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Research Article

Isolation, Identification, and Characterization of Cadmium Resistant *Pseudomonas* sp. M3 from Industrial Wastewater

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The present study deals with the isolation, identification, and characterization of the cadmium resistant bacteria from wastewater collected from industrial area of Penang, Malaysia. The isolate was selected based on high level of the cadmium and antibiotic resistances. On the basis of morphological, biochemical characteristics, 16S rDNA gene sequencing and phylogeny analysis revealed that the strain RZCd1 was authentically identified as *Pseudomonas* sp. M3. The industrial isolate showed more than 70% of the cadmium removal in log phase. The cadmium removal capacity of strain RZCd1 was affected by temperature and pH. At pH 7.0 and 35°C, strain RZCd1 showed maximum cadmium removal capacity. The minimal inhibitory concentration of strain RZCd1 against the cadmium was 550 μg/mL. The resistance against the cadmium was associated with resistance to multiple antibiotics: amoxicillin, penicillin, cephalexin, erythromycin, and streptomycin. The strain RZCd1 also gave thick bands of proteins in front of 25 kDa in cadmium stress condition after 3 h of incubation. So the identified cadmium resistant bacteria may be useful for the bioremediation of cadmium contaminated industrial wastewater.

1. Introduction

Among the list of heavy metals, the cadmium needs special attention because it is identified as significant pollutant due to its high solubility and toxicity in the water [1, 2]. It is one of the most toxic pollutants of the surface soil layer, released into the environment by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels [3]. The wastewaters from the industries and sewage sludge applications have permanent toxic effects to human and the environment. Both terrestrial and aquatic environments have been greatly affected by the cadmium pollution [4]. In human, it affects cell proliferation, differentiation, apoptosis and increases oncogene activation to carcinogenesis. It also causes vertebral osteoporosis and fractures, toxicity to neuron, aging, and peripheral arterial disease. It selectively accumulates in pancreas, bones, renal liver, lungs, and kidneys [5, 6].

The water pollution caused by the cadmium has received increasing attention worldwide. Many conventional approaches have been considered for the removal of cadmium from industrial wastewater, mainly including chemical precipitation, ion exchange, membrane technology, and adsorption [7, 8]. In the field of bioremediation, simpler and relatively inexpensive ways are more focusing than chemical treatment methods. Furthermore, many investigators have reported the bioaccumulation of cadmium onto natural microbial populations like bacteria and algae as the new bioremediation technology [9, 10]. The microbes use various survival strategies to combat the cadmium stress that include metal ions sequestration, active efflux of metals; some use enzymatic detoxification and cadmium accumulation [11, 12].

In this work, a cadmium resistant strain RZCd1 was isolated from industrial wastewater and identified as *Pseudomonas* sp. M3 based on morphological observation, biochemical, physiological characterization, and partial 16S rDNA sequence analysis. The resistance against the cadmium

and the antibiotic was examined. Furthermore, the removal capacity of the strain RZCd1 was also studied.

2. Experimental

2.1. Collection of Water Samples. The wastewater samples were collected from the industrial area of Penang, Malaysia, located in 5°.07617′, 5°.38400′, 5.41264′, and 5°.329379′ "N" longitudes and 100°.386119′, 100°.302104′, 100°.325307′, and 100°.482284′ "E" latitudes. The industrial wastewater samples were collected in the screw capped bottles. The samples ranged in external pH from 6.0 to 7.0, temperature varied from 29°C to 35°C, and the cadmium range was 0.36 ± 0.82 mg/L.

2.2. Isolation of Cadmium Resistant Bacteria. The stock solutions of 10 μg/mL and 100 μg/mL of the cadmium were prepared by dissolving the cadmium chloride in the deionized water and sterilized by using 0.22 μm pore-size sterile filters. The cadmium resistant bacteria were isolated by standard dilution method. The serially diluted water samples were sown on the nutrient agar plates which were loaded with 10 μg/mL of the cadmium solution. The bacterial strains which can tolerate the 10 μg/mL of the cadmium were isolated and further streaked on the agar plates with 100 μg/mL of the cadmium and then finally selected for further experiments.

2.3. Identification of Bacteria. After biochemical tests and staining, the isolated strain was preliminary identified according to the key of Bergey's Manual of Determinative Bacteriology [13]. Further bacterial isolate was characterized on the molecular base. First the bacterial genomic DNA was isolated by Triton-Prep method. The partial 16S rDNA was amplified by the PCR (polymerase chain reaction) with bacterial universal primers 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492R (5′ TACGGYTACCTTGTTACGACTT 3′). The PCR was carried out with an initial denaturation step for 5 min at 94°C, followed by 35 cycles of denaturation at 94°C, 30 sec of annealing at 52°C, and 40 sec of elongation at 72°C. The last step was a final extension at 72°C for 10 sec. The samples were experimented on the gels containing TBE buffer (EDTA 2 mM, Boric acid 89 mM, Tris 89 mM, and pH = 7) and the purification of the PCR products was done by Silica gel kit (Fermentas Co, Germany) and 25 μL of the PCR products sent to Center of Chemical Biology, Universiti Sains Malaysia for sequencing. By using NCBI blast analysis, the 16S rDNA sequences were submitted to the database of GenBank and compared with similar sequences. The phylogenetic tree of partial 16S rDNA was constructed using the software Clustal W.

2.4. Minimum Inhibitory Concentration (MIC) against Cadmium. A stock solution of 1000 μg/mL of the cadmium was prepared. The M9 acetate minimal medium (0.5 g/mL yeast extract, 0.2 g/mL $MgSO_4$, 5.0 g/mL sodium acetate, 0.001 g/mL $FeSO_4$, 0.001 g/mL $CaCl_2$, 0.5 g/mL K_2HPO_4, and 1.0 g/mL NH_4Cl) was used to check the MIC against the cadmium [14]. In each test tube 10 mL of the M9 acetate minimal medium was taken. After autoclaving, the medium was supplemented with 20 μL overnight grown bacterial culture and consecutive addition of the cadmium ranged from 50 to 550 μg/mL and then finally incubated at 37°C for 24 h. The growth was measured as optical density at 600 nm.

2.5. Determination of Antibiotic Resistance. The antibiotic disks (Oxoid, Hampshire, England) contained 8 antibiotics: penicillin (10 IU), tetracycline (30 μg), amoxicillin (10 μg), gentamycin (10 μg), cephalexin (30 μg), erythromycin (15 μg), streptomycin (10 μg), and ciprofloxacin (5 μg). The strain was tested for its sensitivity to the 8 antibiotics. The 0.1 mL culture was spread on the LB agar plates. The antibiotic disks were placed on the plates and incubated at 37°C for 1-2 days.

2.6. Cadmium Removal Capacity. The isolated strain was grown in the Luria Bertani broth containing 100 μg/mL of the cadmium. During the growth period, 1 mL of bacterial culture sample was removed into eppendorf tube after every four hour until 24 h and centrifuged at 6000 rpm (revolutions per min). To check the effect of temperature and pH, the isolated strain was grown at different temperatures (5–45°C) and different pH (1.0–13.0). An extra sample without the addition of bacterial culture was prepared as control. The supernatants were collected and stored at 4°C for the cadmium analysis. The cadmium concentrations in the supernatants were analyzed with a GBC932 atomic absorption spectrometry (Pantech Instruments, Blackburn, Victoria, Australia) at 228.8 nm with a cadmium hollow cathode lamp. The optimal density of each sample was also measured at 600 nm to compare the growth rate of bacteria with the cadmium removal capacity [6].

2.7. Protein Profiling. In the conical flasks, 20 mL of the Luria Bertani broth was taken in triplicate and steam sterilized. The bacterial isolate was stressed with concentration of the cadmium 350 μg/mL and with control. Then these conical flasks were incubated for 3 h at 37°C in shaking incubator and harvest the cells by centrifugation. The pellet was dissolved in 100 μL of the 1x loading dye then heat shock was given for 5 min; eppendorf tube was shifted on ice for 2 min and then was centrifuged at 12000 rpm for 10 min. The supernatant was transferred to a new eppendorf tube, then final centrifugation was done at 12000 rpm for 10 min and the supernatant was shifted to a new eppendorf tube. Initially gel was run at 40 mV after stake formation and the voltage was increased to 80 mV.

3. Results and Discussion

3.1. Bacterium Isolation. There were a few small colonies on the agar plate with 10 μg/mL of the cadmium after incubating for 2-3 days. When the cadmium concentration increased up to 100 μg/mL and incubated for 3 days, only single colony was able to grow on the plate. The colony was streaked on the agar plate to get the purified bacterium and denoted as strain RZCd1.

Table 1: Characteristics of the strain RZCd1.

Characteristics	Strain RZCd1
Morphological observation	
Colony color	Off-white
Gram nature	Gram-negative
Cell shape	Coccus
Colony shape	Round
Motility	Positive
Spore formation	Negative
Biochemical tests	
Catalase test	Positive
Urease test	Negative
Gelatin hydrolysis test	Positive
Carbohydrate test	Negative
MRVP test	Negative
Citrate test	Positive
Blood agar test	Positive
Chocolate agar test	Positive
Antibiotics sensitivity profiles	
Tetracycline (30 μg)	23 mm (sensitive)
Amoxicillin (10 μg)	Resistant
Penicillin (10 units)	Resistant
Gentamycin (10 μg)	20 mm (sensitive)
Cephalexin (30 μg)	Resistant
Erythromycin (15 μg)	Resistant
Streptomycin (10 μg)	Resistant
Ciprofloxacin (5 μg)	40 mm (sensitive)

3.2. Identification of Bacterial Strain. The colony of strain RZCd1 was round and off-white in color. The bacterium was Gram-negative and had polar flagellum, without capsule and spore. The Gram staining observation showed that it was coccus in shape. The morphological and biochemical characteristics of strain RZCd1 are listed in Table 1. According to Manual of Determinative Bacteriology, comparing strain RZCd1 with other bacteria, the strain was similar to *Pseudomonas* sp. The partial 16S rDNA gene sequence of the strain RZCd1 was amplified by PCR. The partial 16S rDNA sequence of strain RZCd1 was submitted to the database of GenBank and the accession number was KF146957. The BLAST analysis showed that the partial 16S rDNA of strain RZCd1 had more than 95% similarity index with that of *Pseudomonas* sp. M3. In order to determine the relationship between strain RZCd1 and the other *Pseudomonas* species, the phylogenetic tree based on partial 16S rDNA was constructed as shown in Figure 1. So based on the results of physiological, morphological, biochemical characteristics and 16S rDNA sequence analysis, strain RZCd1 was identified as *Pseudomonas* sp. M3.

3.3. Resistance against Cadmium. The tolerance test indicated that maximum cadmium tolerance was up to 550 μg/mL as shown in Figure 2. The optical density was decreased with the increase of the cadmium concentration which indicated the toxic effect of cadmium on the growth of bacterium. The bacterium was exposed to the cadmium for a long time, which might change its structure and function to adapt to the cadmium-polluted environment, such as smaller size of bacteria, acquisition of resistance factors, and gene mutation. The high levels of resistance to the cadmium and antibiotics might be associated with its complex survival environment [15]. The strain RZCd1 can grow in the cadmium solution with the high level of resistance. So it is possible to remove the cadmium from wastewater containing cadmium.

3.4. Antibiotic Profile. The susceptibility test of strain RZCd1 against different antibiotics is showed in Table 1. This strain showed only sensitivity against tetracycline, gentamycin, and ciprofloxacin while it was resistant against amoxicillin, penicillin, cephalexin, erythromycin, and streptomycin. The ability of the microorganisms to resist against antibiotics and cadmium seems to be the result of exposure to cadmium contaminated environments that cause coincidental selection of resistance factors for antibiotics and cadmium [16].

3.5. Cadmium Uptake Capacity. The cadmium removal capacity of strain RZCd1 was shown in Figure 3. The lag phase was observed during the initial 0–4 h which was another sign of the cadmium toxicity to strain RZCd1. In this lag phase strain RZCd1 removed only 5.34% of the cadmium. In fact when a microorganism is inoculated into a new culture containing toxic substances, the cells will damage due to pollutant toxicity. In this situation the microorganism expands energy to repair cell damage and adapt its enzymatic pathway to a new condition [17]. After that in log phase (8–16 h), strain RZCd1 removed the maximum amount of the cadmium which is about 74.21% because in this phase bacteria rapidly divide with time so bioaccumulation of the cadmium also increased. The percentage of the cadmium bioaccumulation by strain RZCd1 was increasing from the beginning until 16 h. After 16 h, a slight reduction in cadmium removal was observed in the medium. Some investigators believed that when metal bioaccumulation reaches its maximum level, growth would be decrease and the number of viable cells in the culture would be reduced [11]. If so, it is suggested that glycoprotein materials components in the cell wall of dead bacteria might be degraded by viable cells. This condition leads to the release of the absorbed metals into the aqueous solution.

The capacity of strain RZCd1 to remove cadmium ions from aqueous solutions was also significantly influenced by environmental growth conditions, as pH and temperature as shown in Figures 4 and 5. Studying the effect of different temperatures revealed that 35°C was the optimum temperature for the cadmium uptake by strain RZCd1, because temperature effects are confined to metabolism-dependent metal accumulation [18]. These results were in agreement with Zeng et al. [6] who mentioned that maximum cadmium removal by *Pseudomonas aeruginosa* strain E1 was at 36°C. The removal of the cadmium from nutrient broth media was also influenced by pH values. In the present study maximum cadmium uptake capacity was obtained at pH 7.0, while pH 1.0 and 13.0 were suppressive values for strain RZCd1. The pH of the solution plays a major role in the extent

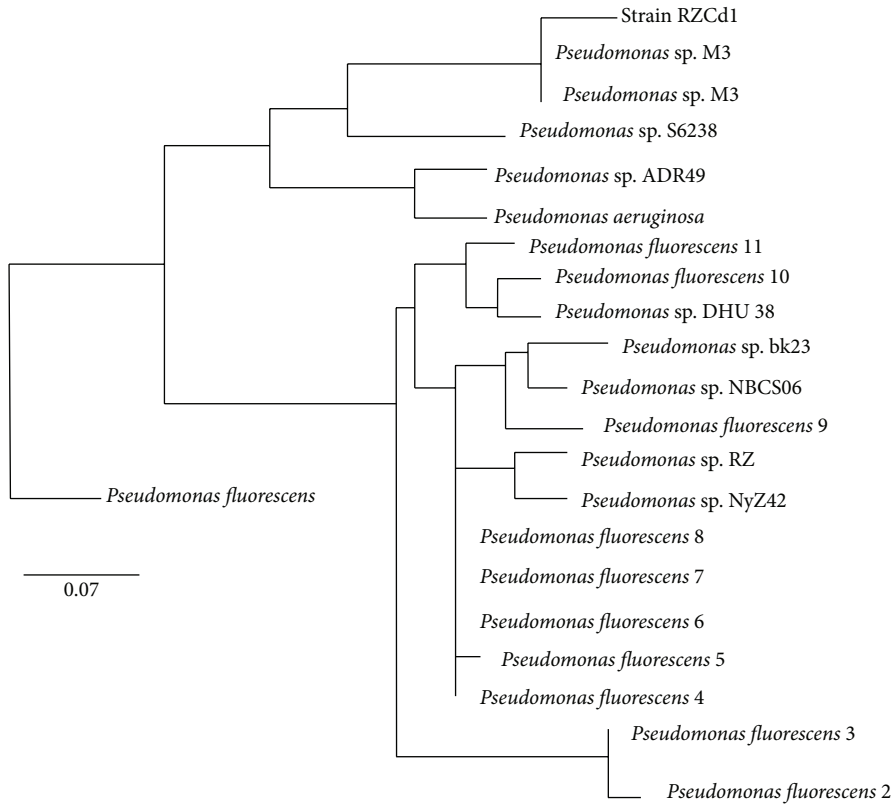

Figure 1: Phylogenetic development trees based on 16S rDNA analysis of strain RZCd1 (*Pseudomonas* sp. M3) while scale bar corresponds to nucleotide sequence difference.

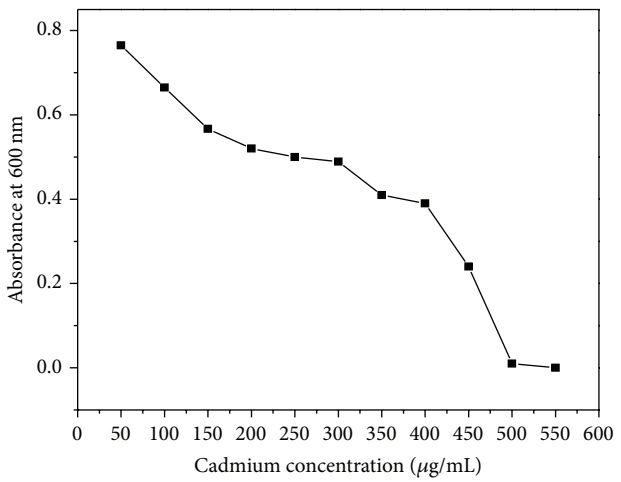

Figure 2: The minimum inhibitory concentration of the strain RZCd1 against cadmium.

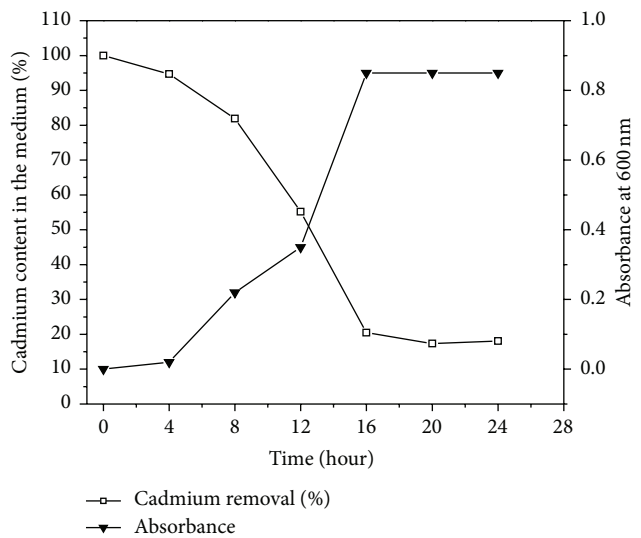

Figure 3: Removal of cadmium after inoculation of the bacterial strain RZCd1.

of metals binding to microorganisms [19]. The variations in external pH can also affect the degree of protonation of potential ligand that contributes to metal binding [20]. The *Pseudomonas fluorescens* also showed maximum cadmium removal at pH 6.8 [21].

3.6. Protein Bands. The proteins bands of strain RZCd1 in stressed and non-stressed medium were shown in Figure 6. The 3 h stressed culture gave thick protein bands of strain RZCd1 in front of 25 kDa. These results were standing with

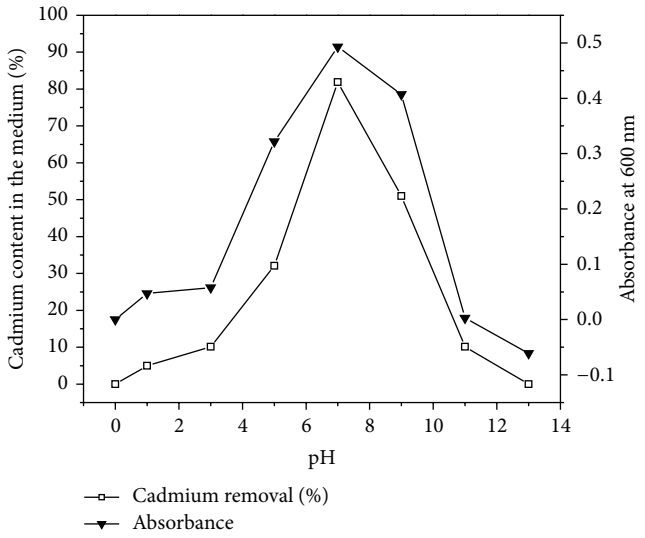

Figure 4: The effect of different pH on the removal of cadmium ions by the strain RZCd1.

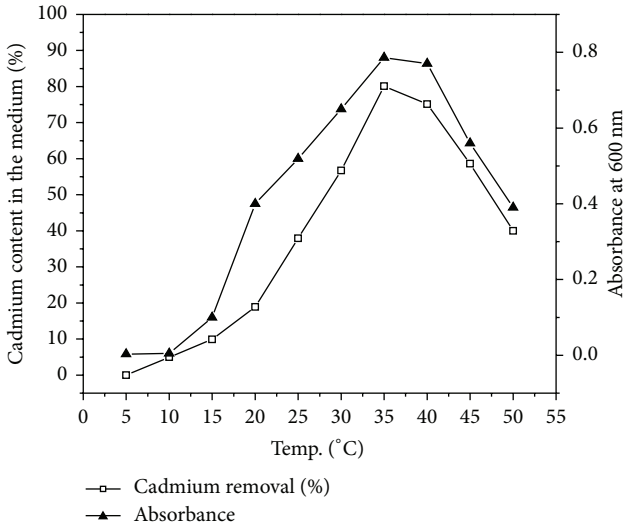

Figure 5: The effect of different temperatures on the removal of cadmium ions by the strain RZCd1.

metaproteomic analysis of a bacterial community response to the cadmium-stressed and nonstressed bands of proteins obtained in front of 25 kDa [22]. So, more than 100 unique differentially expressed proteins were identified through database searching and de novo sequencing. The proteins of importance in the cadmium shock included ATPases, oxidoreductases, and transport proteins. The ability of proteomics to detect the differential regulation of these proteins even during short cadmium exposures shows that it is a powerful tool in explaining cellular mechanisms for a mixed culture [23].

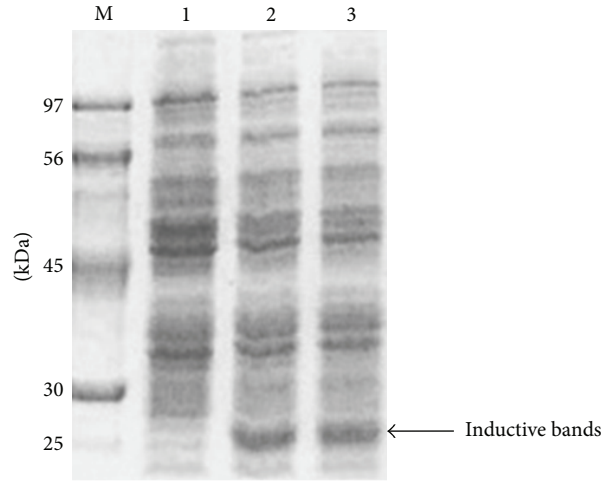

Figure 6: M indicating the marker 1 representing nonstress protein bands of the strain RZCd1 while 2 and 3 lines indicating protein bands of the strain RZCd1 in cadmium stress condition.

4. Conclusions

Due to continued unsustainable levels of human and natural exploitation, search for alternative techniques for treatment of the cadmium contaminated wastewater came into existence. The cadmium bioremediation approach has attracted much attention because it is environment friendly, safe, and economical. We concluded that the Gram-negative strain RZCd1 has resistance against cadmium up to 550 μg/mL and antibiotics. The ability of the strain RZCd1 to tolerate antibiotics and cadmium appears to be the result of exposure to cadmium contaminated environments that cause coincidental selection of resistance factors for cadmium and antibiotics. The elucidation of the exact resistance mechanisms needs further investigation.

Conflict of Interests

The authors declare that they do not have any conflict of interests.

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