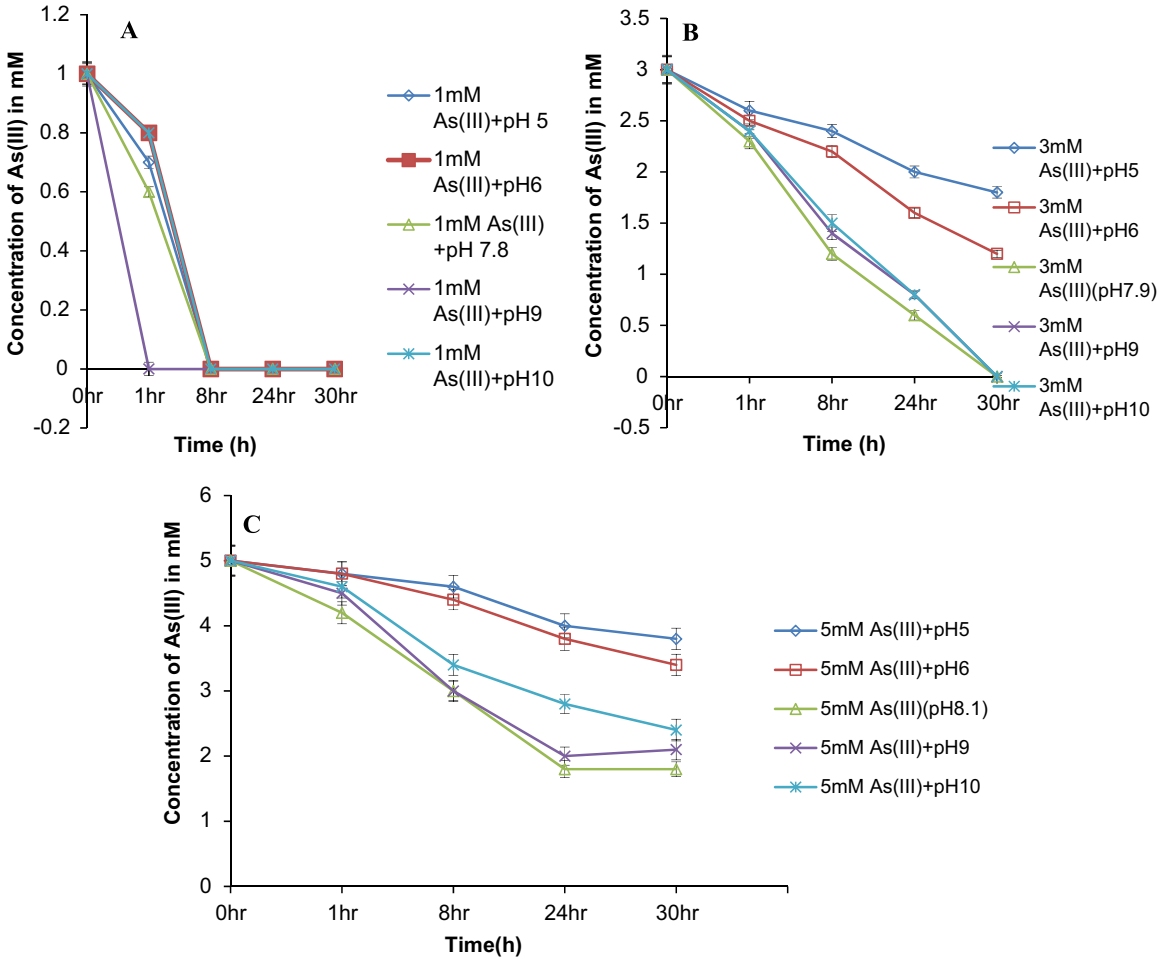

Fig. 4. Removal of As(III) from the growth media by *Bacillus licheniformis* DAS-2. Y-axis represents the concentration of As(III) left in residual media, at different time point (X-axis) of growth phase and at different concentration of As(III) enrichment. (A) 1 mM As(III), (B) 3 mM As(III). All values are mean of three replicates and standard errors (SE) are presented as error bars (±).

presence of different toxicants (Fig. 5) were showing similar pattern of toxicity for *Bacillus licheniformis* DAS-2 in the presence of different stress parameters, as described in above results. On comparing percent inhibition of dehydrogenases in presence of As (V) stress with changed pH conditions of As(V) stress (Fig. 5(A)), it was found that % inhibitions were minimised with alteration of pH (pH 6, 8 and 9), which corresponded to decrease in levels of cellular toxicity. It can also be considered vice versa as % inhibition in case of all pH stresses (pH 5, 6, 8 and 9) were found less on addition of As(V), leading to the conclusion that - As(V) modulates the pH stress. Little contribution of pH in minimising the toxicity of As(III) stress was shown (Fig. 5(B)).

4. Discussion

Changes in geochemical cycle and microbial metabolism ultimately result in intensified level of arsenic in the environment (Tsai et al., 2009; Frankerberger, 2002; Islam et al., 2004). Both the above phenomena are responsible for the release of arsenic into the drinking water in the shallow wells. Ground water of the Indo-Gangetic plain of Bengal Delta has been reported as heavily contaminated by arsenic (Chakraborti et al., 2004; Chowdhury et al., 2009). The site of this study is a region in Indian state i.e Bihar which is a part of Bengal delta and is located at 85° 32′E longitude and 25° 11′N latitude on the Earth. Although arsenic is toxic but it was observed that microorganism can utilize arsenic as a source of energy, resulting into arsenic detoxification or mitigation (Cervantes et al., 1994; Macy et al., 1996; Newman et al., 1997; Santini et al., 2000; Anderson and Cook, 2004). Hence it is important to look for the presence and moderation of arsenic utilizing system in native bacterial species for the amelioration of arsenic contamination.

Bacterium was isolated by the agar plating technique and identified by 16SrDNA sequencing as *Bacillus licheniformis* DAS-2 and was provided with accession number KF664028.

4.1. Growth profile reveals the arsenic toxicity

Gradual decrease in cell growth and increase in the duration of lag phase with the increasing arsenic [As(V) and As(III)] concentration in the medium was the evidence of the arsenic toxicity. Cell growth was reduced up to 98%, in 8 mM As(V)/arsenate and up to 85% in 6 mM As(III)/arsenite enrichment medium which was considered as the MICs and was showing As(III) more toxic which is similar to other reports (Kaltreider et al., 2001). It might be due to the fact that As(III), has very high affinity for protein thiols so it easily gets chelated with the intracellular proteins and directly caused damage to the bio molecules of the cell (Liu et al., 2001). As(V) acts as a substitute of phosphate and inhibits oxidative phosphorylation resulting cell toxicity (Wolfe-Simon et al., 2011; Mukhopadhyay et al., 2002). The other toxic effects of arsenic [As(V) and As(III)] were the significant increase in the length of lag phase of the growth with the increase in arsenic stress (i.e from 2 h to 10 h in higher concentration). During the lag phase, sub population undergoes the biochemical adaptation

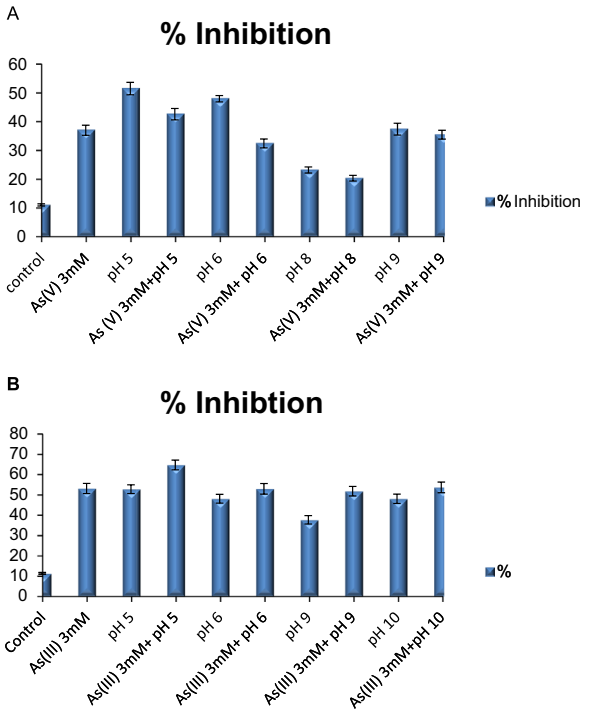

Fig. 5. Percent inhibition of bacterial dehydrogenases by different stresses. (A) As (V) stress, (B) As(III) stress. All values are mean of three replicates and standard errors (SE) are presented as error bars (±).

in new arsenic stress (Zhou et al., 2011). Hence duration of the lag phase was directly proportional to the level of arsenic stress.

Bacillus licheniformis DAS-2 has tolerated both As(V) and As(III) at different concentrations with the MIC for As(V) is 8 mM and for the As(III) is 6 mM. *Bacillus* was reported earlier as As(V) tolerant species and other bacterial species tolerating either As(V) or As(III) are *Paracoccus*, *Alcaligenes* and *Pseudomonas*, (Bachate et al., 2009; Xiong et al., 2006; Clausen, 2000). *Pseudomonas* and *Corynebacterium* were reported as As(III) tolerant species (Anderson and Bruland, 1991; Nagvenkar and Ramaiah, 2010). There are very few reports about a species capable of tolerating both As(V) and As (III) (Banerjee and Santra, 2010). This report on *B. licheniformis* DAS-2 might be new information.

Acidic pH is more toxic than basic pH, near neutral to slightly alkaline TYEG broth was the most suitable growth condition for *B. licheniformis* DAS-2 (Fig. 2(A)). pH significantly affected/modulated the growth of bacterium in all concentrations of As(V) stress (Fig. 2B and C). It might be due to significant decrease in uptake of As(V), in changed acidic and basic pH condition. As stated that availability of As(V) for the uptake by phosphate transporter was higher at near neutral pH (Tu and Ma, 2003). Lesser amount of As (V) uptake had also reduced the cytotoxic effect and hence promoted the growth. But minimum growth was observed in most acidic condition (pH 5) with the As(V) stress, which was the evidence of the intolerance in highly acidic condition (Fig. 5) (Anderson and Cook, 2004). In case of As(III) stress neutral to less alkaline (pH 9) condition was more favourable for the growth of *B. licheniformis* DAS-2. Further changes in pH had diminished the growth (Fig. 2D and E). It might be due to the combined effect of As(III) and pH stress toxicity. Toxicity of both As(V) and As(III) was significantly affected by pH which is also supported by other report. (Fulladosa et al., 2004).

4.2. Potential of Removal/uptake of arsenic species [As(V) and As(III)]

The As(V) removal potential of *Bacillus licheniformis* DAS-2 was dependent on the supplied amount of As(V) in the growth media (Fig. 3.). Maximum removal (100%) was found in 3 mM of As (V) enriched media, and minimum (35%) removal was observed at 7 mM As(V) enrichment. The present strain of bacteria tolerates As (V) up to the significant toxic level with MIC 8 mM. It was noticed by the decreasing level of As(V) in the growth medium, which is the evidence of As(V) transporting capability of the bacteria. With reference to few literature it can be explained in this manner since As(V)/Arsenate ($AsO4^{3-}$) is similar to phosphate ($PO4^{3-}$) in ionic form, hence As(V) might enter into cell through phosphate transport membrane system (Sanders et al., 1997; Wolfe-Simon et al., 2011). As(V) might also act a substitute of phosphate in oxidative phosphorylation resulting into its inhibition and hence causes the cell toxicity. The As(V) toxicity is found as directly proportional to the concentration of supplied As(V).

The pH is also one of the influential factors on the removal of As (V), as change in pH of As(V) enriched medium had caused significant decrement in As(V) removal efficiency. Since pH is highly significant variable in controlling arsenic speciation (Fitz and Wenzel, 2002) so availability of arsenic species for *B. licheniformis* DAS-2 in growth medium depends on pH. At around neutral pH, As(V) is in ionic form as Arsenate ($AsO4^{3-}$) and As(III) is in non ionic condition as $As(OH)_3$ which is most available form for the uptake by the bacteria (Tu and Ma, 2003).

Bacillus licheniformis DAS-2 has tolerated up to 5 mM As(III) (MIC 6 mM), which might be considered as hyper tolerant for As (III). Because in majority of the reports, native As(III) tolerant rhizospheric bacterial species had MIC 1–5 mM (Huang et al., 2010; Liao et al., 2011). As(III) removal potential of *Bacillus licheniformis* DAS-2 was 100% at lower concentration of supplied As(III) in the media (1 mM), which decreased up to 58% at 5 mM of supplied As(III). As(III)/Arsenite (AsO^{2-}) occurs in its hydroxide form as $As(OH)_3$ at neutral pH, which is an inorganic equivalent of non-ionized glycerol, hence As(III) might uses glycerol membrane transport system to move across the cell (Mukhopadhyay et al., 2002; Ramirez-Solis et al., 2004). There was no biotransformation of As(III) observed.

pH significantly effect on As(III) removal efficiency of *B. licheniformis* DAS-2 particularly during the log phase of growth. Maximum removal of As(III) by *B. licheniformis* DAS-2 from the As(III) enriched growth medium was observed at neutral pH. On moving towards acidic or basic pH change of the medium, there was reduction in As(III) removal efficiency. It was due to less availability of As(III) in non-ionic form [$As(OH)_3$] for the uptake via glycerol transporter at changed pH condition than at neutral pH (Tu and Ma, 2003).

4.3. Reduction of As(V) to As(III) by Bacillus licheniformis DAS-2

Almost all microorganism either prokaryote or eukaryote have natural defense mechanism for the arsenic detoxification. This tolerant system of bacteria is mostly governed by the phenomena of arsenic transportation, transformation, and extrusion from the cell or immobilization in the cell (Banerjee and Santra, 2010; Bhattacharjee and Rosen, 2007; Rosen, 1999; Tsai et al., 2009). There are many mechanisms of arsenic transformations by the bacterial cell like oxidation, reduction, and methylation etc. in which at least one type of arsenic transforming mechanism is present in each bacteria. As(V) is the predominant in oxidized environment (Oremland and Stolz, 2003) and microbial reduction of As(V) to As(III) is most common natural phenomena which increases the mobility and bioavailability of Arsenic (Harvey et al., 2002; Macur et al., 2001). In the present study we observed the transformation/reduction of uptaken As(V) to As(III) by *Bacillus licheniformis* DAS-2. Estimation of arsenic by azure B is based on reaction of As(III) radical with the coloured compound, so inorder

to quantify total arsenic in the sample first conversion of all As (V) into As(III) is required and then concentration of total As(III) is determined as total arsenic. Hence speciation of arsenic can be done by quantifying As(V) and As(III) separately. Presence of As(III) in residual media which was previously enriched by As(V) only, signifies the transformation of As(V) into As(III). Non speciation of arsenic in TYEG broth without bacterial inoculation was confirmed as both As(V) and As(III) were found stable in shaking TYEG.

Removal of As(V) as total arsenic, from the growth media was observed at initial phase of growth and addition of As(III) in same growth media was found after few hours (Fig. 3). It can be explained in this manner that *Bacillus licheniformis* DAS-2 had uptake As(V) first and then reduced to As(III) which was extruded in the growth media. This can be inferred by the gradual decrement in the concentration of As(V) and gradual increment in concentration of As(III) in residual media. We can conclude this process of reduction as intracellular because concentration of total arsenic in residual growth media had shown trends of removal and addition (Fig. 3(D)). It can be explained and also supported by other reports in manner that As(V) might first enters into bacterial cell with the help of phosphate transporter system, then reduced to As(III) inside the cell with the help of arsC gene product arsenate reductase (Mukhopadhyay and Rosen, 2002). As(III) accumulated in the cell then extruded by arsB gene product i. e. an antiporter protein channel (Meng et al., 2004). There was very less possibility of volatilization of arsenic from total supply because at the end of experiment average loss of total arsenic was less than 1 mM in lower concentration enrichment and more than 1 mM at higher concentration enrichment which also include approximately 1 mM arsenic accumulated in biomass. Another strain of *B. licheniformis* (DAS-1) had also responded in similar manner against arsenic stress (Tripti et al., 2014). Efficiency of reduction of up taken As(V) into As(III) was also dependent on concentration of As (V) supplied in media. 80% of As(V) was reduced to As(III) in 3 mM of As(V) enrichment which was decreased to 17% in 7 mM As (V) enrichment (Fig. 3.). Effect of pH on efficiency of *B. licheniformis* in the reduction of As(V) to As(III) was also found. Basic change in pH contributes more in the reduction of As(V) to As(III), it might be due to the fact that alkaline condition favoured the reduction of As (V) to As(III) (Anderson and Cook, 2004), Whereas acidic change in pH slightly diminished the reduction of As(V) to As(III).

pH was shown as one of the important influential factor in deciding the arsenic toxicity, as it had been altered the behaviour of growth in arsenic stress, efficiency of removal of As(V) and As (III) from the medium, efficiency of reduction of As(V) to As(III) of *B. licheniformis* DAS-2.

4.4. Chemical toxicity/cellular toxicity

Chemical toxicity to bacteria is normally measured in terms of inhibition of microbial growth rate (Narkis and Zur, 1979) or colony formation on agar plate (Anderson and Abdelghani, 1980). Biochemical test prove to be more sensitive than above mentioned techniques because it is based on interaction between toxicant and enzyme activity. Interaction between toxicant and enzyme results in the inhibition of enzyme activities ultimately which lead to the death of the organism. Dehydrogenases are involved in vital anabolic and catabolic reactions and hence considered ideal for chemical toxicity studies (Liu, 1981). Percentage inhibition of bacterial dehydrogenases by different types of stress (Fig. 5) reproduced the similar observations as presented in above results and discussions. Measurement of cellular toxicity had strongly favoured the role of pH in modulating arsenic toxicity in *B. licheniformis* DAS-2.

5. Conclusion

Bacillus licheniformis DAS-2 is a native soil bacteria which was isolated from the arsenic contaminated region located at 85° 32′ E longitude and 25° 11′N latitude on the Earth. It is unique that the bacterium had shown the capability to tolerate both As(V) [MIC 8 mM] and As(III) [MIC 6 mM]. The bacteria had also removed/uptaken good enough amount of As(V) and As(III) (i.e 100%) from the growth medium particularly at the lower concentration of arsenic enrichment. As(III) was determined in the same media which was previously enriched by As(V) only, along with concentration of As(V) was found decreasing from the media. This phenomenon signified transformation/reduction of uptaken/removed As(V) into As(III) which might be one of the survival strategy of *B. licheniformis* DAS-2 to tolerate arsenic toxicity. Efficiency of arsenic tolerance (growth, removal, transformation [As (V) to As(III)]) of *B. licheniformis* DAS-2 were significantly altered with different concentrations of arsenic and pH. Results support the conclusion that pH modulate the arsenic toxicity in *B. licheniformis* DAS-2 which can play better role in amelioration of arsenic contamination.

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