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ISOLATION AND IDENTIFICATION OF
A MERCURY RESISTANT STRAIN

A mercury resistant strain 2 was isolated. It was identified as *Pseudomonas putida* and designated as *Pseudomonas putida* 2 on the basis of morphological and biochemical analysis in combination with phylogenetic analysis. Strain 2 could tolerate and aerobically grow in the medium containing up to of 50 mg·dm^3 Hg(II). The highest Hg(II) removal of strain 2 was 85.2% at an initial concentration of 15 mg·dm^3 Hg(II). The highest Hg(II) removal of strain 2 was obseved at 30 °C and pH 7.

1. INTRODUCTION

Mercury is highly toxic to environment and human beings, even in a low concentration [1]. It can accumulate along the food chain due to its non-biodegradability [2]. Therefore, toxic effects of mercury are mostly pronounced on animals with higher nutrient levels [1]. Mercury sources are of both natural and anthropogenic origin. Natural sources include volcanoes, soil erosion and oceans, whereas anthropogenic sources are even diverse and are changing with time [3]. These mercury pollutants are derived from a growing number of diverse anthropogenic sources, such as industrial effluents and wastes, urban runoff, sewage treatment plants, agricultural fungicide runoff, domestic garbage dumps and mining operations [4]. Atmosphere, some agricultural lands and water bodies have been affected and destroyed by industrialization process and continuing release of mercury [5]. Mercury contamination is progressively affecting more and more different ecosystems [6].

As a consequence of its toxicity and risks to the environment and human health, studies on mercury pollution and its remediation have received more and more atten-

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tion. Mercury removal from wastewater is one of the scientific topics attracting scientists globally [1]. Some physical and chemical treatments such as adsorption, chemical oxidation and incineration, have been suggested [5]. However, due to their lower costs and less pollution to the environment, biological treatments are preferred [5]. Many microorganisms resistant to mercury exist in the environment. They can be isolated and then be used to remove mercury. This paper describes the isolation and characterization of a mercury resistant bacterial strain from the mercury polluted soil at a waste treatment plant in Hefei, Anhui Province, PR China. Its anti-mercury capabilities were investigated.

2. EXPERIMENTAL

Isolation of the bacterial strain. Soil samples were collected [4] at the Waste Treatment Plant in Hefei, Anhui Province, PR China. Most industrial wastes of Hefei were managed in the Waste Treatment Plant. These wastes contained toxic metals, such as Hg(II), Cr(VI), Cd(II). The quality and quantity of these wastes containing toxic heavy metals were dependent upon their industrial sources. Some surface soil was polluted by these wastes. The studied region was divided into a grid of 10 square cells using a systematic grid sampling method with regularly spaced intervals of about 50 m. Within each grid cell, two samples were randomly collected to a depth of 0–15 cm and then mixed thoroughly to give a composite sample. These samples were used for the isolation of the mercury resistant strain [7]. All samples were dried at room temperature and sieved through a < 2 mm screen. All samples were mixed for use.

5 g mixed samples were dissolved in 500 cm^3 sterilized distilled water. 5 cm^3 of the supernatant was aseptically added to a shaking flask containing 100 cm^3 of LB medium [8] supplemented with 10.0 mg·dm^{-3} of Hg(II) and was incubated on a rotary shaker (120 rpm) at 30 °C. During the incubation, the optical density at 600 nm (OD$_{600}$) of the culture broth was monitored every five hours. After 24 h of incubation, an obvious OD$_{600}$ increase was observed and subsequently 10 cm^3 of the inoculants were transferred to a new shaking flask containing 100 cm^3 of fresh LB medium supplemented with 20.0 mg·dm^3 of Hg(II) . Such operation was repeated with the concentration of Hg(II) increased to 50.0 mg·dm^3. The final culture broth was serially diluted with 1× phosphate-buffered saline and spread onto agar plates containing LB medium supplemented with 50.0 mg·dm^3 of Hg(II). Colonies forming on agar after 24 h of incubation at 30 °C were picked and sub-cultured several times to obtain pure cultures. The pure colony was classified according to its cultural characteristics. Pure culture was transferred to slant LB medium and stored at 4 °C. For longer storage, the bacterium was kept in 20% glycerol stock at –80 °C.

Identification of the isolate. The bacterium isolated was inoculated on several media for biochemical testing. The morphological and biochemical characteristics of the isolate were carried out as described in International Streptomyces Project [9]. Gram stain, catalase- and oxidase-activities, methyl-red, V-P, arginine, protease, glucose, citrate, indol and starch hydrolysis tests were made. Motility was assessed by testing the ability of the strain to migrate from the point of inoculation through semisolid agar plate.

DNA of the isolate was isolated for molecular detection [1]. DNA was then incited and dissolved into 100 µl of TE buffer and stored at –20 °C for further use. A whole-cell direct lysis PCR amplification method was used to amplify 16S r DNA of the strain. Previously described primer pairs (5′-AACTGAAGAGTTTGATCCTGGCTC-3′ and 5′-TACGGTTACCTTGTTACGATT -3′) were adopted in the detection [4]. 16S r DNA sequence of the strain was determined for both strands using the dideoxy chain termination method [4]. The similarity and homologue of the sequence was compared with that of existing sequences available in the data bank with BLAST search [9]. DNA sequences were aligned and a phylogenetic tree was made by the neighbour-joining method [9]. The construction of phylogenetic tree based on the maximum parsimony method was also carried out using MEGA 4-biologist-centric software for evolutionary analysis of DNA and protein sequences.

Effects of Hg(II) on the growth of the isolate. Batch experiments were performed in duplicate to clarify the mercury resistant potentials of the isolate. Before each experiment, the same concentration of bacterial levitation liquid was made by bacteria maintained on nutrient agar slant. For each experiment, the same amount of bacterial levitation liquid (1 cm^3) was transferred aseptically into 250 cm^3 flasks with 99 cm^3 of sterilized LB liquid medium (pH 6.8–7) supplemented with various amounts of Hg(II) (0, 5, 15, 30, 50 and 70 [mg·dm^3]). The inoculants were incubated for 24 h in flasks on a New Brunswick rotary shaker (at 30 °C, 120 rpm). Parallel test was carried out. Samples were detected periodically for cell density at hourly intervals. Cell growth [9] was monitored by the absorbance at 600 nm with a 760CRT Dual-beam UV-visible spectrophotometer (Shanghai the Third Analysis Instrument Plant).

Hg(II) removal by the isolate. Biological sorption experiments were carried out with different concentrations of Hg(II) (5 mg·dm^3, 15 mg·dm^3, 30 mg·dm^3, 50 mg·dm^3 and 70 mg·dm^3, respectively). All other factors, such as pH, the temperature and the biomass, were kept the same with the experiment of Effects of Hg(II) on the growth of the isolate. After 24 hours the culture was centrifuged at 8000 r·min^{-1} at room temperature for 10 min and the supernatant was used for Hg(II) measurement. Hg(II) was analyzed by using the F732-VJ Cold Vapor Atomic Absorption Mercury Analyzer (Shanghai Huaguang Instrument and Meter Factory) to investigate Hg(II) removal by the isolate [1]. Hg(II) removal was calculated as follows:

$$\text{Removal} = \frac{C_i - C_f}{C_i} \times 100\%$$

where C_i and C_f are the initial and final Hg(II) concentrations, respectively.

Impacts of temperature and pH on Hg(II) removal by the isolate. Various incubation temperatures (10, 20, 30, 40 and 50 °C) were applied to investigate the efficiencies of Hg(II) removal by the isolate [10]. The concentration of Hg(II) in the LB medium was kept 30 mg·dm^3. All other factors such as pH and the biomass were the same for all the samples The shaking flask (250 cm^3) containing LB medium was kept shaking in an electrically thermostatic reciprocating shaker at 120 rpm for 24 h at various temperatures. Then concentration of Hg(II) and OD_{600} were measured for 24 h. The experiment was repeated three times.

Various pH values (5, 6, 7, 8 and 9) of the medium were tested to compare the efficiencies of Hg(II) removal by the isolate. All the other procedures were the same as the above mentioned.

3. RESULTS

3.1. ISOLATION AND IDENTIFICATION OF THE ISOLATE

A bacterial strain (strain 2) was successfully isolated from the soil samples which could survive in LB containing 50 mg·dm^3 of Hg(II). Colonies of strain 2 on LB solid medium after 24 h of incubation at 30 °C were smooth and translucent, with no pigment. Gram staining revealed that strain 2 was Gram-negative. Cells were around 2 μm long and 0.8 μm in diameter. Substrate-utilization experiments were performed and showed that strain 2 was capable of using a variety of substrates, including oxidase, V-P, citrate, arginine, protease and glucose.

Strain 2 was identified by 16S r DNA sequencing, which is a widely used method for the identification of isolates. The 1.5 kb 16S r DNA gene (Fig. 1a) was PCR amplified using primers mentioned in the method sections and was sequenced by these primers. The sequence was matched with NCBI database. The sequence of strain 2 showed 98% similarity to *Pseudomonas putida* isolate PSB30 and a phylogenetic tree was constructed with bootstrap values (Fig. 1b). A neighbour-joining tree based on 16S r DNA sequences showed that the isolate occupied a distinct phylogenetic position within the radiation including representatives of the *Pseudomonas* family. Based on morphological and biochemical analysis in combination with phylogenetic analysis, strain 2 was identified as *Pseudomonas putida* and designated as *Pseudomonas putida 2*.
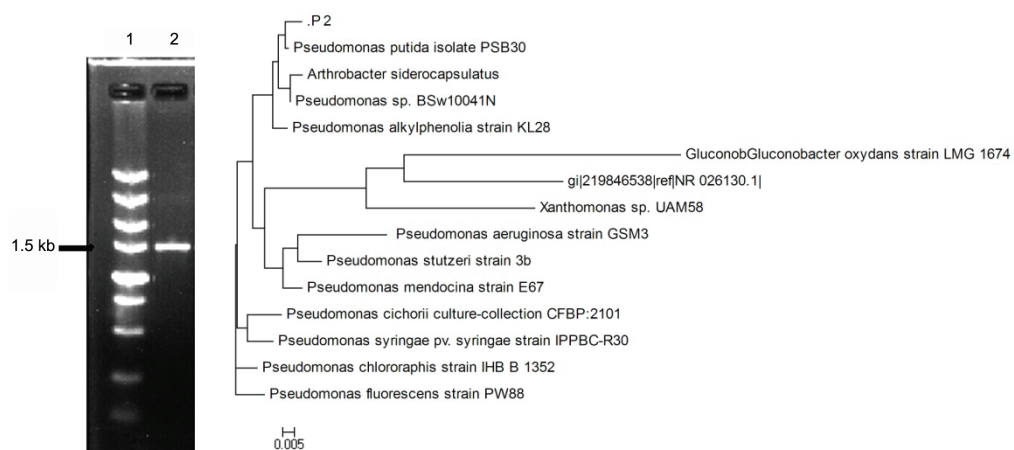
Fig. 1. 16S r DNA analysis of the mercury resistant strain 2: a) electrophoresis
of amplification of strains 2′ 16S r DNA; lane 1 presents DL5000^{TM} DNA marker from TaKaRa,
lane 2 – 16S r DNA of strain 2, b) phylogenetic tree of 16S r DNA of strain 2

3.2. EFFECTS OF Hg(II) ON THE GROWTH OF STRAIN 2

Results showed that strain 2 could survive on LB medium containing no more
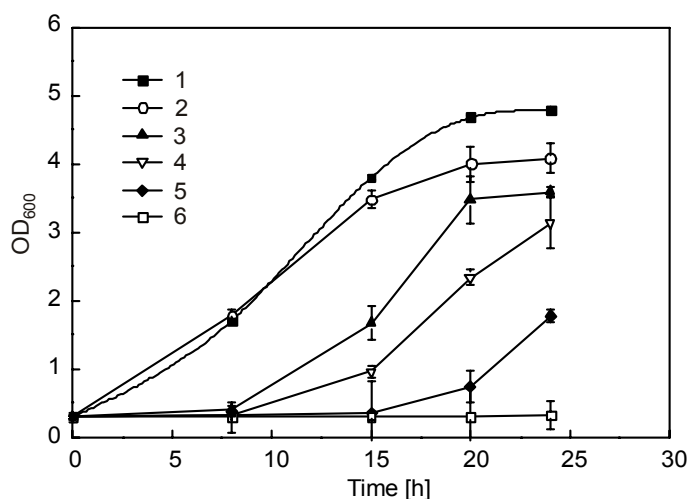than 50.0 mg·dm^3 of Hg(II) (Fig. 2).

Fig. 2. Effects of Hg(II) on the growth of strain 2

At the initial Hg(II) concentration of 70.0 mg·dm^3, no growth was observed. The lag phase of strain 2 increased with the increase of Hg(II) concentration, especially when the initial Hg(II) was higher than 30.0 mg·dm^3 and an obvious delay in the lag phase, the exponential growth phase and the stable phase was observed. When the concentration of Hg(II) was 30.0 mg·dm^3, strain 2 could survive well but its lag phase extended. When the concentration of Hg(II) was 50.0 mg·dm^3, strain 2 could survive with serious inhibition and its OD$_{600}$ diminished by 50% after 24 h. Thus the most appropriate tolerated concentration of strain 2 for Hg(II) was 30.0 mg·dm^3. Low level of Hg(II) showed less toxicity to the growth of strain 2 [4].

3.3. Hg(II) REMOVAL BY STRAIN 2

Removals by strain 2 at different concentrations of Hg(II) are given in Table 1. At concentrations of 5 mg·dm^3 and 15 mg·dm^3, however, there was no significant difference in strain 2 to absorb Hg(II) in the medium. At the initial Hg(II) concentration of 15 mg·dm^3, strain 2 gave the highest Hg(II) removal capacity of 85.2%. Low level of Hg(II) showed less toxicity to the growth of strain 2 and its metabolism by strain 2 was quicker. Upon the increase of initial Hg(II) concentration, the removal efficiency decreases (Table 1). This finding is consistent with the results presented by other authors [11]. With the increase of initial Hg(II) concentration, Hg(II) removal capacity decreases as the available binding sites on the biomass surface are restricted [11].

Table 1

Hg(II) removals of strain 2 for 24 hours

Initial Hg(II) concentration [mg·dm^3]	Removal [%]
5.0	81.2 ± 0.04a
15.0	85.2 ± 0.01a
30.0	73.8 ± 0.02b
50.0	61.9 ± 0.04c
70.0	0.0 ± 0.03d

Values in the columns marked with different letters differ significantly (ANOVA, Tukey' post hoc test, $P = 0.05$)

3.4. EFFECTS OF TEMPERATURE AND pH ON Hg(II) REMOVAL CAPACITY OF STRAIN 2

Hg(II) removal and cell growth of strain 2 were increased gradually with the increase of temperature until 30 °C and then decreased (Fig. 3). There existed a positive correlation between culture temperature and the removal of Hg(II) and cell growth of strain 2. The effect of pH on Hg(II) removal by *Pseudomonas putida* is shown in

Fig. 4. The highest values were observed at pH 7. When pH increased or decreased, the removal and cell growth were dramatically decreased.
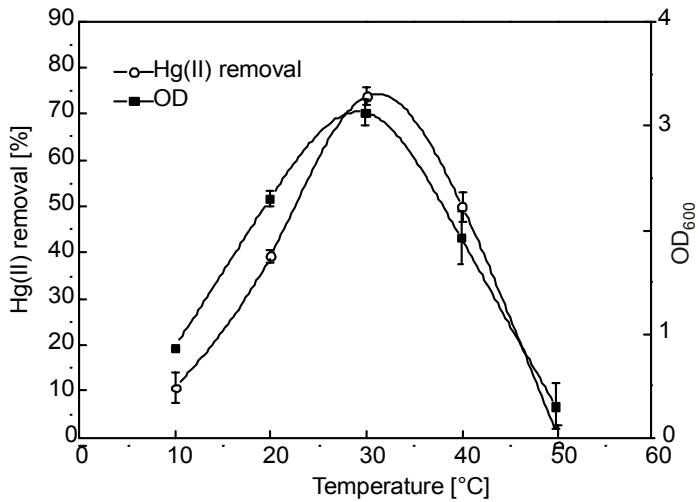

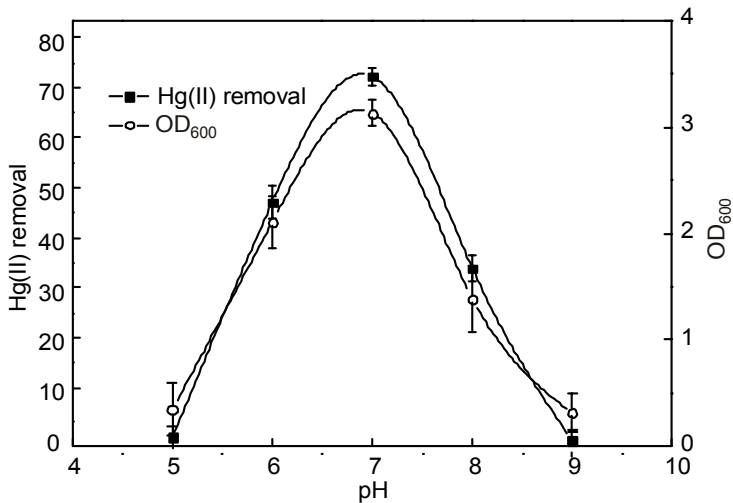Fig. 3. The impact of temperatures on Hg(II) removal of strain 2

Fig. 4. The impact of pH on Hg(II) removal of strain 2

Our results have shown that the highest Hg(II) removal of strain 2 occurred at 30 °C and pH 7. The metal-binding properties of bacteria were largely due to the existence of specific anionic polymers in the cell wall structure, consisting primarily of peptidoglycan, teichoic or teichuronic acids [12]. The activity of these anionic polymers could be affected by different temperatures and pH values. Earlier studies on

heavy metal biological absorption have shown that temperature and pH are important parameters affecting the process [10].

4. DISCUSSION

Isolation of bacterial strain through the enrichment technique has been extensively used. Sampling environment containing elevated concentrations of heavy metals was a potential source for toxic metal-tolerant bacteria [13]. By constant isolation and domestication in this study, strain 2 was successfully isolated from the soil samples which could survive in LB containing 50 mg·dm^3 of Hg(II). Strain 2 was identified as *Pseudomonas putida*. Until now there are several reports on isolation of mercury resistant bacteria belonging to family *Pseudomonas* [14]. For example, the results of this study are similar with those for strain KHg$_2$ isolated by Yan Zeng and Qiang Chen [14]. Strain KHg$_2$ was isolated from the river sediment of Liangshui river in Beijing and could grew on LB medium plate containing 70 mg·dm^3 of Hg(II). The morphological characteristics and the biochemical properties of strain KHg$_2$ were in agreement with those of *Bacillus silvestris*. One PCR fragment obtained from strain KHg$_2$ shared 99% sequence identity with P*seudomonas putida* [14]. However, this is the first report on isolation of a mercury resistant strain which reached the highest Hg(II) removal (85.2%).

The next step we will study the mechanism of mercury removal and the development in the long term of strain 2. And the gene which decides strain 2 to remove mercury should be located. We will also construct gene engineering bacteria to be used in a continuous process for the removal of mercury in industrial effluents.

5. CONCLUSIONS

By a culture enrichment technique, a new mercury-removing bacterium (strain 2) was isolated from contaminated soil at the Waste Treatment Plant in Hefei, AnHui Province, PR China. Strain 2 was identified as *Pseudomonas putida* and designated as *Pseudomonas putida 2* based on the morphological and biochemical analysis in combination with phylogenetic analysis. The maximum tolerated concentration of strain 2 for Hg(II) was 30 mg·dm^3. When initial Hg(II) concentration was 15 mg·dm^3, strain 2 reached the highest Hg(II) removal (85.2%). And optimum Hg(II) removal was observed at pH 7.0 and 30 °C. *Pseudomonas putida 2* had a high tolerance to mercury toxicity and enriched the knowledge on *Pseudomona*s species that can grow on mercury compounds.

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