Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2019.23279

138 (2019) 128–133
January

Isolation and characterization of mercury-resistant bacteria from industrial wastewater

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Received 24 April 2018; Accepted 13 October 2018

ABSTRACT

Mercury (Hg) is present in the environment due to the natural processes and from anthropogenic sources. The amount of Hg mobilized and released into biosphere has increased with the increase of industrial age. The aim of this study was to isolate and characterize the Hg-resistant strains from industrial wastewater of Penang, Malaysia, in terms of Hg processing and uptake ability. These bacterial isolates were designated as CZ1 and CZ2 after isolation. These were identified as *Acinetobacter junii* and *Pseudomonas stutzeri* on the basis of morphological, biochemical and 16S rDNA characterization. The optimum pH for *Acinetobacter junii* and *Pseudomonas stutzeri* was 7.0 and 8.0, respectively. The optimum temperature for both bacterial strains was 35°C. The growth patterns of both isolates were similar with control (without Hg stress) but greatly affected by Hg. Both strains were mostly resistant against antibiotics but sensitive against penicillin. The *Acinetobacter junii* and *Pseudomonas stutzeri* could remove the Hg up to 70% and 90%, respectively. The Hg bioaccumulation ability of *Acinetobacter junii* and *Pseudomonas stutzeri* was 76% and 90%. The Hg induced bands were observed with molecular weight of 28 kDa (*Pseudomonas stutzeri*) and 98 kDa (*Acinetobacter junii*). This study shows that these bacterial strains can be employed as an efficient bioremediation tool to recover and remove the Hg from industrial wastewater.

Keywords: Antibiotics; Bioaccumulation; Mercury; Resistance

1. Introduction

Mercury (Hg) is a chemical element placed in group 12 period 6 in a periodic table, with atomic number 80 and 200.592 standard atomic weight. Due to the high conductivity physical property, Hg is used in electronic equipment, Hg relays and Hg switches as they are easy to make and very efficient. Hg is used in agriculture to manage fungus-caused diseases in seeds, bulbs, plants, fruits and vegetation [1]. Hg in the environment occurs naturally in the earth's crust. It enters the environment through volcanic activities and weathering of rocks. The sedimentary rocks contain more Hg than the igneous rocks [2]. On the other hand, human activities also contribute to the Hg releases to the environment, such as industrial processes, waste incinerators, coal burning and mining for Hg, gold and silver. The anthropogenic Hg is inorganic, such as mercuric sulphide or mercuric chloride; whereas the organic Hg, such as methyl-Hg, is the Hg transformed by bacteria that bioaccumulate in living organism such as fish. Hg is one of the most perilous and omnipresent pollutants [3]. Workers in factories might face chronic exposure to Hg through inhalation of Hg vapours during industrial processes that cause harmful effects on nervous, digestive and immune systems. The mercurial compounds can lead to Parkinson's and Alzheimer's disease in

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long term. The inorganic salt of Hg, such as mercuric chloride, is corrosive to the epidermis of eyes and gastrointestinal tract. Acute exposure, which is a short-period exposure to Hg, can cause brain or liver damage, decrease in memory strength and heart problem [4].

Aldroobi et al. [5] found that hair Hg concentration in Penang Island populations is in the range of 2.2–17.5 mg/kg by using XRF technique on scalp hair. The industrial wastewater of Penang, Malaysia, is polluted with Hg which is dangerous for aquatic ecosystem and also for human health. The contamination of Hg in water will affect the aquatic living organisms. There are various conventional methods to remove the Hg from wastewater such as oxidation, membrane filtration and osmosis, which are not eco-friendly as they produce secondary pollutants and are less efficient for the removal of Hg in the contaminant water when the concentration is low. So the biological method is an alternative method to remove the Hg from wastewater. In this study, Hg-resistant bacteria were isolated and characterized from industrial wastewater collected near Sungai Pinang.

2. Material and methods

2.1. Sampling

Industrial wastewater samples were collected from point source of industry and stored in screw-capped sterilized bottles at the Sungai Pinang 5° 24.262′N 100° 19.570′E. The samples were stored in ice box at 4°C during the transportation. The collection and preservation of water samples are according to the requirement of APHA Standard Method 3010B [1].

2.2. Isolation of Hg-resistant bacteria

About 10 μL of industrial wastewater sample was spread on Luria Bertani (LB) agar which contained 50 μg/mL of Hg^{2+} was mixed with LB agar and was autoclaved at 121°C and 15 lbs for 15 min before solidification. The growth of bacterial colonies was observed after 24 h of incubation at 37°C. The individual colonies were picked up and streaked onto 100 μg of Hg^{2+}/mL of Hg agar plates by using a loop, then incubated for 24 h of incubation at 37°C. The process was repeated with successively higher concentration of Hg^{2+}: 150, 200, 250, 300, 350, 400, 450, 500 μg of Hg^{2+}/mL of Hg, until the minimum inhibitory concentration of each isolate was obtained.

2.3. Physical, biochemical and molecular characterization

For biochemical characterization, the bacteria isolated were tested for MacConkey agar test, motility test, urease test, oxidase test and tributyrin agar test. For biochemical and physical characterization of bacterial strains, the Benson (1994) and Bergey's Manual of Determinative Bacteriology criteria were adopted [6].

For molecular characterization, DNA of bacterial strains was isolated and the 16S rRNA gene of bacteria was amplified by polymerase chain reaction with universal bacterial primer 27F (5′ TACGGYTACCTTGTTACGACTT 3′) and 1492R (5′ AGAGTTTGATCMTGGCTCAG 3′) using the following thermo cycling conditions: 3 min at 95°C for initial denaturation followed by 30 cycles of 2 s at 98°C for denaturation, 15 s at 62°C for annealing and 1 min at 72°C for extension; the last cycle was flowed by 1 min at 72°C. For sequencing, the sediment samples were sent to the Centre of Chemical Biology, Universiti Sains Malaysia (CCBUSM). The obtained sequences were blasted by using online tool NCBI Blast by comparing with GenBank and ribosomal RNA [7,8].

2.4. Phylogenetic analysis

The GenBank database was used to compare the 16S rDNA sequences with the similar sequences. The phylogeny of bacterial strains was constructed by Molecular Evolutionary Genetics Analysis (MEGA) software.

2.5. Screening process to determine the best growth condition

The temperature and pH were considered as optimum growth conditions of bacterial strains. For determination of optimum pH, 5 sets of 5 mL of LB broth test tubes were prepared, for each isolate and their pH was adjusted from 1.0 to 13.0. About 10 μL of freshly prepared culture was inoculated into the test tubes which have been autoclaved at 121°C and 15 lbs for 15 min. The absorbance was taken at 600 nm after incubation of 24 h. The graph was plotted between absorbance and time.

For determination of optimum temperature, 4 sets, each 6 test tubes of 5 mL LB broth were prepared and autoclaved at 121°C and 15 lbs for 15 min. About 10 μL of freshly prepared culture were inoculated in each test tube and 4 sets of test tubes were incubated at 15°C, 25°C, 35°C, 45°C and 55°C. After 24 h, their absorbance was taken at 600 nm. The graph was plotted between absorbance and time.

2.6. Growth curves of isolates

The most suitable pH and temperature for each strain were taken to determine the growth curves of isolates. Each isolate consisted of three flasks of nutrient broth which were autoclaved at 121°C and 15 lbs for 15 min, inoculated with 50 μL of freshly prepared inoculums and 50 μg/mL of Hg^{2+} was also added. These cultures were incubated at pH 7.0 and temperature 37°C in a shaker at 150 rpm. An aliquot of culture was taken out in the clean bench at every hour for 24 h. Absorbance was taken at 600 nm wavelength and the growth was plotted graphically.

2.7. Antibiotic sensitivity

The bacterial culture was spread on the agar plates and incubated at 37°C for 24 h. When the bacterial culture was grown on the agar plates then antibiotic disks were placed on this bacterial culture. The zone of inhibition was used to measure the antibiotic sensitivity against bacterial strains. The antibiotics used were carbenicillin (100 μg), chloramphenicol (30 μg), ampicillin (10 μg), penicillin (10 μg), gentamicin (10 μg) and oxytetracycline (30 μg).

2.8. Hg bioaccumulation capability of the bacterial strains

The Hg bioaccumulation ability of bacterial strains was measured by adding Hg^{2+} at a concentration of 100 μg/mL in

the growth medium. The control (without Hg stress) was also run parallel that contains with same concentration of metal but without bacterial culture. The cultures were incubated for 40 h and from each growth medium (treated and control), 5 mL culture was drawn under sterilized states after 0, 10, 20, 30 and 40 h, respectively, for getting the bacterial pellets. The cultures were centrifuged for 5 min at 3,000 rpm and the supernatants were capitalized for the evaluation of Hg^{2+} by atomic absorption spectrophotometer (A3G graphite furnace AAS, United States) at wavelength 253.7 nm. The amount of Hg^{2+} in the supernatants was measured by using standard curve. The percentage reduction in the amount of Hg^{2+} in the medium was calculated.

2.9. Uptake ability of Hg by bacterial isolates

The bioaccumulation of Hg by bacterial isolates was measured by acid digestion [1]. About 50 μg/mL of Hg^{2+} was added into the growth medium and incubated for 24 h. After incubation, the cells were collected and washed with saline solution three times and acid digested (HNO_3:H_2SO_4: 1:1). The metal content after digestion was estimated by atomic absorption spectrophotometer (A3G graphite furnace AAS, United States) at 253.7 nm wavelength. The standard curve was used to calculate Hg uptake in μg/mL.

2.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

About 20 mL broth was taken in triplicate flasks and 100 μg/mL of Hg^{2+} stress was given to bacterial isolates with control. These bacterial cultures were incubated in shaking incubator for 16 h at 37°C. The cell pellet was collected by centrifugation. The 1X (100 μL) loading dye was used to dissolve the pellet. Then for 5 min heat shock was given, for 2 min eppendorfs were transferred on ice and then was centrifuged for 10 min at 12,000 rpm. Supernatant was shifted to new eppendorfs, then the final centrifugation was given at 12,000 rpm for 10 min, and the supernatant was transferred to new eppendorfs. The bacterial proteins were resolved by SDS-PAGE. Electrophoresis was practiced in vertical mini-slab gel (Mini-Protean III; BioRad, Penang, Malaysia) with a gel size 8 × 7 cm and gel thickness of 0.75 mm. The gels were consisted of stacking gel (4%) and resolving gel (10%) and run for 50 min at constant voltage of 200 V.

Each well of the gel was loaded with about 10 μL of bacterial protein extracts. After electrophoretic separation, the gel was stained with 0.01% Coomassie blue solution, methanol (45% v/v) and glacial acetic acid (10% v/v) for 30 min at room temperature and consequently placed in the destaining solution methanol (50% v/v) and acetic acid (2% v/v) for 1 h. The gel image was analyzed and captured by using VersaDoc Imaging System (BioRad)

3. Results and discussion

3.1. Physicochemical properties of industrial wastewater

The physiochemical properties of industrial wastewater are shown in Table 1. The temperature ranged from 19°C to 25°C, pH varies between 6.4 and 8.0 at sampling points and concentration of Hg^{2+} ions ranged from 1.12 ± 0.05 to 1.70 ± 0.09 μg/mL.

3.2. Isolation and molecular identification of bacterial isolates

Only two rough bacterial colonies were observed on the agar plates with 50 μg/mL of Hg^{2+} after incubation for 24 h. The colonies were re-streaked on the agar plates to get the purified bacterial strains and were assigned as CZ1 and CZ2.

The NCBI Blast of 16S rRNA gene revealed that CZ1 was 100% homologous to *Acinetobacter junii* strain lzh-X15 (accession no: CP024632.1) while CZ2 had 100% similarity index with *Pseudomonas stutzeri* strain NP4 (accession no: MG917743.1).

3.3. Phylogenetic analysis

The blasting of nucleotides sequences of 16S rRNA gene is very fast and accurate method to determine the position of bacterial strains in the phylogenetic tree. The full-length phylogenetic trees of both CZ1 and CZ2 strains were constructed as shown in Fig. 1. It was found that CZ1 was classified under proteobacteria class with 100% similarity with 0.0001 nm neighbour joining distance as shown in Fig. 1(a). The CZ2 was grouped with *Pseudomonas stutzeri* strains with 100% similarity index about 0.00006 nm neighbour joining distance (Fig. 1(b)).

3.4. Bacterial growth patterns against pH and temperature

The optimum pH of *Acinetobacter junii* was 7.0 while the *Pseudomonas stutzeri* showed maximum growth at 8.0 (Fig. 2). The ionization state of the functional groups (phosphate, carboxylate and amino groups) of the microbial cell and metals solubility effected by medium pH [9].

The optimum temperature of both strains was 35°C as shown in Fig. 3. At low temperature, the bacterial growth is retarded due to chelation of internal organelles and at high temperature, the bacterial enzymes denature which result in death of cells. At high and low temperatures the binding of metals with microbes is through passive process which needs energy [10].

The growth patterns of both isolates were observed with control. The growth patterns of both isolates were similar to the control but the growth was highly inhibited by Hg^{2+} ions. The lag phase in the *Acinetobacter junii* was extended about

Table 1
Physicochemical properties of industrial wastewater collected from industrial area near Sungai Pinang

Parameters	Point 1	Point 2	Point 3	Point 4	Point 5
Temperature	25.60 ± 0.40	24.59 ± 0.51	21.01 ± 0.45	24.00 ± 0.75	19.50 ± 0.50
pH	6.44 ± 0.06	7.45 ± 0.09	8.05 ± 0.15	6.48 ± 0.06	7.09 ± 0.11
Hg (μg/mL)	1.12 ± 0.05	1.55 ± 0.04	1.70 ± 0.09	1.45 ± 0.05	1.60 ± 0.11

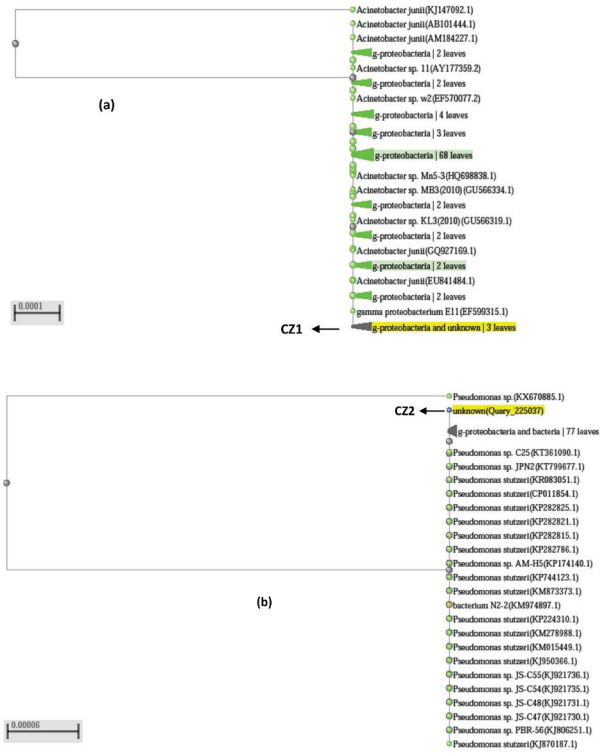

Fig. 1. Phylogenetic trees of bacterial isolates based on 16S rDNA analysis: (a) *Acinetobacter junii* and (b) *Pseudomonas stutzeri*.

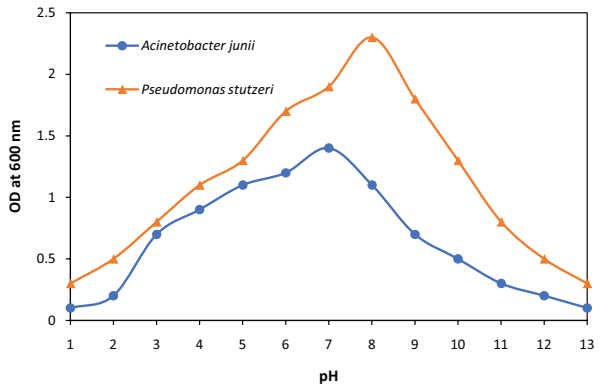

Fig. 2. Effect of different ranges of pH on bacterial growth.

0–12 h and in *Pseudomonas stutzeri* was about 0–8 h as shown in Fig. 4. The initial Hg concentration and growth phase of cells are important parameters for Hg detoxification. The initial higher concentration normally inhibits the essential metabolic reactions and retards the growth [11]. The prolonged lag phase in both bacterial isolates may be due to higher initial concentration, adjustment with the new environment or repairing the cell parts damaged by Hg.

3.5. Antibiotics sensitivity test

The Hg-resistant bacterial strains also checked against antibiotics resistance as shown in Table 2. Both strains showed resistance against carbenicillin, chloramphenicol and ampicillin. Both strains were sensitive against penicillin. *Acinetobacter junii* was sensitive against gentamicin and oxytetracycline while *Pseudomonas stutzeri* was resistant. The plasmids of many bacteria encode different resistance systems against metals such as Cr^{6+}, As^{2+}, Ag^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+} and Hg^{2+} [12]. The plasmids and transposons of many Gram-negative and Gram-positive bacteria contain the resistance genes of Cr^{6+}, Cd^{2+}, Hg^{2+} and Cu^{2+} [13]. Abbas et al. [14] reported that the metal-resistant bacteria are mostly resistant to antibiotics due to mediation of same plasmids resistance genes.

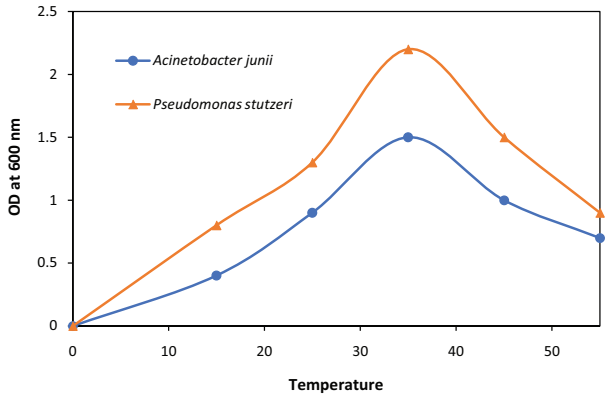

Fig. 3. Effect of different ranges of temperature on bacterial growth.

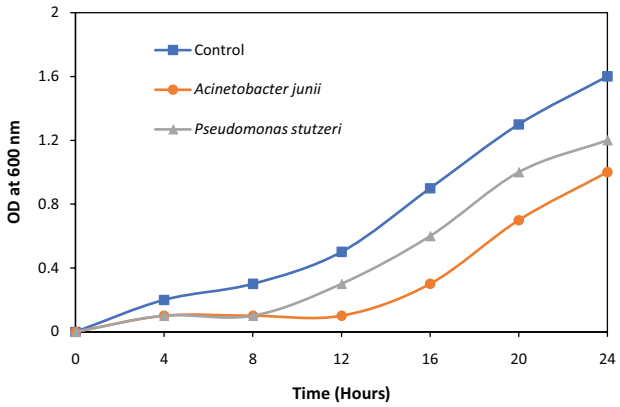

Fig. 4. Growth patterns of *Acinetobacter junii* and *Pseudomonas stutzeri* against control with 50 μg/mL of Hg^{2+}.

Table 2
Antibiotics sensitivity test of Hg-resistant bacterial strains

Antibiotics	Inhibition zones (mm)	
	Acinetobacter junii	*Pseudomonas stutzeri*
Carbenicillin (100 μg)	R	R
Chloramphenicol (30 μg)	R	R
Ampicillin (10 μg)	R	R
Penicillin (10 μg)	S (18)	S (17)
Gentamicin (10 μg)	S (20)	R
Oxytetracycline (30 μg)	S (22)	R

S, sensitive; R, resistant.

3.6. Hg^{2+} reduction ability of bacterial isolates

The Hg processing ability of both strains was measured by giving the stress of 100 μg/mL of Hg^{2+} to bacterial culture as shown in Fig. 5. The *Acinetobacter junii* could reduce the Hg^{2+} 30%, 40%, 55% and 70% after 10, 20, 30 and 40 h, respectively. The *Pseudomonas stutzeri* could also efficiently remove Hg after 10, 20, 30 and 40 h about 35%, 60%, 75%, and 90%. The bacteria have large surface area due to small size so easily provide large surface area to contact with metal [15].

3.7. Bioaccumulation of Hg^{2+} by bacterial isolates

The pellets of *Acinetobacter junii* and *Pseudomonas stutzeri* had ability to bioaccumulate the Hg about 38% and 45%, respectively. So, the Hg^{2+} bioaccumulation ability of *Acinetobacter junii* was observed about 76% and 90% of *Pseudomonas stutzeri* as shown in Table 3. Two main mechanisms are involved in bioaccumulation of Hg as follows: the first is enzymatic detoxification of Hg in which organic Hg is converted to inorganic Hg and upon further detoxification degraded into methyl-Hg that normally volatilizes from the bacterial surface to atmosphere. Matsui and Endo [16] reported that MerA and many other enzymes are involved in the reduction of Hg^{2+}. Dash et al. [17] reported four types of enzymatic detoxification mechanisms in the bacteria as follows: production of insoluble HgS, enzymatic reduction of Hg^{2+} to Hg°, volatilization and biomineralization of Hg^{2+}.

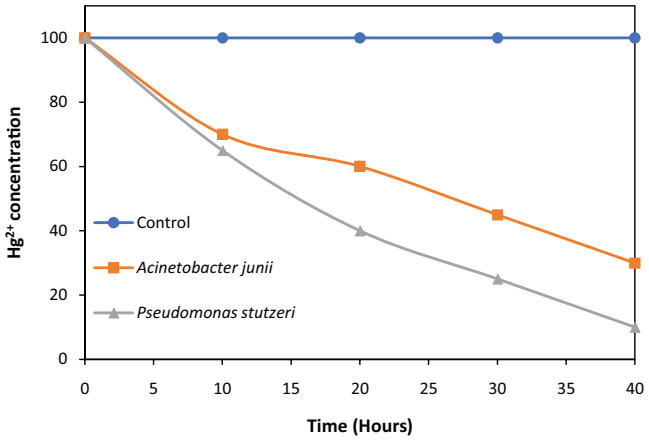

Fig. 5. Hg^{2+} bioaccumulation ability of *Acinetobacter junii* and *Pseudomonas stutzeri* against control which did not contain bacterial culture.

Table 3
Bioaccumulation ability of bacterial strains with the initial concentration of Hg^{2+} at 50 μg/mL and at optimum growth conditions

Bioaccumulation percentage of Hg^{2+}	Bacterial strains	
	Acinetobacter junii	*Pseudomonas stutzeri*
Supernatant (μg/mL)	12	5
Pellet (μg/mL)	38	45
Bioaccumulation (%)	76	90

In the presence of NADPH, mercuric reductase reduces Hg^{2+} into sulfhydryl compounds and Hg°. Due to high vapour pressure, Hg° volatilizes from the system. Many bacteria belonging to the genera *Staphylococcus*, *Bacillus* and *Pseudomonas* have been found to reduce Hg^{2+} to Hg° [18]. The second mechanism is biosorption in which Hg adsorb on bacterial surface or accumulate inside the bacteria. The biosorption of Hg involves different chemical reactions such as chelate, ion exchange and adsorption. The *Pseudomonas* strains normally use these strategies for the removal of Hg from wastewater. The biosorption process also needs the energy provided by microbial metabolism [19].

3.8. Protein bands analysis

The cell pellets of stressed and control were collected after 16 h just to make sure that bacteria entered into log phase. The proteins bands of Hg-stressed bacterial culture were compared with the control (non-stressed). The sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that protein bands were induced in the bacterial strains after Hg stress was given. The molecular weight of these proteins bands were 28 kDa (*Pseudomonas stutzeri*) and 98 kDa (*Acinetobacter junii*) as shown in Fig. 6 with pointed arrows. The thickness of *Acinetobacter junii*-induced band is slightly less than the induced band of *Pseudomonas stutzeri* may be due to the already-present transposons on plasmids. The production of these induction bands in adapted may be the main reason of prolonged lag phase and resistance against Hg [20]. This SDS-PAGE analysis was in agreement analysis by Helmann et al. [21] and François et al. [22]. They also reported the induced bands in the Hg-resistant bacteria.

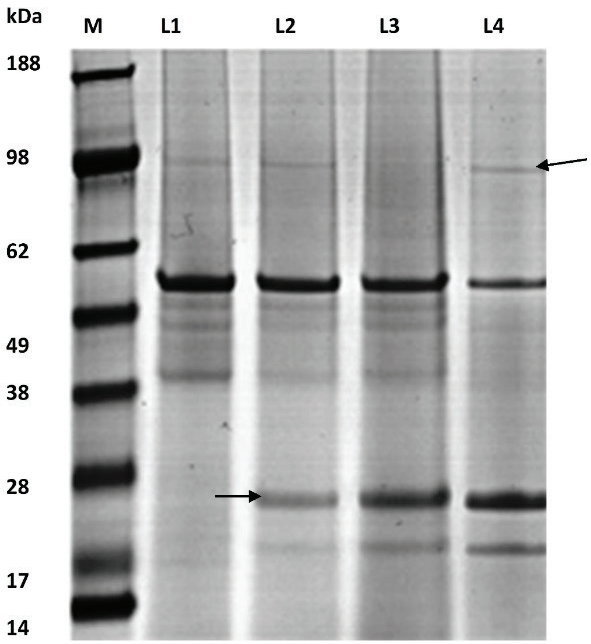

Fig. 6. "M" indicating the marker lane 1 and 2 representing non-stressed (control) and stressed proteins bands of *Pseudomonas stutzeri*, while lane 3 and 4 indicating non-stressed (control) and stressed protein bands of *Acinetobacter junii*, respectively.

4. Conclusions

In this study, both strains *Acinetobacter junii* and *Pseudomonas stutzeri* could remove and bioaccumulate more than 70% of Hg. So these strains can be employed for the bioremediation of waste-containing Hg due to their high resistance against Hg and potential to remove the Hg from industrial wastewater. Further research is needed to know more what kind of mechanisms or genes mediation are adopted by these strains after bioaccumulation/biosorption of Hg.

Acknowledgements

The authors would like to express their appreciation to Universiti Sains Malaysia Global Fellowship (USMGF) and Bridging Research Grant (304/PTEKIND/6316043) for the support and research facilities for this project.

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