Screening of Potent Arsenic Resistant and Plant Growth Promoting *Bacillus* species from the Soil of Terai Region of Nepal

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ABSTRACT

Objectives: To isolate arsenic resistant *Bacillus* spp. and to determine plant growth promoting activities.

Methods: Eighteen soil samples were collected from the agricultural soil of Terai region of Nepal. Selective isolation of *Bacillus* species was done by heating the soil at 80 ^{o}C for 15 minutes before the isolation. Nutrient agar was used as an isolation medium. Screening of arsenic resistant *Bacillus* species was done using nutrient agar supplemented with 100 ppm sodium arsenate and sodium arsenite. For plant growth promoting activity; IAA production was detected taking 0.1% tryptophane and measuring absorbance at 540 nm, NH_3 production was tested by Nessler's reagent and phosphate solubilization activity was detected by growing colonies on Pikovskaya's agar. Sugar assimilation test was performed to identify the isolates. Most potent arsenic resistant isolate was identified by 16S rRNA gene sequencing.

Results: Among 54 randomly selected isolates, 42 were found to be Gram-positive rod-shaped, spore-forming while 12 isolates were Gram-negative bacteria. The isolates $IN_1$2a, $M_1$2a and $BG_3$4a showed growth on 100 ppm sodium arsenite containing NA. Only isolate $M_1$2a tolerated up to 1000 ppm and 15000 ppm of sodium arsenite and sodium arsenate respectively, while other isolates could not grow above 400 ppm sodium arsenite. The isolates $IN_1$2a and $M_1$2a were able to produce IAA and solubilize phosphate while $BG_3$4a could not. Both the isolates $IN_1$2a and $M_1$2a were able to utilize the sugars glucose, fructose, lactose, sucrose, galactose, mannose, mannitol, maltose and xylose. Based on the 16S rRNA gene sequencing, isolate $M_1$2a was identified to be *Bacillus flexus* with highest similarity of 99.2%.

Conclusion: Arsenic resistant and plant growth promoting *Bacillus* spp. was isolated from the agricultural soil of Terai region of Nepal.

Key words: Soil, Arsenic resistant *Bacillus*, Plant growth promotion, Bioremediation

INTRODUCTION

Arsenic (As) is a poisonous heavy metalloid present in the soil as well as in the water as arsine (III), elemental arsenic (0), arsenite (III) and arsenate (V). It has been reported that, arsenite and arsenate forms are poisonous to environment as well as human health (Dey et al. 2016). The activity that leads to As pollution are mining, smelting, ore processing, and utilization of arsenic-based pesticides or herbicides, and arsenic contaminated water when irrigated posed heavy contamination of soil, especially in cultivated land ecosystem (Shagol et al. 2014). Arsenic consumption beyond a threshold level i.e. 0.05 ppm pose a serious health risk to humans (Dey et al. 2016). Exposure

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of arsenic to body parts cause cancer. nervous and cardiovascular problems (Ghosh et al. 2011), weight loss, loss of appetite, weakness, lethargy and easily fatigued limits the physical activities and working capacities, chronic respiratory disorder, gastrointestinal disorders like anorexia, nausea, pain in abdomen, enlarged liver and spleen as well as anemia (Dey et al. 2016).

In Nepal, safe drinking water supply is one of the major issues. Groundwater is the foremost source of drinking water in Terai region of Nepal which is usually contaminated with arsenic (Shakya, et al. 2012). Such groundwater when irrigated in crop land increases the concentration of As in crop fields and soil get contaminated. It leads to severe threats for bio amplification by entering into the food chain (Mallick et al. 2014). According to Nepal standard and World Health Organization (WHO) the concentration of arsenic in drinking water are 50µg/L and 10µg/L respectively. Nawalparasi district is placed as a prone to the Arsenic problem where the arsenic concentration is significantly beyond the safe limit (Smith et al. 2009).

Moreover, the presence of Arsenic and its forms in the environment has developed a many bacteria Arsenic resistance mechanisms like arsenite methylation, arsenite oxidation, etc. (Mallick et al. 2014). Currently the detoxification of arsenic by using bacteria has become an interest due to environmental issues immersed by other conventional chemical processes (Banerjee et al. 2013). Bacteria play a significant role in the biochemical cycle of arsenic and changes to different oxidation states with different solubility, mobility and toxicity (Banerjee et al. 2013). The important enzyme i.e. arsenic oxidase is present in the protoplasm of arsenic oxidizing bacteria which oxidizes arsenite to arsenate (Dey et al. 2016). Some bacteria such as *Bacillus subtilis, Deinococcus indicus*, *Pseudomonas fluorescens, Thermus aquaticus, Thermus thermophilus, Yersinia enterocolitica, Bacillus arsenicus* have efficiently removed soluble and particulate forms of metals, especially from dilute solutions via bioaccumulation (Pepi et al. 2011).

Bacillus species are becoming interest due to its role in wide variety of fields such as bioremediation, enzyme production, plant-growth-promoting (PGP) traits, organic acid production etc. (Poudel et al. 2016). Till now, there are limited research findings on Arsenic resistant and plant growth promoting Bacillus species regardless of their tremendous applications in Nepal. The main propose of this study is to explore the arsenic resistant *Bacillus* species having plant growth promoting traits so as to minimize Arsenic pollution and increase the yield of crops. Furthermore, potent *Bacillus* strains could be applicable as a bio fertilizer for sustainable bioremediation in agriculture.

MATERIALS AND METHODS

Sample collection, isolation and screening of arsenic resistant bacterial species

Twenty soil samples were collected from Terai region of Nepal. About 20 g of soil samples was collected in a polythene bag and processed for isolation. For the selective growth of spore forming Bacillus species, 10 g of soil sample was mixed with 100 mL of 0.85% saline solution and placed in 80°C water bath for 10 minutes (Travers et al. 1987). It was serially diluted and spread on to nutrient agar plates. All the plates were incubated at 37°C for 48 h. Bacterial colonies were sub-cultured onto Nutrient Agar plates supplemented with different concentration of sodium arsenite (up to 800 ppm) and incubated. The media plates, after incubation, were observed for growth of bacteria colonies. The colonies that showed growth were sub-cultured on nutrient media and incubated. After incubation, these colonies were subjected to further tests and studies (Selvi et al. 2014). Silver nitrate test was performed in NA plates supplemented with sodium arsenite. A single line streak (perpendicular) of the screened organism was drawn on the agar surface and incubated at 37˚C for 48 h. After incubation, the plates were flooded with 0.1 M silver nitrate solution and observed for brownish precipitate (Simeonova et al. 2004).

Phenotypic characterization of arsenic-resistant bacteria

The bacterial isolates that tolerated arsenate and arsenite concentration were selected and characterized by the morphological and biochemical features (Indole production, MR-VP test, Citrate utilization, Oxidase test, Catalase test, Starch hydrolysis, Gelatin hydrolysis, Triple sugar iron test, Mannitol salt agar, Urea hydrolysis test, Dextrose, Sucrose, Maltose, Rhamnose, Arabinose and Sorbitol tests).

Effect of arsenic on bacterial growth

Growth of arsenic resistant bacterial strains was determined in NB medium. From an overnight pure culture, 1% inoculum was added to 50 ml of NB medium supplemented with 200 ppm, 400 ppm, 600

ppm, 800ppm, 1000 ppm sodium arsenite. The cultures were incubated at 37 °C in an orbital shaker at 120 rpm for 72 h. The growth of the isolate was monitored by measuring optical density at OD 600 nm using spectrophotometric method.

Effect of NaCl and pH in the growth of arsenic resistant isolates

The freshly prepared culture was inoculated into the 5 mL of sterile NB and incubated at 37°C for 24 h. The optical density of the culture broth was measured at 600 nm using spectrophotometric method. The pH of the broth was maintained by using 1N NaOH and 1N HCl. The NaCl concentration in the broth ranged from 1-8%.

Determination of plant growth promoting (PGP) activities

The Arsenic resistant *Bacillus* species were tested for PGP activities based on whether the isolates is capable to solubilize phosphates, produce indole acetic acid, siderophores and NH_3. Phosphate solubilization activity was examined by growing isolates in modified Pikovskaya's medium with 0.5% of tricalcium phosphate (TCP) and incubated at 30°C for 5 days. Quantitative analysis of IAA was performed using the method of Loper et al. (1985) at different concentrations of tryptophan (0, 50, 150, 300, 400 and 500 mg/ml). Isolates were grown for 48 h on their respective media at 37°C. Fully-grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of per chloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink color indicated IAA production. For the ammonia production, freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48–72 h at 28°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow color indicated a positive test for ammonia production.

Molecular identification of potent arsenic resistant *Bacillus* species

Genomic DNA was extracted by using phenol-chloroform assay method. DNA Amplification of the 16S rRNA gene was performed using the following universal primer sets: 8f (5′ AGA GTT TGA TCC CTC AG 3′) and 1492r (5′ GGT TAC CTT GTT ACG ACTT 3′). The amplification conditions were as follows: 30 cycles of DNA denaturation at 98°C for 10 s, primer annealing at 55°C for 5 s, and elongation at 72°C for 1 min. Polymerase chain reaction products was purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions. Sequence homology was compared with 16S rRNA gene sequences available in the DDBJ/EMBL/GenBank DNA database using the FASTA algorithm (http://www.ddbj.nig.ac.jp/), and all reference sequences was obtained through the Ribosomal Database Project II (http://rdp.cme.msu.edu/). Sequences were aligned using CLUSTAL W ver.2.01 (http://clustalw.ddbj.nig.ac.jp/) and phylogenetic tree was constructed using MEGA ver.7 by neighbor-joining method with bootstrap values calculated from 1,000 replications.

RESULTS

In total, 54 colonies were randomly selected and sub-cultured on NA media. Among this, 42 isolates were Gram positive rods and 12 isolates were Gram negative. Twelve isolates were excluded for further studies. Forty-two isolates showed moist, flat, irregular, and slightly convex colonies on Nutrient agar and were endospore forming rods (Table 1). Among these, 6 isolates had terminal spores, 14 had sub-terminal, and central spore was seen in 22 isolates. *Bacillus* spp. were isolated from soil having pH ranging from 5.2-6.3.

Table 1: Position of endospores in bacterial isolates

Location	pH of soil	Total no. of isolates	Endospore position		
			Terminal	Sub-terminal	Central
Sarlahi	5.6	3	1	2	-
Biratnagar	5.9	4	1	3	-
Saptari	6.1	9	-	1	8
Palpa	6.3	11	2	5	4
Parsa	5.3	2	1	-	1
Bara	5.8	2	-	-	2
Yangjakot	5.7	2	-	1	1
Birgunj	5.2	5	1	-	4
Nawalparasi	6.0	4	-	2	2

In total, only three isolates were able to resist 100 ppm and 1500 ppm of sodium arsenite and sodium arsenate respectively. These isolates were subjected to biochemical tests (Table 2).

Table 2: Biochemical tests of bacterial isolates

Test	Isolate		
	$IN_1$2a	$M_1$2a	$BG_3$4a
Catalase	Negative	Negative	Negative
Oxidase	Positive	Positive	Positive
O/F	Fermentative	Fermentative	Fermentative
Indole	Negative	Negative	Negative
MR	Positive	Positive	Positive
VP	Negative	Negative	Negative
Citrate	Negative	Negative	Negative
Urease	Negative	Negative	Negative
TSIA	Acid/Acid	Acid/Acid	Acid/Acid
Motility	Motile	Motile	Motile
H_2S	Negative	Negative	Negative

Two isolates $M_1$2a and $IN_1$2a were able to oxidize arsenite to arsenate whereas isolate $BG_3$4a was not able to do so (Figure 1). So, BG34a was not tested for PGP activities.

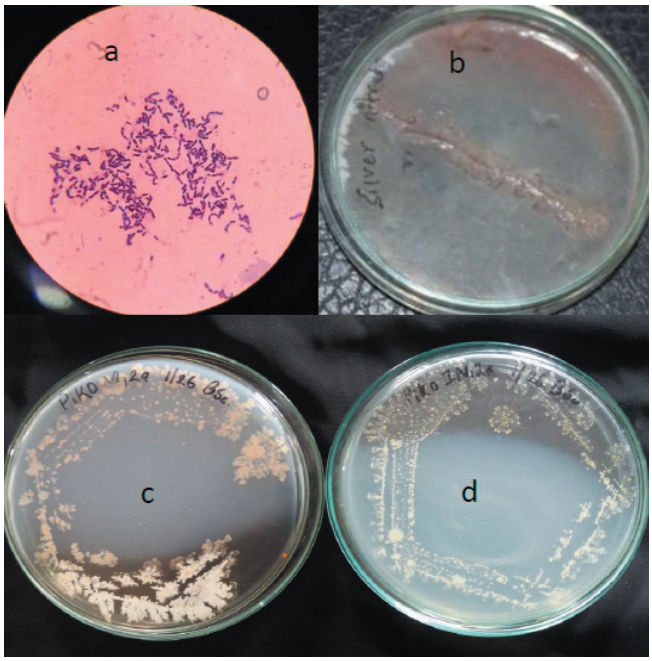

Figure 1: Typical features of Isolates; Gram stain of isolate $M_1$2a (a), Detoxifiction of arsenite to arsenate by isolate $M_1$2a (b), Phosphate solubilization activity of isolates $M_1$2a (c) and $IN_1$2a (d).

Table 3 describes the IAA production by isolates $IN_1$2a and $M_1$2a. Maximum absorbance of 0.105 was observed in $IN_1$2a culture broth containing tryptophan at 0.05 g/L and the lowest value of -0.015 was seen at concentration of 0.22 g/L. In Isolate $M_1$2a, absorbance of 0.423 was observed at 0.5 g/L of tryptophan and lowest of -0.02 at 0.25 g/L of tryptophan.

Table 3: IAA production ability of the isolates after 24 h of incubation at 37°C

Organism	Concentration of tryptophan (g/L)	Absorbance
$IN_1$2a	0.05	0.105
	0.12	0.047
	0.18	0.03
	0.22	-0.015
	0.25	-0.014
$M_1$2a	0.05	0.423
	0.12	0.190
	0.18	-0.007
	0.22	-0.012
	0.25	-0.02

Tested isolates ($IN_1$2a and $M_1$2a) could not produce ammonia after addition of reagent. Both the Isolates $M_1$2a and $IN_1$2a were able solubilize phosphate in Pikovskaya's agar (Figure 1).

Isolates $IN_1$2a and $M_1$2a were able to ferment sugars like glucose, fructose, lactose, sucrose, galactose, mannose, mannitol, maltose and xylose. Based on the sugar assimilation pattern, test isolates could be *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. brevis*, *B. stearothermophilus.*

Table 4: Sugars assimilation pattern of isolates

Organism	Sugars	Result	Possible organisms
$IN_1$2a	Glucose	Positive	*Bacillus subtilis*
	Fructose	Positive	*Bacillus licheniformis*
	Lactose	Positive	*Bacillus pumilus*
	Sucrose	Positive	*Bacillus brevis*
	Galactose	Positive	*Bacillus stearothermophilus*
	Mannose	Positive	
	Mannitol	Positive	
	Maltose	Positive	
	Xylose	Positive	
$M_1$2a	Glucose	Positive	*Bacillus subtilis*
	Fructose	Positive	*Bacillus licheniformis*
	Lactose	Positive	*Bacillus pumilus*
	Sucrose	Positive	*Bacillus brevis*
	Galactose	Positive	*Bacillus stearothermophilus*
	Mannose	Positive	
	Mannitol	Positive	
	Maltose	Positive	
	Xylose	Positive	

As describe in Table 5, only isolate M12a was able to tolerate the sodium arsenite up to 1500 ppm.

Table 5: Growth of isolates on different concerntration of sodium arsenite containing NA media after incubation at 37 °C, 48 hours

Isolates	Concentration of Sodium arsenite (ppm)	Absorbance (λ=610 nm)
$M_1$2a	**0**	**1.723**
	200	1.649
	400	1.140
	600	1.323
	800	0.923
	1000	0.351
	1500	0.124
$IN_1$2a	0	1.213
	200	1.132
	400	0.762
	600	0.000

16Sr RNA gene sequence analysis result indicated that the isolate $M_1$2a showed 99.2% similarity with *Bacillus flexus*. The phylogenetic analysis clearly showed the isolate grouped to *Bacillus* species (Figure 2).

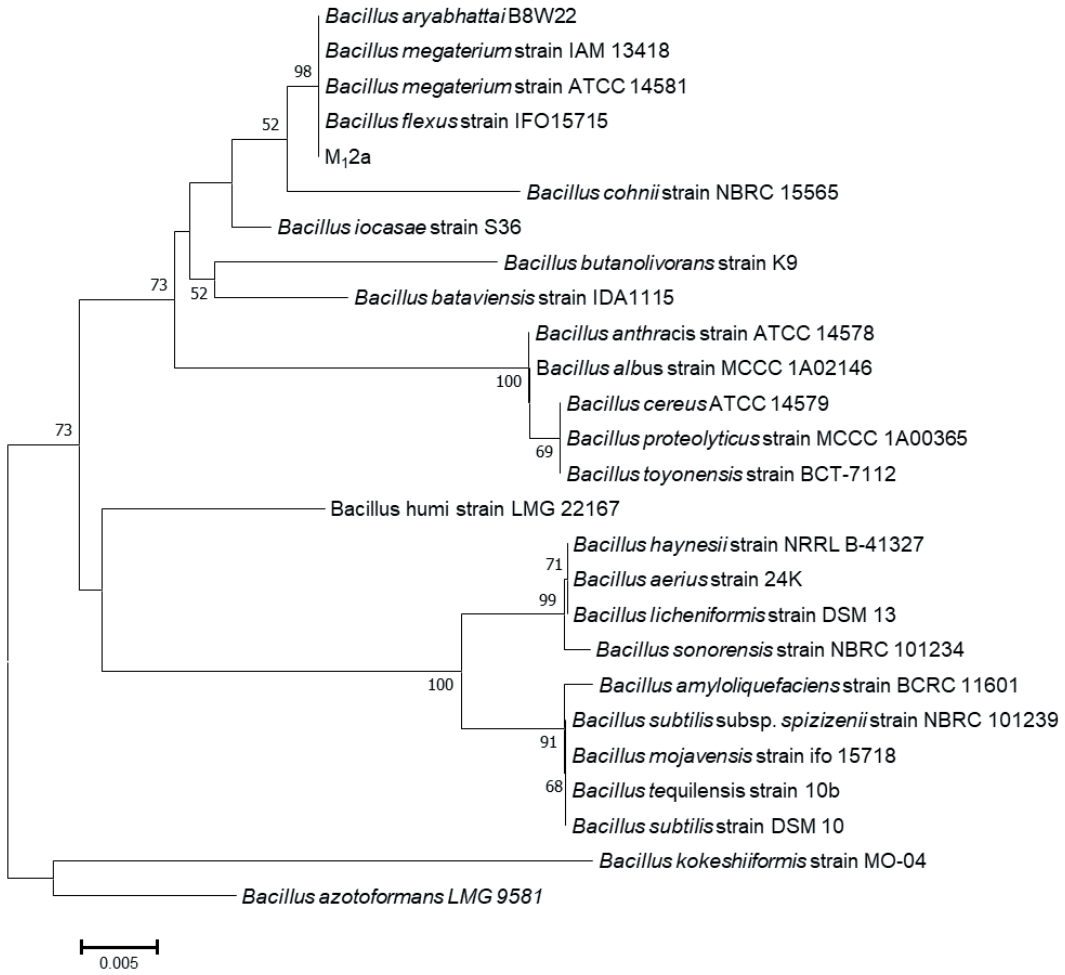

Figure 2: Neighbor-joining phylogenetic tree of isolate $M_1$2a and other closely related reference strains based on 16S rRNA gene sequences.

The growth pH of the isolate $M_1$2a and $IN_1$2a ranged from 5.0 - 9.0. The optimum pH for the growth of isolate $M_1$2a and $IN_1$2a was 7.0. Similarly, the optimum NaCl concentration for the growth of isolate $M_1$2a and $IN_1$2a was 2% (Figure 3).

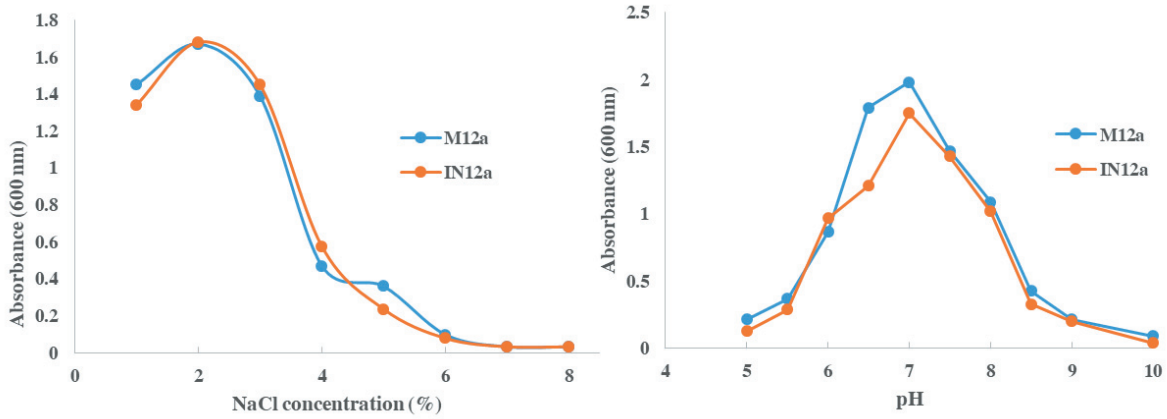

Figure 3: Growth of arsenic resistant isolates on different pH values and NaCl concentrations

DISCUSSION

From the results it can be observed that the pH of the soil was slightly acidic which is suitable for the growth of the most of plants (Kumar et al. 2019). In addition, some useful microorganisms can tolerate acidic pH and are applicable for crop enhancement (Kumar et al. 2019). Soil is the largest deposit of heavy metals such as arsenic and their compounds. These compounds may be harmful to other components of soil such as animals and plants. However, a solution to this problem can also be found in soil in the form of microorganisms that can utilize and degrade these harmful metals. Mostly the soil inhabitant microbes such as *Bacillus* species have been reported by many researchers (Schallmey et al. 2004; Radhakrishnan et al. 2017). In this study, Gram positive, endospore forming rod-shaped bacteria was isolated and tentatively identified as genus *Bacillus*. Travers et al. (1987) have reported the isolation of *Bacillus* species from various soil samples.

Isolation of arsenic resistance *Bacillus* species have been reported previously (Satyapal et al. 2016; Shakya et al. 2012; Selvi et al. 2014; Dey et al. 2016). Arsenic resistance *Bacillus aryabhattai* was isolated from the Indian soil and water samples (Singh et al. 2016). In this study, forty-two isolates were found to be Gram positive spore forming rod shaped bacteria. Shakya et al. (2011) also reported techniques of identification of *Bacillus* spp. based on cultural, morphological, and biochemical characteristics. On screening of these isolates for arsenic tolerance, 3 isolates produced colonies on Nutrient agar. The resistance was determined by inoculating the isolates on NA supplemented with sodium arsenite (Selvi et al. 2014). Colony formation in NA indicated the tolerance of arsenite and could possibly determine the toxic arsenite is utilized and converted to non-toxic forms. Only the arsenic tolerance isolates were vertically streaked on arsenic-supplemented NA and incubated. After incubation, the plates were flooded with freshly prepared silver nitrate solution which cause formation of yellowish brown precipitate which is suggestive of metabolic activity on the arsenite in the medium. This test confirmed the utilization of arsenic by the isolates $IN_1$2a and $M_1$2a. Selvi et al. (2014) also reported similar precipitation seen in media plates supplemented by arsenic and flooded with $AgNO_3$.

The arsenic resistance isolates were then tested for plant growth promoting activities. Indole acetic acid production was measured highest when the concentration of tryptophan was 0.05%. At higher concentration of tryptophan, there was low accumulation of IAA. This might be due to inhibitory effect of tryptophan against growth of *Bacillus* species. Ahmad et al. (2005) reported a contradictory result where IAA production increased with the increase in concentration of tryptophan in the medium when inoculated with *Psuedomonas* and *Azotobacter* isolates. The difference in results may be due to difference in type of microbes and their sensitivities to differing compounds. None of the isolates tested were capable of producing ammonia, both isolates were able to

solubilize phosphate in the medium. As a result, a clear zone of hydrolysis was observed around the fully developed colonies of isolates $IN_1$2a and $M_1$2a when grown on Pikovskaya's agar (Figure 1). According to Kitpreechavanich et al. (2016) *Bacillus* spp. have the ability to produce a clear zone around their colonies on Pikovskaya's agar by solubilizing phosphate in the medium.

Arsenic resistance isolates $IN_1$2a and $M_1$2a were able to assimilate all of the sugars tested. *Bacillus* spp. are well characterized and are able to assimilate the sugars as shown in Table 4. According to Bergey's manual of determinative bacteriology (1957) probably the isolates could be *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. brevis*, *Geobacillus stearothermophilus*.

Isolates $M_1$2a tolerated up to 1000 ppm of sodium arsenite and 15000 ppm sodium arsenate, which is the highest reported in Nepalese soil. In addition, mild growth was observed even up to 1500 ppm of sodium arsenite. However, further studies are necessary in this regard. The isolates showed growth from acidic to alkaline pH and at high concentration of NaCl, which is a typical characteristics of many *Bacillus* species (Poudel et al. 2016). Based on the 16S rRNA gene sequencing, high resistance isolate M12a was identified as *Bacillus flexus*. To our knowledge, this is the first report of isolation of arsenic resistant and plant growth promoting *Bacillus flexus* in Nepalese soil. These tests provide evidence that the isolates $IN_1$2a and $M_1$2a could be useful for the preparation of effective biofertilizer having PGP activity and bioremediation behavior. However, further testing is required to determine more characteristics of the isolates and also their best possible use in bioremediation of arsenic.

CONCLUSION

This is the first report to show the isolation of arsenic resistant and plant growth promoting *Bacillus flexus* in Nepalese soil. The result indicated that the isolates could be useful for the preparation of effective biofertilizer having PGP activity and bioremediation behavior. However, other experiments on arsenic resistant genes are necessary at the molecular level to understand the whole mechanism.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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