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Isolation and characterization of arsenic resistant bacteria, isolated from industrial wastewater of Pakistan region and bacterial biomass bioremediation activity in arsenic bioremediation

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Abstract

Arsenic is a toxic metalloid and ubiquitous, means found everywhere on earth. Due to toxicity it is necessary for scientist to remove or reduce it from the environments. So through bioremediation, by using the bacteria arsenic can be reduced from the environments. In this present study arsenic resistant bacterium was isolated from wastewater of industrial origin, in District Sheikhupura, Pakistan. Optimum growth conditions, growth curve, minimum inhibitory concentration regarding arsenic and other heavy metals, biochemical testing, 16S rRNA analysis, glutathione and non-protein thiol contents and bacterial biomass bioremediation activity was determined for isolated bacterial strain. The isolated strain showed best growth at 37 °C and pH 7. The minimum inhibitory concentration (MIC) regarding arsenite and arsenate in isolated strain was 32 mM and 220 mM respectively. Cross metals resistance profile was (Pb; 6 mM, Cd; 5 mM, Cr; 6 mM, Hg; 2 mM, Se; 6 mM, Co; 2 mM and Ni; 2.5 mM). On 16S rRNA sequence and biochemical basis bacteria was closely related to *Staphylococcus warner* Strain AW 25. The significant alternation in reduced glutathione level was observed under 15 mM arsenite stress. The ratio of GSH and GSSG was increased 33.33 % while Non-protein thiol was increased 55.55 % due to 15 mM arsenite stress in isolated bacterial strain. The bioremediation efficiency of isolated bacterial biomass was 92 % after 10 h. So, due to its better bioremediation activity, the isolated bacterial strain can be used in the bioremediation of arsenic from arsenic contaminated sites.

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Introduction

Arsenic is a metalloid with high toxicity (Sher *et al.,* 2019). It is found in various kind of environment due to release from natural sources as well as from anthropogenic activities (Koechler *et al.,* 2010). It is well known carcinogenic for living organism especially for human beings. Arsenic was discovered by Albertus Magnus in 1250 and in ancient time it was called by poisons of king and a king of poisons (Sher and Rehman, 2019). There are two main factor on which arsenic toxicity depend, one is its chemical form and other is its oxidation states (Rosen, 2002). Inorganic form of arsenic is more toxic than organic one, while arsenite with oxidation state +3 is hundred time more toxic than arsenate with oxidation state +5 (Mujawar *et al.,* 2019). It is reported that water contamination with arsenic is increasing day by day (Smith *et al.,* 2000). Arsenic is not a local problem for Pakistan, it has been reported that it effect on human beings in major part of the world specially northeast India, northwest part of USA and Bangladesh(Muller *et al.,* 2003) . The environmental protection agency of USA places it at the top of list for Hazardous Substances due to its toxicity (Zhang *et al.,* 2016). The drinking of arsenic containing water for long period of time cause various health related problems in human beings like, change in color of skin or cancer, diabetes, Hypertension, chromosomal aberrations, amplification of gene, alternation in eukaryotic cell morphology as well as some disorder which are linked to reproduction system (Sher and Rehman, 2019).

Microorganisms are found in every kind of environment and these microorganisms have a potential for the remediation of arsenic from contaminated environment (Dey *et al.,* 2016). Arsenic can be reduced or oxidized with the help of microorganisms (Oremland and Stolz, 2003). The conversion of arsenate (As^{+5}) into arsenite (As^{+3}) is called reduction and is carried out by the gene (*arsC*) located on chromosomal DNA or Plasmid inside the Bacteria (Mujawar *et al.,* 2019). While the oxidation of arsenic invovled arsenite into arsenate carried out by genes (*aioA, aioB*) present in bacteria (Li *et al.,* 2014). Arsenic toxicity can also be reduced with the process of methylation, in which methyltransferase gene (*arsM*) used S-adenosylmethionine (SAM) as a source of methyl group for the addition in arsenic (Li *et al.,* 2016).

The main natural source of arsenic is volcanic activities and weathering of rocks while anthropogenic sources are use of arsenic containing compounds such as pesticides, dyes and preservation of wood through which arsenic is increasing in water bodies (Prasad *et al.,* 2013). Different conventional method can be used for the removal of arsenic from water, such as membrane filtration, coagulation, ion exchange method, nanoparticles, phytoremediation and some other chemical methods (Mohanty, 2017; Ng *et al.,* 2004). These methods cannot be used further because of non-cost effective and production of secondary toxic compounds (Tariq *et al.,* 2019). The best approach for arsenic detoxification is bioremediation, in which bacteria or other microorganisms used toxic compound as a source of energy in their metabolism process and convert toxic form into less or nontoxic form (Qin *et al.,* 2006; Tariq *et al.,* 2019).

Materials and methods

Sampling

In this present study wastewater sample was taken in an already sterilized glass tube from an industrial area of Pakistan named as District Sheikhupura, is located in Province Punjab, Pakistan. The geographical coordinates of Sheikhupura are 31° 42' 59.9796" North and 73° 59' 6.0828" East at an altitude 207 m (679 feet). The geographic map as shown in (Fig. 1). 10 ml wastewater sample in glass tube was transferred to Department of Microbiology and Molecular Genetics, University of the Punjab, for further research work. Sample was kept at 4 °C until for further research works.

Physiochemical parameter of wastewater sample

Physiochemical characteristics of wastewater sample like color, temperature, pH, Electrical conductivity, turbidity, and total dissolved solid (TDS) and arsenic concentration were measured.

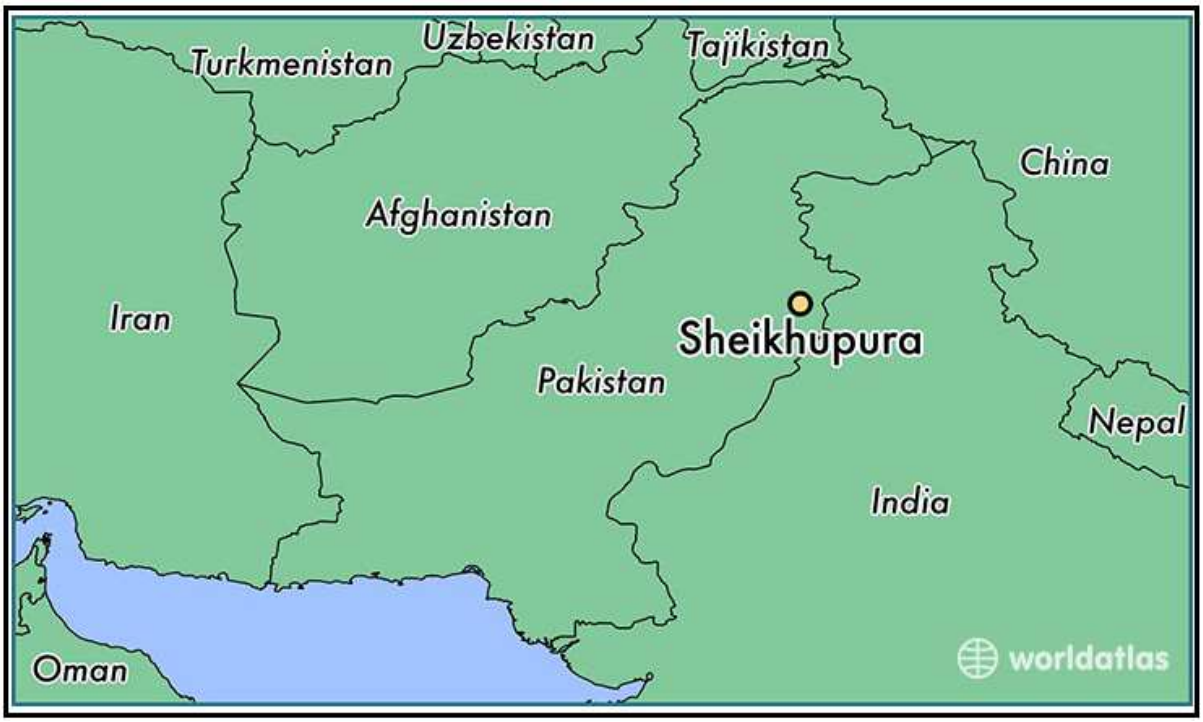

Fig. 1. Geographical map of District Sheikhupura (sampling site), Pakistan.

Temperature and pH were measured by thermometer and pH meter respectively. While digital conductivity meter and turbidity meter were used for the measurement of electrical conductivity and turbidity in the sample. Total arsenic concentration in wastewater was determined through flame atomic absorption spectrophotometer (AAS).

Optimum growth condition

The isolated bacterial strain "AS6" was grown at different temperature (20,25,30,37,42) and pH (5,6,7,8,9,10) to determine its optimum temperature and pH. For the determination of optimum temperature strain –AS6 was inoculate in five set of 250 ml flask with 100 ml Luria Bertani broth (LB-broth) in each flask, there were further three flasks in each set. But for optimum pH, bacteria were grown in five set of flask; each set was with different pH but optimum temperature. After 24 hours of incubation, 2ml of sample was taken in cuvette and determine its optical density at 600 nm through spectrophotometer for all the temperatures and pH.

Growth curve

For determination of growth curve the isolated bacterial strain was grown in the presence (15 mM As^{+3}) and absence (control) of sodium arsenite. A set of three 250 ml flask, containing 100 ml MS-broth were autoclaved and added arsenic (15 mM As^{+3}) in first two flasks, third one was used as a control (without arsenic). Each flask was inoculated with 1% freshly prepared inoculum and incubated it at optimum temperature in a shaking incubator (100 rpm). The third flask was worked as a positive control because of no arsenic stress. After every 4 h interval, growth was determined by taking 2 ml of aliquot from each flask and absorbance was taken at 600 nm through spectrophotometer. Growth curves were plotted between absorbance and time.

MIC against arsenic

For the determination of minimum inhibitory concentration against arsenic, the isolated bacterial strain was grown in different flasks, containing MS-broth (Naureen and Rehman, 2016). Each flask was supplemented with different concentration of arsenite (5 mM to 50 mM) and (5mM to 250 mM) for arsenate separately. Each flask was incubated at 37 °C in a shaking incubator with 100 rpm. After 48 h an aliquot of 2 ml from each flask was taken and optical density was taken at 600 nm through spectrophotometer.

Multiple metal resistance assays

The resistance of isolated strain against heavy metals ions (lead, cadmium, chromium, mercury, selenium, cobalt and nickel) was determined by growing it in MS-broth supplemented with respective metal ions. The concentration of stock solution was 1M for each metal ions salt (lead nitrate, cadmium chloride, potassium dichromate, mercuric chloride, sodium selenite, cobalt chloride and nickel chloride) was used. The concentration of each metal ion was increased 1 mM, each time in a stepwise manner until the growth of strain was inhibited. The bacterial growth was determined by taking optical density (OD) at 600 nm after 24 h of incubation at 37 °C.

Physical and biochemical characteristics of bacteria

The isolated bacterial strain was identified on the bases of colony morphology as well biochemical testing such as Gram staining, catalase test, oxidase test, coagulase test, urease test, fermentation of lactose, production of H2S, reduction of nitrate, production of indole, methyl red test, VP-test and growth on manitol salt agar was observed for isolated bacterial strain (James and Natalie, 2014).

Phylogenetic analysis

The 16S rRNA sequence of the isolated strain AS6 obtained from complete genome sequence was BLAST (tblastn) to find the closely related sequence. Phylogenetic analysis was performed by working on (phylogeny.fr), for making phylogenetic tree of 16S rRNA gene from isolated bacteria was aligned through multiple sequence alignment from GenBank database.

Measurement of glutathione and other non-protein thiol contents

Under the absence and presence of arsenic, reduced glutathione (GSH), oxidized glutathione (GSSG) and other non-protein thiol contents were estimated for the isolated strain (Shamim and Rehman, 2015).

Non-living biomass preparation of arsenic resistant bacteria

Non- living biomass of arsenic resistant bacteria was obtained by growing the bacterial culture in one liter flask of LB-medium and incubates it at optimum temperature and pH to obtain high density growth (Tariq *et al.,* 2019). After that broth culture was centrifuge at 4000 rpm for 10 min and washed the pellet with deionized water for several times. The bacterial pellet was incubated in oven at 70 °Cto obtain bacteria in powder form. The process was repeated 15-20 times to obtain huge amount of bacterial biomass.

Bioremediation analysis

Bioremediation activity for the isolated bacterial strain was performed by biosorption process under 10000 mM arsenic stress (Tariq *et al.,* 2019). Biosorption is a physiochemical process through which metals or metalloids bind with the cellular structure of bacteria. The biomass 1g/L of bacterial strain was used initially. The isolated bacterial strain was mixed in 1 liter of arsenic solution in flasks, which were already supplemented with 10000 mM arsenic stress. Then flasks were incubated on shaker at optimum temperature and pH for 10 h. After every 2 h an aliquot of sample was taken, followed by filtration through 0.22µm filter paper and frozen the sample until for the determination of arsenic through atomic absorption spectrophotometer. The following formulas were used for arsenite biosorbed, by the gram of biomass (q) and bioremediation efficiency.

formula1: $$q = \left(\frac{Ci - Cf}{m}\right) V$$

formula2: $$E = \left(\frac{Ci - Cf}{Ci}\right) * 100$$

Ci = arsenite ions concentration (initial used)
Cf= arsenite ions concentration (final)
m = biosorbent mass in reaction
V = reaction mixture volume

Statistical analysis

All the experiments were carried out in triplicates and results were observed. For each treatment three flasks were maintained. 3 readings were taken for each experiment, their mean and standard error of the mean were calculated.

Results

Physiochemical properties of wastewater

Physiochemical properties of wastewater sample are shown in Table-1. The temperature was 27°C and pH was 7.5. Color was light black and arsenic concentration was 355 µg/L.

Screening of arsenic resistant bacteria

The isolated bacterium AS6 was selected due to high resistance against arsenic.

Optimum growth condition

The optimum temperature for isolated strain was 37 °C and bacterium showed optimum growth at pH 7. The growth of isolated bacterium at different temperature is shown in fig. 2. And growth at different pH is shown in fig. 3.

Table 1. Physiochemical properties of wastewater sample.

Physiochemical parameter	Results
Color	Light black
Temperature	27
pH	7.5
Electrical conductivity (µS/cm)	1452
Turbidity (NTU)	11.8
TDS (total dissolved solids) (µg/L)	574
Arsenic concentration (µg/L)	355

Effect of arsenite on bacterial growth

The effect of arsenite on bacterium was checked by growing it in the absence and presence of arsenite. The 15 mM arsenite stress was used. It was observed that arsenite affect the growth of bacteria. Growth pattern of bacteria in the absence and presence of arsenite is shown in fig. 3.

Table 2. MIC value (in mM) against different metal ions in isolated bacterium.

Metal ions	MIC (mM)
Arsenite (As^{+3})	32
Arsenate (As^{+5})	220
Lead (Pb)	6
Cadmium (Cd)	5
Chromium (Cr)	6
Mercury(Hg)	2
Selenium (Se)	6
Cobalt(Co)	2
Nickel (Ni)	2.5

MIC of arsenic and other heavy metal ions

Apart from arsenite and arsenate the isolated bacterial strain also showed resistance against other heavy metal ions. The MIC against arsenic and other heavy metal is shown in Table-2. The order of resistance against different metal ions is Arsenate >Arsenite> Lead = chromium = Selenium > Cadmium > Nickel > Cobalt = Mercury. The value of minimum inhibitory concentration against arsenate and arsenite was 220 and 32 mM. While the MIC against lead, cadmium, chromium and mercury was 6, 5, 6 and 2 mM respectively. Selenium, Cobalt and Nickel MIC in *staphylococcus* sp. Strain AS6 was 6, 2 and 2.5 mM respectively.

Table 3. Morphological and biochemical characteristics of isolated bacterial strain AS6.

Morphological and biochemical tests	Results
Form	circular
Surface	smooth
Color	yellow
Margin	entire
Elevation	covex
Opacity	opaque
Cell shape	cocci
Motality	non-motile
Gram staining	Gram positive cocci
Catalase	positive
Oxidase	negative
Coagulase	negative
Urease	positive
Citrate	positive
Lactose fermentation	negative
H2S	negative
Nitrate reduction	positive
Indole	negative
Methyl red	positive
Vogesproskauer (VP)	positive
Manitol salt agar growth	Positive

Morphological Characteristics and biochemical tests

The isolated bacterial strain was identified on the bases of morphology, biochemical testing and 16S rRNA sequencing. The 16S rRNA genes of strains AS6 showed 99.46 % similarity to 16S rRNA of *Staphylococcus warner* Strain AW 25 (accession number NR_025922.1). The morphological and biochemical characteristic of isolated strain is shown in Tab-3.

Table 4. Glutathione and non-protein concentration under 15 mM arsenite stress in isolated bacterium.

Arsenite concentration (mM)	GSH (mM g^{-1} FW)	GSSG (mM g^{-1} FW)	GSH+ GSSG (mM g^{-1} FW)	GSH/GSSG	% increase in GSH/GSSG	Non-protein thiols	% increase in non-protein thiol
0	5.5	0.4	6.10	13.75	4.58:13.75*100 = 33.33 %	1.8	1:1.8*100 = 55.55 %
15	11	0.6	12.5	18.33		2.8	

Phylogenetic tree

The isolated strain taxonomy was determined by aligning the 16S rRNA sequence obtained from complete genome sequence of isolated strain to already known most similar sequences data available on NCBI GenBank. The 16S rRNA genes of strains AS6 showed 99.46 % similarity to 16S rRNA of *Staphylococcus warner* Strain AW 25 (accession number NR_025922.1). The phylogenetic tree of isolated starin as shown in fig. 5.

Glutathione and non-protein thiols

There was varied response of reduced glutathione, oxidized glutathione and non-protein thiol under the

presence of arsenite stress (15 mM) in isolated bacterial strain AS6. The level of GSH, GSSG, total glutathione, and GSH/GSSG and non-protein thiol is shown in Tab-4.

Bioremediation activity of bacterial biomass

Bioremediation activity was determined for 10 h with each 2 h of interval. It was determined that after 2 hours of incubation 54 % of arsenite was decreased and 72 % after 4 h and 82%, 89% after 6 and 8 h respectively. Almost 92 % bioremediation efficiency was estimated after 10 hours.

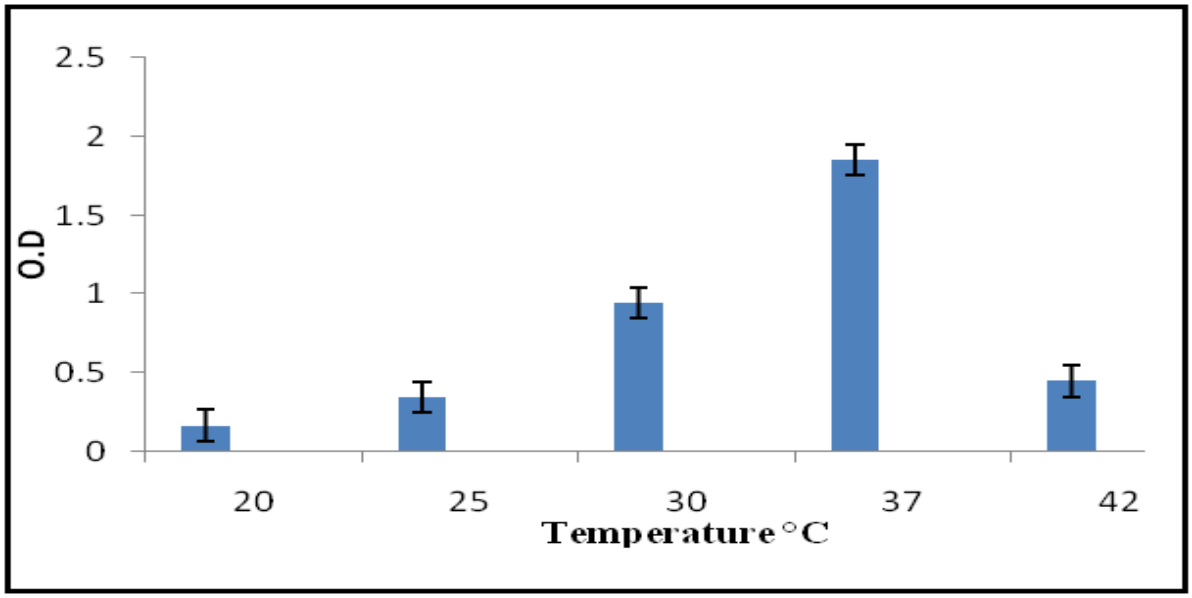

Fig. 2. Optical densities (OD) of isolated bacterium at different temperature.

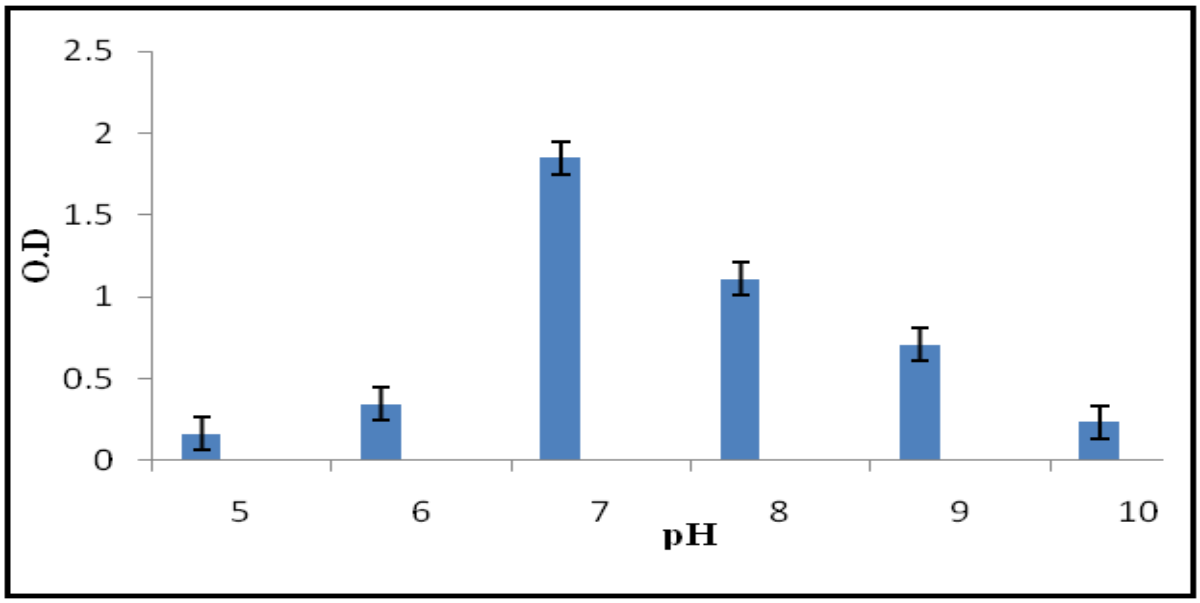

Fig. 3. Optical densities (OD) of isolated bacterium at different pH.

Discussion

In this present study arsenic resistant bacteria *Staphylococcus* sp. strain AS6 was isolated from wastewater of industrial origin in an area District Sheikhupura, Pakistan. The isolated bacterium has MIC 32 mM against arsenite (a toxic form of arsenic) while the MIC of arsenite in *Klebsiella pneumonia* is 21 mM (Mujawar *et al.,* 2019). *Bacillus cereus* has 40 mM MIC against arsenite (Naureen and Rehman, 2015).

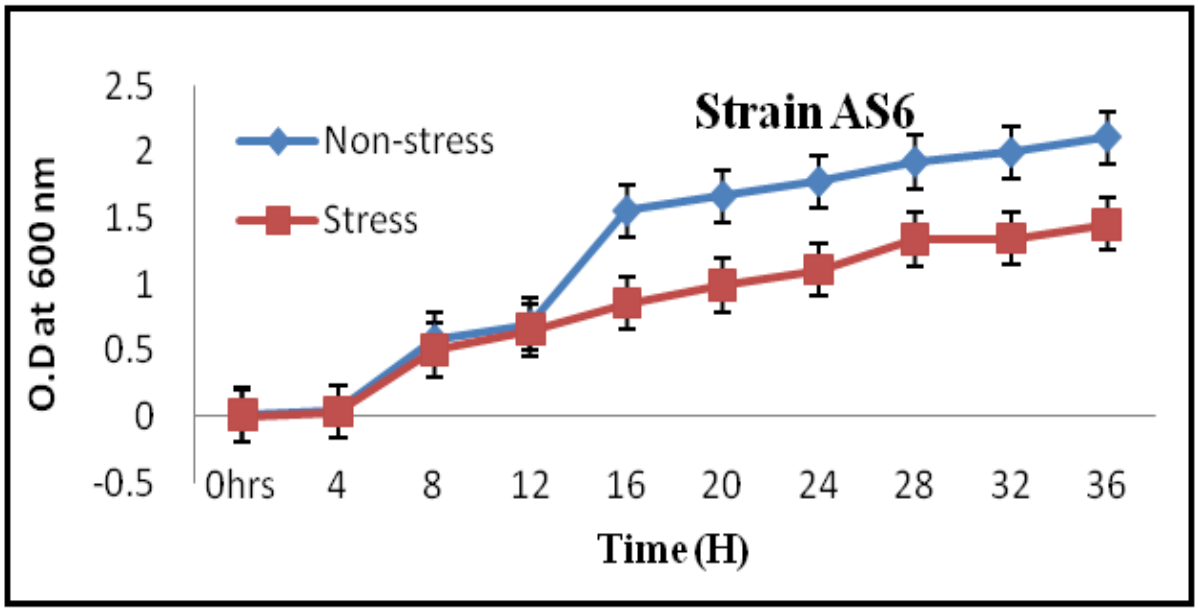

Fig. 4. Growth curves of isolated strain in the absence and presence of arsenite at 37 °C and pH 7.

The MIC of isolated strain for arsenate was 220 mM while in another study it is reported that the MIC for arsenate in *Brevibacterium* sp. strain CS2 and *Micrococcus luteus* strain AS2 is 275 and 280 mM respectively (Sher *et al.,* 2019). Isolated bacterial strain also has cross metal resistance apart from arsenite and arsenate while *Pseudomonas* sp. strain PG-12 has resistance against multiple metal ions apart from arsenic such as its MIC against Cadmium (cd) is 0.6 mM and 10 mM against Lead (Pb) (Manzoor *et al.,* 2019). The cross metal resistance in *Bacillus cereus* is 10 mM Pb; 8 mM Cd; 6 mM Cr and 10 mM Cu (Naureen and Rehman, 2019).

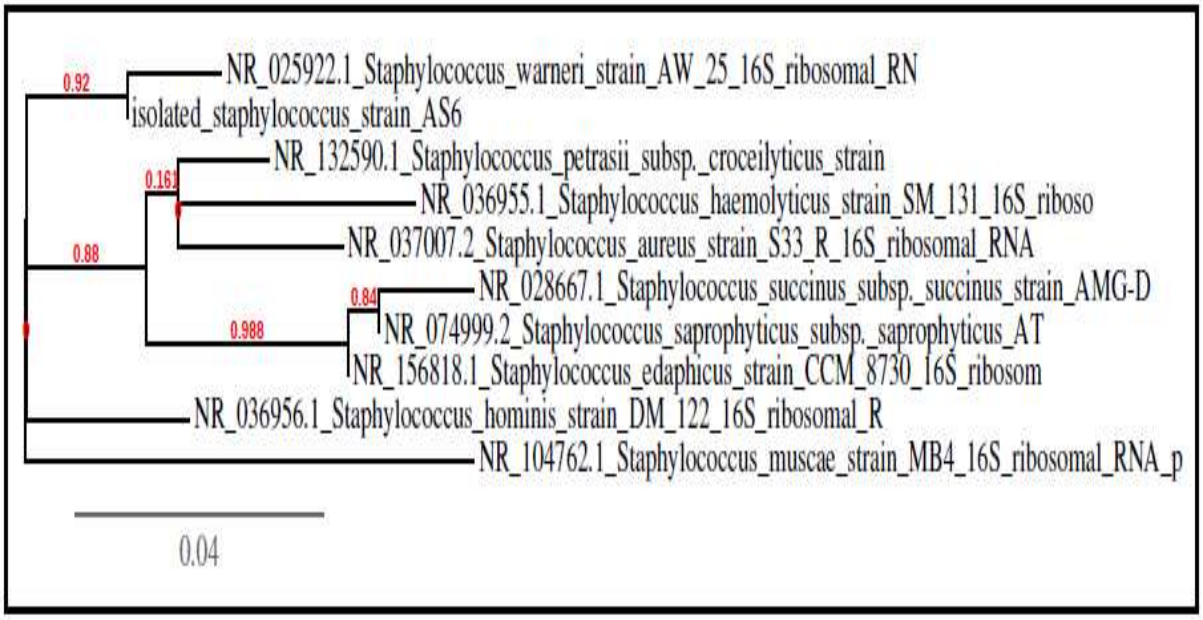

Fig. 5. Phylogentic tree of isoalted strain.

The lag phase was extended due to presence of arsenite in isolated strain while same Scenario was observed in *Klebsiella pneumonia* (Mujawar *et al.,* 2019). These were significant increase was observed in Glutathione level in isolated strain. The bioremediation efficiency of isolated bacterial

biomass was 54 % after 2 h and 94 % after 10 h. While in *Pseudomonas aeruginosa* ATCC27853 strain have remediation efficiency 90.72 % after 30 min and 98% after 2 h (Tariq *et al.,* 2019). The biosorption capacity of the *Arthrobactor* sp. biomass for As^{+3} and As^{+5} 74.91 mg/ and 81.63 mg/g, respectively using 1 g/L biomass with contact time of 30 min (Prasad *et al.,* 2013). In one of the other studies it is reported that, the dried biomass of bacterium *Bacillus* sp. KUJM2 can removed potentially toxic elements such as arsenic, copper, nickel and chromium up to 90.17% to 94.75% from water (Mondal *et al.,* 2019).

Conclusion

In this present study arsenic resistant bacteria *Staphylococcus* sp. Strain AS6 was isolated from industrial waste of District Sheikhupura, province Punjab, Pakistan. The isolated bacterial strain Staphylococcus sp. strain AS6 has resistance against arsenic and other heavy metal such as Zinc (Zn), Cadmium (cd), Mercury (Hg), Nickel (Ni) and cobalt (Co) Chromium (Cr). So this bacterium can be used for the detoxification of arsenic as well as other heavy metals. The dried biomass of isolated bacterium can removed 92 % arsenite after 10 h. A large number of industries in Pakistan released arsenic containing wastes in environment. So the isolated bacterium is a potential candidate for the decontamination of arsenic contaminated sites.

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