Proteomics and transcriptomic analysis of *Micrococcus luteus* strain AS2 under arsenite stress and its potential role in arsenic removal

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ABSTRACT

The proteomics and transcriptomic analysis of *Micrococcus luteus* strain AS2 was carried out through 2D gel electrophoresis and RT-PCR. Seven protein spots were selected randomly from the gel and identified through mass spectrometry. Four proteins including putative metal-dependent hydrolase TatD, thioredoxin reductase, DNA-directed RNA polymerase subunit alpha and chaperone protein DnaK were upregulated while superoxide dismutase [Mn], 3-oxoacyl-[acyl-carrier-protein] reductase FabG, and putative alkyl/aryl-sulfatase YjcS were down-regulated under arsenite stress. No significant difference was observed in *aioB* gene expression analysis in the presence and absence of arsenite. The optimum arsenite processing ability was determined at 37°C (90%) and at pH 7 (92%). The maximum metal processing ability was determined at 250 mM arsenite/L (90%) while the minimum was estimated at 1250 mM arsenite/L (42%). The maximum arsenite removal ability of strain AS2 determined after 8 days was 68 and 82% from wastewater and distilled water, and the organism can be a good bioresource for green chemistry to eradicate environmental arsenite.

1. Introduction

Arsenic is a metalloid with high toxicity (Sher and Rehman, 2019). It is found in various kinds of environments (Koechler et al., 2010). Arsenic contamination is a problem for whole world because of natural geochemical and anthropogenic activities (Islam et al., 2004; Kumari et al., 2018). It is well known carcinogenic for living organisms especially for human beings (Mead, 2005). More than 40 million people are being exposed to arsenic almost above 50 ppb while the limit fixed through the Environmental Protection Agency (EPA) is 10 ppb (Prasad et al., 2013). 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 factors on which arsenic toxicity depends, 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 times 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 effects on human beings in major parts of the world especially northeast India, northwest part of the 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, causes

various health related problems in human beings like, change in color of skin or cancer, diabetes, hypertension, chromosomal aberrations, amplification of genes, alternation in eukaryotic cell morphology as well as disorders which are linked to reproductive system Dey et al. (2016).

Microorganisms are found in every kind of environment and these microorganisms have potential for arsenic remediation from the 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 genes (*arsC* and *arrA*) located on chromosomal DNA or plasmid inside the bacteria (Mujawar et al., 2019; Han et al., 2019). On the other hand, oxidation of arsenic involves conversion of arsenite (As^{+3}) into arsenate (As^{+5}) which is carried out by genes (*aioA, aioB*) present in bacteria (Li et al., 2014). Arsenic toxicity can also be decreased 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., 2014).

The natural sources of arsenic are volcanic activities and weathering of rocks while anthropogenic sources are use of arsenic containing compounds such as pesticides, dyes and preservation of wood. Both sources are responsible for increasing arsenic concentration in water bodies (Prasad et al., 2013). Different conventional methods can be used for the removal of arsenic from water, such as membrane filtration, co-

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agulation, ion exchange method, nanoparticles, phytoremediation, and some other chemical methods (Ng et al., 2014; Mohanty, 2017). 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 non-toxic form (Qin et al., 2006; Tariq et al., 2019).

In the present study, arsenic resistant bacterium, *Micrococcus luteus* strain AS2, was used for proteomics and transcriptomic studies under the presence and absence of arsenite stress to analyze proteins response. The evaluation of bacterium ability regarding arsenic oxidation and arsenic removal from the industrial wastewater was also carried out. *M. luteus* strain AS2 could be a potential candidate for arsenic decontamination from the metal polluted sites.

2. Materials and methods

2.1. Microorganism used

Micrococcus luteus strain AS2, already identified by Sher et al. (2020a), was used in the present study. The organism has been deposited to First Culture Bank of Pakistan (FCBP), in the Department of Agricultural Sciences, University of the Punjab, Lahore, Pakistan with the accession number of FCBP-B-730. The strain was maintained in LB agar plate at 37°C.

2.2. Qualitative test for arsenic oxidation

The *M. luteus* strain AS2 having resistance against arsenite was inoculated on acetate minimal agar plates (having 5 mM sodium arsenite) for 2 days. Then bacterial growth on plates was flooded with silver nitrate solution (0.1%) and brownish precipitates were formed indicating the ability of microorganism to convert arsenite into arsenate (Simeonova et al., 2004).

2.3. Protein extraction and precipitation

Protein was extracted from bacterial cells with slight modification in B-PER reagent procedure for protein extraction. Bacterial pellet was obtained after centrifugation at 5000 xg for 10 min. Enzymes i.e., lysozyme (2 μL), DNase 1 (2 μL), and viscolase (0.8 μL) were added in each 1 mL of B-PER reagent. For each 1 g of pellet, 4 mL of B-PER reagent was used and homogenized by pipetting it up and down. After homogenization, bacterial cells with B-PER reagent were incubated at room temperature for 15 min. After incubation, the mixture was centrifuged at 15,000 xg for 5 min and supernatant was used for protein estimation. After extraction, proteins were precipitated by using protein a precipitation kit (A&A Biotechnology, innovating life science). Solution containing proteins (400 μL) was shifted into 1.5 mL of eppendorf tube, mixed with 800 μL of acetone, and incubated at room temperature for 5 min. Sample was loaded on already an assembled mini-column tube and centrifuged at the speed of 12,000 rpm for the duration of 1 min. Filtrate was removed, mini-column was re-assembled, and centrifuged at 12,000 rpm for 1 min. The mini-column was transferred into a new eppendorf tube and 20 μL of 1X Laemmli buffer was applied onto the center of the resin. The tube was incubated at room temperature for 3 min and was centrifuged at 12,000 rpm for 1 min. Finally, the column was removed and protein was stored at -80°C (Naureen and Rehman, 2016; Sher et al., 2020a).

2.4. Estimation of protein through Qubit

After extraction of protein from bacterial cells, protein was estimated through Qubit^R 2.0 fluorometer. In this technique, mixture was prepared (199 μl Qubit^{TM} protein buffer, 1 μL Qubit^{TM} protein reagent) along with three protein standards of different concentration. Then 10 μL of each standard was mixed in 190 μL of above mixture separately in a small tube. For sample estimation, 2 μL of protein sample was mixed with 198 μL of mixture. Firstly, three standards were placed in qubit to take value after one and other, then protein sample (for protein estimation), and finally protein concentration in μg/mL was determined. SDS-PAGE was performed according to Laemmli (1970).

2.5. Two dimensional gel electrophoresis

2.5.1. Sample preparation for 1st dimension

Proteins were extracted from bacterial cells under arsenite and without arsenite stress after overnight growth at 37°C and pH 7 (Khan et al., 2017b).

2.5.2. Preparation of gel of big size (12-gels)

First of all, 40.8 mL of dd H$_2$O was taken in a beaker and 22.2 mL of buffer was added to it. Then 26.4 mL of 40% acrylamide was added in the beaker. All these contents were mixed in a beaker. Two mL of the mixture was added in a small eppendorf tube and 50 μL ammonium phosphate sulphate (APS) and 5 μL TEMED was added in eppendorf. Pippeted 450 μL of mixture from eppendorf was poured on gel plates to seal all four corners and left to polymerize the gel. Rest of the TEMED and APS were added in the beaker and loaded 32 μL from the beaker to each gel. One ml of isopropanol was added at the top of each gel to prevent the bubble formation at the top of the gel. After gel polymerization, gel-containing plates were inverted to release isopropanol and rinsed with H$_2$O. Gel tray was placed at 4°C with wet towel to prevent gel from dryness (Khan et al., 2017b; Sher et al., 2020a).

2.5.3. Rehydration step

Strip chamber, working area, and pipette were cleaned with methanol. First two 1.5 mL tubes were taken. Rehydration buffer (RB) was prepared and 9 mg dithiothreitol (DTT) and 8 μL ampholyten were added. Then sample (325 μL) was prepared in RB. For sample 1, 90 μL of protein sample was mixed in 235 μL of buffer while for sample 2, 87 μL of sample was mixed with 238 μL of buffer. Short spin was given to a sample to mix it. Then 7 cm immobilized pH gradient strip was taken from -20°C freeze. Sample 1 was poured in the first line of chamber tray and sample 2 in the second line from positive to negative terminal of the chamber. Two strips on two lines in the chamber were placed and on strips, 2 mL of mineral oil was added to prevent dehydration of samples. Lid was placed on a chamber and was gently placed in the machine with focusing of 200 V 1 h, 500 V 1 h, 1000 V 1.5 h, finally 8000 V 2 h reaching a total 12,000 V 7 to 8 h at the end (Khan et al., 2017b).

2.5.4. Equilibration for second dimension

IPG strips were horizontally incubated in a shaker with buffer 1 (6 M urea, 2% SDS, 30% glycerin, 0.375 M tris base with pH 8.8, 2 0.5% IAA, 10 μL BPB) for 5 min of 2.5 mL. Then followed by incubation in equilibrium buffer 2 (6 M urea, 2% SDS, 30% glycerin, 0.375 M tris base with pH 8.8, 2% DTT, 10 μL BPB) for 25 min with 2.5 mL (Khan et al., 2017b).

2.5.5. Second dimension electrophoresis by SDS-PAGE

Equilibrated strip was placed on the top of 12.5% SDS-PAGE. One μL of protein marker was applied on the bloating paper strip and put on the positive side of the strip. Gel was run at 120 V for 20 min and at 100 V for overnight (Khan et al., 2017b).

2.5.6. Fixation of gel

Gel was fixed in a fixative solution (methanol 250 mL, acetic acid 60 mL, and 190 mL dH$_2$O). After fixation, gel was floated into 250 mL of 50% ethanol, then in 30% ethanol. Then gel was dipped in a sensitization buffer (100 mg Na$_2$S$_2$O$_3$ in 500 mL ddH$_2$O) for 1 min. After sanitization of gel, it was washed 3 times with ddH$_2$O. Then gel was

stained with AgNO$_3$ (0.2%) for 20 min and washed 3 times with water. The Developing buffer 250 mL (30 g Na$_2$CO$_3$, 250 μL of fresh formaldehyde, 20 μL Na$_2$SO$_3$ and ddH$_2$O) was used to visualize the protein spot. Fixative solution was used as a stopping solution at this point. Again the gel was washed with ddH$_2$O and left the gel for overnight to gain its original size. Gel was scanned and finally a picture was taken (Khan et al., 2017b).

2.5.7. Staining and imaging of gel

Gel was stained through the procedure of silver staining. Fixer solution called formaldehyde having acetic acid (1%) was used initially for the placement of gel. Then further remaining steps were followed according to Blum et al. (1987). Scanning of the gel was carried out through CanoScan 8400F (Japan). Delta 2D software version 3.6 (Decodon GmbH, Greifswald, Germany) was used for densitometric studies. Some spots on gels were selected, which showed some alteration under arsenite stress. Then those spots were cut and proceeded through in-gel protein digestion. The separated peptides were analyzed with Q-TOF Ultima Global (Micromass, Manchester, United Kingdom) mass spectrometer equipped with nanoflow ESI Z-spray source in positive ion mode and data was collected by using MassLynx (v 4.0) software and data processed on Protein-Lynx-Global-Server (v 2.1), (Micromass, Manchester, United Kingdom) (Ramljak et al., 2008).

2.5.8. In-gel protein digestion

Firstly, spots on silver stained gels were selected and excised. After cutting from the gel, it was distained with the solution of potassium ferricyanide 15 mM and sodium thiosulfate 50 mM. After distaining, equilibration was carried out with acetonitrile (50%) and 50 mM NH$_4$HCO$_3$. Vacuumed concentrator was used for its drying process. Afterwards, DTT (10 mM) and ammonium carbonate (100 mM) was used for rehydration. Then proteins bands were incubated at 56 °C for the duration of 45 min followed by incubation of bands at room temperature (RT) for 30 min with the mixture of ammonium bicarbonate (100 mM) and 55 mM iodoacetamide. Then washing was carried out NH$_4$HCO$_3$ (100 mM) followed by acetonitrile dilution in ratio of (1:1) and placed at room temperature for 15 min. Again dried and rehydrated the samples digestion solution called trypsin 20 μL (0.1 $\mu g/\mu L$). While in digestion solution (without trypsin), after overnight incubation at 37 °C with, extracted the peptides for 30 min firstly with trifluoroacetic acid (0.1%) followed by 30% and 60% of acetonitrile in 0.1% trifluoroacetic acid. Through the drying process, the peptides were extracted from the eluted solution and 0.1% formic acid was used as a dissolving solution for further use (Shevchenko et al., 1996; Asif et al., 2006).

2.6. Extraction of RNA

Extraction of RNA from arsenic resistant bacterium was performed through an RNA isolation kit named as Total RNA Mini (A & A Biotechnology innovating life science). First of all, bacterial cells were grown overnight at the shaker with optimum conditions. Overnight bacterial culture (2 mL) was centrifuged at 8000 xg for 1 min to get bacterial pellet. Pellet was suspended in 800 μL fenozol to lyse it by repeating pipetting and incubated at 50 °C for 5 min. Then 200 μL of chloroform was added in Eppendorfs, mixed by inverting the tubes several times and again incubated for 3 min at room temperature. After this, tubes were centrifuged at 10,000 rpm for 10 min and the supernatant (450 μL) was transferred into the new tube and 250 μL isopropanol was added to it. After mixing, it was poured onto a mini-column. Then again centrifuged at 10,000 rpm for 1 min, the mini-column was transferred into a new tube followed by 700 μL of A1 wash solution. Then again centrifuged at 10,000 rpm for 1 min and flow through was discarded. Then again washing was done with A1 solution and centrifugation was performed at 10,000 rpm for 2 min. Afterward mini-column was shifted into a new 1.5 mL of elution tube and 100 μL of DEPC-treated water was added. The tube was incubated for 3 min at room temperature and was centrifuged

for 1 min at 12,000 rpm. Then mini-column was removed and RNA in tube was stored at -20°C for further research work (Chomczynski and Sacchi, 1987).

2.6.1. Synthesis of cDNA from mRNA

ProtoScript first strand cDNA Synthesis kit (New England BioLabs Inc.) was used for the synthesis of cDNA. First of all, RNA template (1 μg) was taken in PCR- tube and following components were added in the given amount (Random primer mix 2 μL, M-MuLV reaction mix 2X, 10 μL, M-MuLV enzyme mix 10 × 2 μL, and nuclease free water up to 20 μL). After mixing these components, the tube was incubated at 25°C for 5 min and then at 42°C for 60 min. The enzymes were inactivated at 80 °C for 5 min.

2.6.2. Real time PCR

For mRNA quantification, level of arsenite oxidizing gene *aioB*, real time PCR was performed. Primers for *aioB* gene were designed using primer 3 web version 4.0. Initially, three different sets of primers against *aioB* gene having three different product sizes were designed (Table S1). Each 20 μL reaction mixture contained 10 μL enzyme 2X qPCR Bio SYBR Green PCR master mix, 1.6 μL master mix, 5 μL cDNA and 3.4 μL of PCR grade water. Applied bio-system 7500/7500 (fast real time PCR system) was used for qPCR which was further attached with software for data analysis (Arshad et al., 2015; Khan et al., 2017a).

2.7. Assessment of arsenite oxidizing ability of bacterial strain

The bacterial strain was preceded for its arsenic oxidizing ability by culturing it in LB broth (100 mL) in flasks of 250 mL. Experiments were run in 3 flasks for the strain already containing arsenite 250 mM. One flask was used as control, having arsenite with the same concentration but not inoculated with bacterial strain. Arsenic oxidation potential was estimated at three different parameters [Temperature, pH, and arsenite concentration (mM)]. Temperature (20°C, 25°C, 30°C, 37°C, and 42°C), pH (3, 5, 7, 9 and 11) and arsenic concentrations (250, 500, 750, 1000, and 1250 mM mL^{-1}) were maintained during the arsenic oxidizing potential experiment. The arsenite oxidizing activity of the isolated bacterial strains was estimated for 96 h with the interval of 24 h. The 5 mL of broth sample was drawn from each and every flask after 24, 48, 72, and 96 h. Each time the centrifugation of sample was carried out at 3000 rpm for 5 min to harvest the bacteria cells. For the estimation of arsenite and arsenate, supernatant was used. Arsenite was estimated through the P S Analytical Millennium Excalibur Method (Department of ENVS, Biosciences Section Aarhus University, Denmark) (Sher et al., 2020b).

2.8. Arsenite removal from industrial waste water by M. luteus strain AS2

An experiment was designed to evaluate the arsenic removal potential of isolated bacterial strain from the waste water of industrial origin at room temperature 25°C ± 2 and with arsenite stress of 250 mM/L. Initially, 3 containers were used, in 1st container 5 L of waste water along with 500 mL growth of bacteria was taken, in the second container 5 L of distilled water along with 500 mL of bacterial growth was taken and in the third container waste water was taken as control. The experimentation was piloted for 6 days and 5 mL of sample was taken after each 48 h' interval and proceeded to centrifuge at 4000 rpm for 10 min to take the bacterial pellet. Supernatant was put in a separate sterilized tube for the estimation of arsenite through P S Analytical Millennium Excalibur Method.

3. Results

3.1. MIC and arsenite oxidizing ability of bacterial strain

The bacterium showed 50 and 275 mM resistance against arsenite and arsenate, respectively. The appearance of brownish color revealed
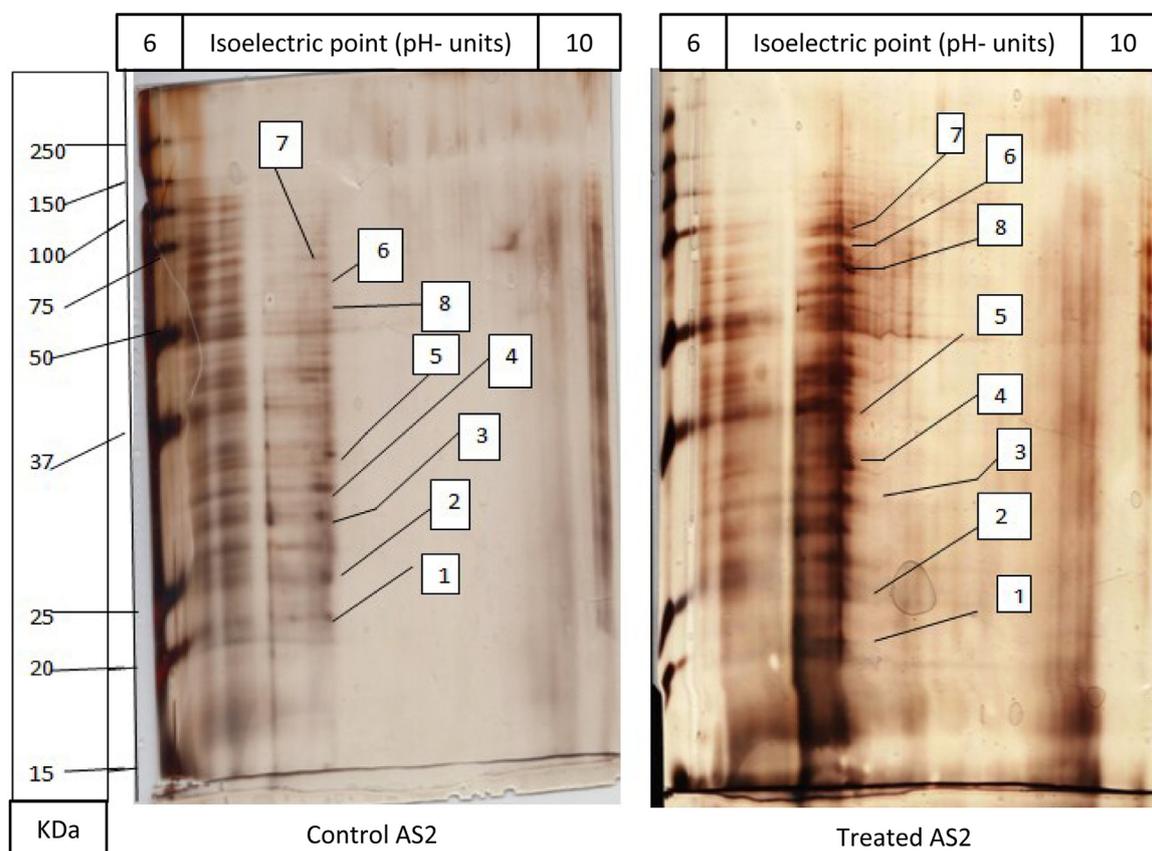
Fig. 1. 2D Gel electrophoresis of *M. luteus* strain AS2 with and without arsenite stress.
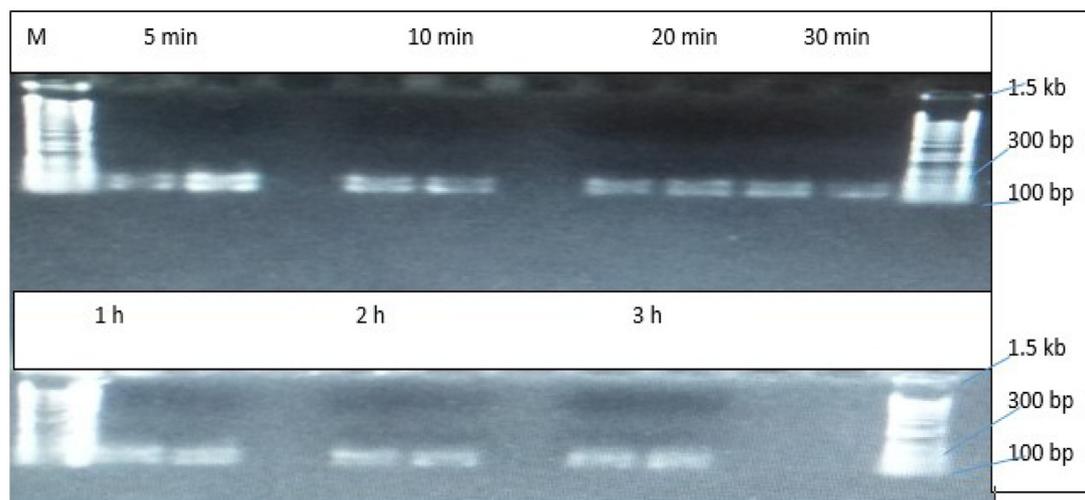
Fig. 2. Expression analysis of *aioB* gene in *M. luteus* strain AS2.

that the isolated bacterial strain has potential to oxidize arsenite into arsenate (Fig. S1).

3.2. Proteomic analysis through 2D gel electrophoresis

Total cell proteins from *M. luteus* strain AS2 were resolved through SDS-PAGE (Fig. 1) and for the proteomic studies 2-D gel electrophoresis was carried out. Total 8 spots on gel were selected (Fig. 2) and mass spectrometry analysis revealed 7 proteins which were up- and downregulated under arsenite stress including superoxide dismutase [Mn], 3-oxoacyl-[acyl-carrier-protein] reductase FabG, putative

metal-dependent hydrolase TatD, thioredoxin reductase, DNA-directed RNA polymerase subunit alpha, chaperone protein DnaK, and putative alkyl/aryl-sulfatase YjcS. The proteins names and their molecular weights along with change in folds under arsenite stress are given in Table 1.

3.3. Expression analysis of aioB gene

Transcription analysis was performed to confirm the expression of *aioB* gene in the presence and absence of arsenite (20 mM) after a certain interval of time in the log phase. The mRNA was taken from the growing

Table 1
Protein identification in *Micrococcus luteus* strain AS2, with differential expression under arsenite stress (15 mM) through mass spectrometry.

Biological samples name	Protein name	Protein M.W(kDa)	Fold change treated	Regulation	Number of unique peptides	Number of amino acids	Coverage (%)
1	Superoxide dismutase [Mn]	23.1	2.1	Down regulation	4	208	24
2	3-oxoacyl-[acyl-carrier-protein] reductase FabG	25.2	1.2	Down regulation	5	250	42
3	Putative metal-dependent hydrolase TatD	31.7	2.4	Upregulation	6	292	39
4	Thioredoxin reductase	34	2.8	Upregulation	5	324	26
5	DNA-directed RNA polymerase subunit alpha	35.7	1.4	Upregulation	7	329	29
6	Chaperone protein DnaK	66.5	1.6	Upregulation	45	620	29
7	Putative alkyl/aryl-sulfatase YjcS	69.9	1.2	Down regulation	1	637	2
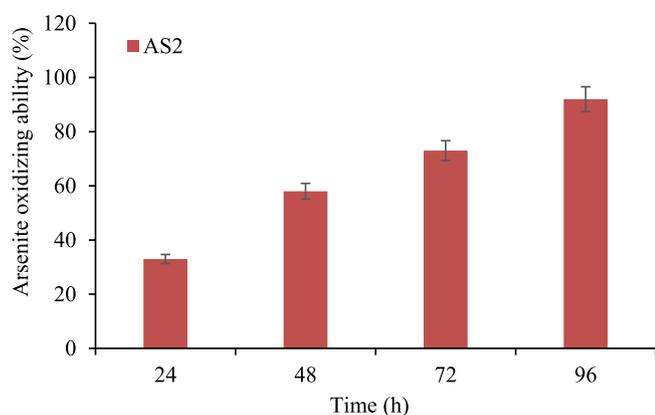
Fig. 3. Arsenite oxidizing ability of bacterial strain at optimum growth conditions.

culture at log phase by supplementing the arsenic stress at the time of 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 4 h, and 5 h and converted into cDNA. It was seen that *aioB* gene was equally expressed in the absence and presence of arsenite with the product size of 214 bp (Fig. 2). The first two bands in the upper row are representing control and stress after 5 min, then 10 min and so on up to 3 h. At each corner of the gel, there is a ladder of 100 bp. The expression analysis of *aioB* gene in *M. luteus* strain AS2 is shown in Fig. 2.

3.4. Arsenite processing ability

The arsenite oxidizing ability of bacterial strain AS2 was 33, 58, 73, and 92% after 24, 48, 72, and 96 h of incubation under optimum conditions (37°C, pH 7) when the arsenite concentration in the medium was maintained at 250 mM/L (Fig. 3).

3.5. Effect of factors on arsenite processing ability

The strain AS2 was studied for arsenite processing ability at different temperatures, pH, and initial arsenite concentration for 2 and 4 days of incubation.

3.5.1. Temperature effect

It was noted that strain *M. luteus* strain AS2 showed optimum arsenite processing ability at 37°C. The strain AS2 showed 58 and 90% arsenite oxidizing ability after 48 h (Fig. S3a) and 96 h (Fig. 4a), respectively. The arsenite oxidizing ability of AS2 was 8% after 48 h at 20°C and 12% after 96 h. At 25°C, the arsenic oxidizing ability of strain was 18 after 48 h and 32% after 96 h. At 30°C, strain AS2 showed 42 and 58% arsenite oxidizing ability after 48 and 96 h. Moreover, at 42°C the strain AS2 showed 13% arsenic oxidizing ability after 48 h while it was 23% after 96 h (Fig. 4a).

3.5.2. pH effect

It was noted that no oxidation was determined at pH 3 after 48 and 96 h at pH 3 while optimum arsenic oxidizing ability was determined at pH 7 after 48 (57%) and 96 h (92%). At pH 5, 9% arsenite oxidizing ability after 48 h (Fig. S3b) and 21% after 96 h were determined. At pH 9, it was 32% after 48 h and 16% after 96 h in the bacterial strain. At pH 11, strain AS2 showed 8 and 16% arsenite oxidizing ability after 48 and 96 h, respectively (Fig. 4b).

3.5.3. Effect of arsenite concentration

It was ascertained that maximum arsenite oxidizing ability was determined at 250 mM/L (90%) under optimum conditions (37°C, pH 7) while the minimum was determined at 1250 mM/L (42%). At 500 mM/L arsenite, AS2 arsenite processing ability was 55% after 48 h (Fig. S3c) and 82% after 96 h. At 750 mM/L, the arsenic processing ability was 50% after 48 h and 68% after 96 h. At arsenite stress of 1000 mM/L, the arsenite oxidizing ability of the strain was 40 and 58% after 48 h and 96 h, respectively (Fig. 4c).

3.6. Arsenic removal from industrial waste water

It was noted that strain AS2 has the ability to remove 10% arsenite from waste water and 15% from distilled wastewater after 2 days. At day 4 its removal capacity was 18 and 32% from industrial waste water and distilled water, respectively. At day 6, arsenite removal ability of bacterial strain AS2 was 42 and 65% from waste water and distilled water. The maximum arsenite removal ability of strain AS2 was determined after 8 days, which was 68 and 82% from waste water and distilled water, respectively (Fig. 5).

4. Discussion

The waste water from industrial origin contains chromium, cadmium, nickel, arsenic, and cobalt (Gunatilake, 2015). As far as arsenic is concerned it is extra poisonous metalloid, carcinogenic, ubiquitous and accumulating in our surroundings with the passage of time (Sher and Rehman, 2019). The best approach for the arsenic decontamination is bioremediation. Arsenite oxidation is a promising way to detoxify arsenic. The more toxic form of arsenic i.e. arsenite converted into less toxic form (arsenate due to its less mobility) in the presence of arsenite oxidase. The two genes regulating arsenite oxidation are *aioA* and *aioB*. In the present study, strain AS2 has an efficient ability to oxidize arsenite into arsenate and hence can effectively be used in environmental arsenic remediation.

It has been reported that several bacterial strains are being used for the remediation of arsenic (Aguilar et al., 2019; Bhakat et al., 2019; Han et al., 2019; Mu et al., 2019; Sher et al., 2020a). The *M. luteus* strain AS2 strain showed MIC against arsenite 50 mM and arsenate 275 mM. *Brevibacterium* sp. strain CS2 showed MIC against arsenite 40 mM and 275 mM against arsenate, respectively (Sher et al., 2019). In another

Fig. 4. Arsenic oxidizing ability of *M. luteus* strain AS2 at different temperature (a), pH (b), and arsenic concentration (c) after 96 h of incubation.

Fig. 5. Arsenite removal ability of *M. luteus* strain AS2 from original industrial waste water after 2, 4, 6, and 8 days of incubation.

In this investigation, in *M. luteus* AS2 under arsenite stress, seven spots on 2D gel were selected because of differential expression. Four proteins i.e. putative metal-dependent hydrolase TatD, thioredoxin reductase, DNA-directed RNA polymerase subunit alpha and chaperone protein DnaK were upregulated. Putative metal-dependent hydrolase TatD (upregulated 2.4) is cytoplasmic Mg^{2+} -dependent protein and it may play a role in the H_2O_2-induced DNA repair (Chen et al., 2014). Another upregulated protein is thioredoxin reductase, a central component of thioredoxin system, and is involved in reduced disulfide bond formation. The mass spectroscopy (MS) analysis revealed 2.8-fold upregulation of this protein which also plays an important role in arsenic resistance. In another study, it is reported that Leptospirillum ferriphilum ML-04 produced 65 significant differential proteins under arsenic stress and 25 of them were known function while 40 were unknown function (Li et al., 2010). The genomic studies of *Rhodococcus aetherivorans* BCP1 revealed that the phylogenetic analysis of thioredoxin reductase is closely related in function with *ArsC1* which encodes mycothiol transferase protein for arsenic resistance (Firrincieli et al., 2019).

The upregulated DnaK protein is involved in preventing protein misfolding. These chaperones capture a misfolded protein, unfold it, and then by utilizing ATP, fold it properly (Khan et al., 2015). DNA replication and RNA polymerase alpha subunit is also upregulated (Table 1). In Exiguobacterium sp. S17, it has been reported that 25 proteins are differentially expressed under the presence of arsenic and were upregulated, involved in stress, energy metabolism, transports, and protein synthesis (Belfiore et al., 2013). In Dunaliella salina, 65 proteins were differentially expressed in the presence of arsenic stress. Out of 65, 45 were significantly induced while 20 were declined proteins (Ge et al., 2016). These proteins are mainly involved in energy metabolism, protein synthesis and folding, ROS defense, phosphate transport, and amino acid synthesis (Ge et al., 2016).

Mass spectrometry analysis revealed that three proteins down regulated including 3-oxoacyl-[acyl-carrier-protein] reductase, also known as FabG, is involved in fatty acid biosynthesis and its down regulation indicates some sort of cell membranes destruction during metal stress. Another down regulated protein is superoxide dismutase (SOD) is responsible to convert superoxide into hydrogen peroxide and dioxygen. The decreased production of SOD under metal stress was due to inhibitory effect of arsenite on SOD. Another study reported that H_2O_2 can decrease the activity of SOD (Scandalios, 1997). Another down regulated protein during arsenite stress is putative alkyl/aryl-sulfatase YjcS. It is outer membrane-bounded periplasmic protein and is involved in sulfur metabolism including sodium dodecyl sulfate degradation.

In RT-PCR analysis of the *aioB* gene it was seen that this gene is equally expressed in the presence and absence of arsenite in *M. luteus* strain AS2. This is because of strain ability to detoxify arsenic naturally in wastewater from where it was originally isolated. From the growth curves, it was evident that there is no such significant difference in the optical densities of the bacterial strain in the presence and absence of

study, it is reported that *Klebsiella pneumoniae* MIC against arsenite was 21 mM (Mujawar et al., 2019). The *M. luteus* strain AS2 produced brown color precipitate as a result of arsenite oxidation (Fig. S1). Brown color precipitation is due to interaction of silver nitrate and arsenate ions (Lett et al., 2001). Several researchers have reported arsenic resistant bacterial strains with efficient arsenite oxidizing ability (Table 2).

The 2D analysis of bacterial strain showed that there is up and down regulation of some proteins under arsenic stress. It has been reported that in Staphylococcus sp. 8 proteins which were upregulated under arsenic stress, play a key role in the detoxification of arsenic (Srivastava et al., 2012). Both transcriptomic and proteomic approaches are important in determining the cellular response against heavy metal ions (Teitzel et al., 2006).

Table 2
Arsenite resistance and oxidation potential in some reported bacterial strains.

Sr. #	Organism	As^{3+} resistance (mM)	As^{3+} oxidation potential (%)	Reference
1	*Acinetobacter jejuni* 1.3S	40.0	88	Naureen and Rehman (2016)
2	*Klebsiella pneumoniae* strain SSW7	21	–	Mujawar et al. (2019)
3	*Brevibacterium* sp. strain CS2	40.0	–	Sher et al. (2019)
4	*Micrococcus* sp. EIKU8	25.0	86	Bhakat et al. (2019)
5	*Micrococcus luteus* strain AS2	75.0	90	This study

arsenic. It was observed that in all log phases the *aioB* gene is equally expressed. Abbas et al. (2018) reported that in arsenite oxidizing bacteria such as *Enterobacter* sp. MNZ1 and *Kliebsilla* sp. MNZ4, MNZ6 that genes are equally expressed in the absence and presence of sodium arsenite. In *Pseudomonas xanthomarina* S11, the arsenite oxidizning genes have constitutive expression either the bacteria were grown in the presence or absence of arsenite (Koechler et al., 2015).

The arsenite oxidase, Aio, contains a high potential 3Fe-4S cluster in the large catalytic subunit, *AioA*, rather than the more common 4Fe-4S cluster, and a Rieske centre in the small subunit, *AioB*, homologous to the Rieske protein in the bc1 complex. It has been suggested that the electrons from arsenite oxidation pass to the molybdenum center, to the 3Fe-4S cluster, the Rieske cluster, and finally to an electron acceptor. The present investigation represents the constitutive expression of the *aiob* gene with or without the presence of arsenite.

Arsenic processing ability for *M. luteus* strain AS2 was determined for 4 consecutive days with the interval of 24 h. After every passing day the arsenic processing ability was increased. The strain showed arsenic oxidizing ability 92% after 4 days of incubation. It is reported that *Bacillus cereus* and *Acinetobacter jejuni* can oxidize 92 and 88% of arsenite after 6 days, respectively (Naureen and Rehman, 2016). One of the bacterial strains *Thermus* sp. HR13 can oxidize arsenite 100% with 16 h of incubation (Gihring and Banfield, 2001). Bahar et al. (2012) reported that *Stenotrophomonas* sp. MM-7 can oxidize 500 μM with 12 h. Some of the factors that affect arsenite oxidation rate are temperature, pH, and initial arsenic concentration (Johnson and Pilson, 1975).

In the present study, *M. luteus* strain AS2 showed arsenic oxidation potential in temperature range of 20°C to 42°C. The strain showed optimum arsenic oxidation potential at 37°C which is an optimum growth temperature for this strain. The arsenite oxidation potential of bacterium determined at 20 and 42°C was low. From 20 to 37°C, the arsenite oxidation rate was increased but after 37 to 42°C, the arsenic oxidation rate was decreased. The maximum oxidation rate was estimated at 37°C. Same observation was made in arsenite oxidizing bacterial population named as CASO1 (combination of strain b3, *Ralstonia pickettii* and strain b6 *Thiomonas*) with the optimum temperature 25°C. It has been reported that CASO1 strain showed arsenic oxidation in the range of 10°C to 35°C. At 10°C, the arsenite oxidation rate was minimum but maximum at 25°C but again decreased after 25°C to 30°*C* (Battaglia-Brunet et al., 2002).

The effect of pH on arsenite oxidation was determined in the pH range of 3–11. There was no significant arsenite oxidation noted at pH 3. The bacterial strain showed significant arsenite oxidation in pH ranged of 5–11 but maximum potential was estimated at pH 7. In CASO1, pH was observed in the range of 3–10 but the maximum arsenite oxidation potential was determined at pH 5–7, an optimum pH for CASO1 (Battaglia-Brunet et al., 2002). The optimum pH range for *Stenotrophomonas* sp. MM-7 was 5–7 (Bahar et al., 2012). The bacterial strain showed good arsenite oxidation potential at 250 mM/L initial arsenite concentration. The arsenite oxidation potential was decreased by increasing the initial arsenite concentration in the culturing medium. Same observation was made in CAS01, showed optimum arsenite oxidation at 500 mg/L but arsenite oxidation rate was decreased by increasing arsenic concentration (Battaglia-Brunet et al., 2002).

It was determined that *M. luteus* strain AS2 can remove 68% arsenic from waste water and 82% from distilled water after 8 days. With each passing day the arsenic removal capacity of the bacterial strain in-

creases, because with passing days the number of bacterial cells increase and it means there will be more active sites for arsenic attachment which will remove more arsenic from the waste water. At day 2, the number of active sites for arsenic binding was low as a result less part of arsenic will be removed from the waste water. Prasad et al. (2013) reported that biomass (1 g/L) *Arthrobacter* sp. removed 74.91 and 81.63 mg/g of As^{+3} and As^{+5}, respectively with exposure time of 30 min. Another study reported that the mixture of sulfate reducing bacteria (SRB) can remove 27% arsenic in the absence of Fe^{+2} from industrial waste water while in presence of Fe SRB can removed 98% arsenic from industrial waste water after 12 days (Liu et al., 2018). It has been reported that *Acidothiobacillus ferrooxidans* NCIM No.5370 can remove 80–85% of arsenic (Kamde et al., 2018).

5. Conclusions

In conclusion, maximum upregulation (2.8 fold) was determined in thioredoxin reductase while least upregulated was DNA-directed RNA polymerase subunit alpha (1.4 fold). Superoxide dismutase (2.1 fold) and protein 3-oxoacyl-[acyl-carrier-protein] reductase (1.2 fold) were down regulated under arsenic stress. No significant difference was observed in the expression analysis of *aioB* gene in arsenite stress. *M. luteus* strain AS2 showed 92% arsenite oxidizing ability after 96 h at optimum conditions. The maximum arsenite processing ability was determined at 37°C (90%), pH 7 (92%), and at 250 mM/L initial arsenite concentration (90%). The maximum arsenite removal ability of *M. luteus* strain AS2 was determined after 8 days (68 and 82% from wastewater and distilled water). The presence of proteins involved in resistance and genetic determinant indicates that the bacterium has high potential to decontaminate metal polluted sites.

Author's contribution

SS conducted experiments, compiled results, and wrote the manuscript. AR contributed in the design, write up and final editing of the manuscript. Both authors approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2021.100020.

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