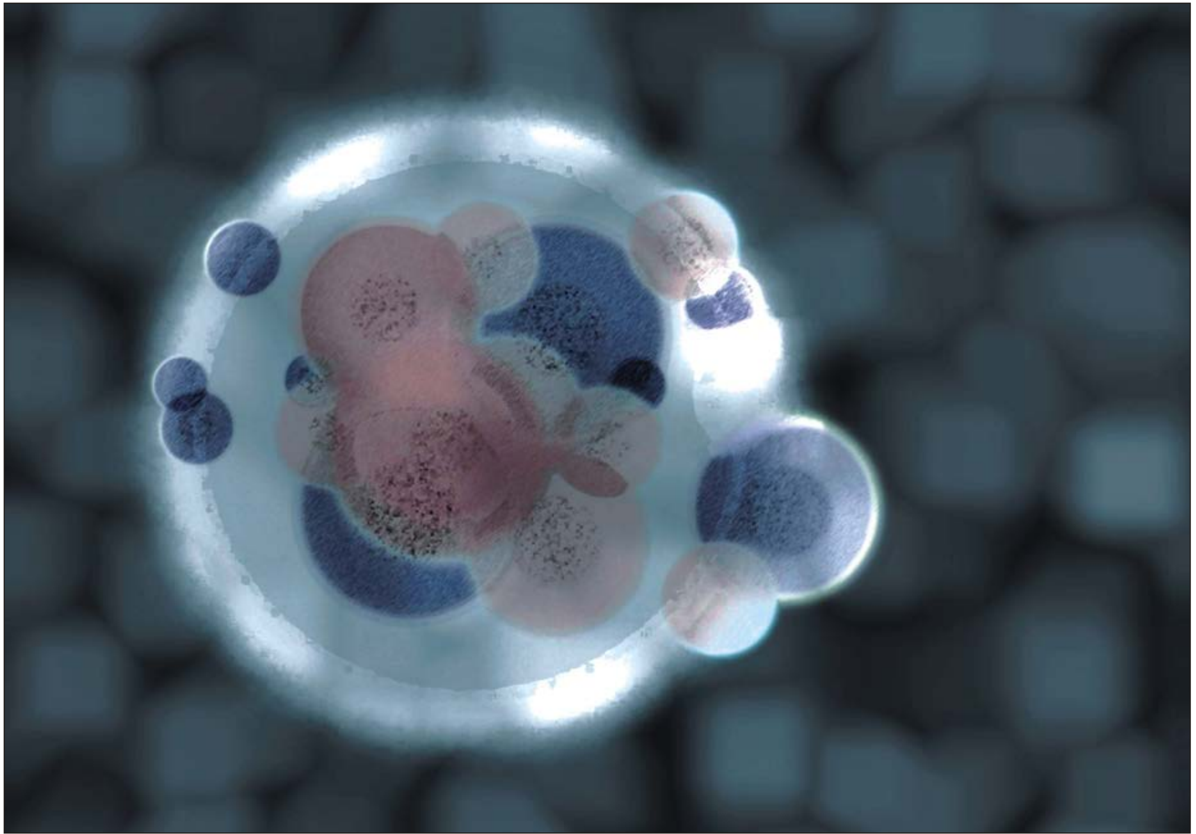

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 5 (3): 269-273, 2005
ISSN 1727-3048
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Removal of Mercuric Chloride by a Mercury Resistant *Pseudomonas putida* Strain

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Abstract: In the present study, *Pseudomonas putida* strain, a mercury resistant bacterium, which is able to reduce ionic mercury to metallic mercury was isolated from chloralkali wastewater and used to remediate ion mercury in liquid medium.The bacterium exhibits high Minimal Inhibitory Concentrations (MICs) for mercuric Chloride. Removal of mercuric chloride by *Pseudomonas putida* was studied using pepton water medium in the concentration range 1-120 mg L^{-1}. Two processes, adsorption on the cell surface and bioaccumulation have been observed. Maximum removal capacity for the bacterium was found to be 98%. Thus, bacterial removal of mercury is a potential biological treatment for mercury environmental pollutants

Key words: Mercury, *Pseudomonas putida*, wastewater, biosorption, chloralkali

INTRODUCTION

The discharge of heavy metals into aquatic systems has been a matter of worldwide concern over the last few decades. These pollutants are introduced into aquatic system significantly as a result of various industrial operations[1]. Mercury is one of the heavy metals of concern and has been found in the wastewaters coming from chloralkali manufacturing industry, oil refinery, paint, pharmaceutical, paper and battery manufacturing industries. Mercury and mercurial compounds are highly toxic contaminants in aquatic system and soils. They are dangerous pollutants because they are able to disperse widely into the environmental due to their high mobility and are potentially concentrated through the food chain[2]. In an aqueous environment, Hg^{2+} in sediment is subject to methylation, forming more toxic methylmercury. Bioaccumulation of methylmercury through the food chains is a potential risk to consumers of contaminated fish or shellfish[3]. At high concentrations, mercury vapor inhalation produces acute necrotizing bronchitis and pneumonitis, which can lead to death from respiratory failure. Long term exposure to mercury vapor primarily affects the central nervous system. Mercury also accumulates in kidney tissues, directly causing renal toxicity, including proteinuria or nephritic syndrome[2]. High concentration of Hg^{2+} cause inpairment of pulmonary function and kidney, chest pain and dyspnousea[4]. Purification of areas polluted by heavy metals such as merury is difficult, because the metals cannot be transformed into hamless elements. over a few decades, community is devoting concentrated efforts for the treatment and romoval of heavy metals in order to combar this problem[1]. Various types of technology is available for removing of mercury in water and wastewater including chemical precipitation, conventional coagulation, reverse osmosis, ultrafiltration, magnetic filtration, ion exchage and activated carbon adsorption and chemical reduction[5]. Biological systems have been thought to be adapted for the removal of toxic heavy metals from industrial aqueous waste[6]. Bioremoval, i.e., the use of biological systems for the removal of metal of metal ions from polluted waters, has the potential to achieve greater performance at lower cost than nonbiological wastewater treatment for the removal of heavy metals[7]. Development in the field of environment biotechnology indicate that bacteria, fungi, yeasts and algae can remove heavy metals from aqueous solutions by adsorption[8]. In bacteria resistance to mercury is related to enzymatic reduction of Hg^{2+} to volatile Hg^{0}[9]. Mercury detoxification process originated from *mer* operon located on either plasmids or transposable elements in the mercury resistant microorganisms. Specific transport of bulk mercury across the cell membrane is achieved by two *mer* operon genes *mer*P and *mer*T, which express cysteine-rich proteins to deliver ambient mercuric toward intracellular mercuric reductase for subsequent reduction of mercuric ions to volatile Hg^{0}[3]. In the present investigation after isolation bacteria from petrochemical wastewater the ability of isolated bacterium,

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Pseudomonas putida, has been assessed for removal, biosorption and up take, mercury ion.

MATERIALS AND METHODS

Isolation of mercury resistant bacteria: Mercury contaminated sludges were collected from the wastewater outlet of chloralkali plant. To minimize the potential for volatilization or biodegradation between sampling and isolation of bacteria, samples were keep in ice without freezing. Aliquts of 0.1 mL of 10^{-1}, 10^{-2} and 10^{-3} dilutions were spread onto brain hearth infusion broth, Trypticase soy broth and nutrient broth. All tubes were incubated at 37°C for 2 days. Isolated bacteria from wastewater were inoculated into 20 mL pepton water in various concentrations of mercuric chloride (1-120 mg L^{-1}) then incubated with shaking at 130 rpm for 24 h at 37°C. Bacterial identification was done by standard biochemical analysis. *Pseudomonas putida,* which has the highest mercury resistance in both liquid and solid media, was selected to study the removal of mercury.

Determination of Minimal Inhibitory Concentration (MIC): The MIC was determined in fluid medium (Pepton water, Merck) using serial twofold dilutions and an inoculum of 10^6 bacteria/mL; results were read after incubation at 37°C for 24 h. The MIC was defined as the lowest concentration of mercury allowing no visible growth.

Mercury measurements: All the glass and plastic wares used were kept in 1.0 N HNO_3 solution overnight and then thoroughly rinsed with deionized water. Mercury stock solution (1000 ppm) were prepared from dissolve chloride mercuric in mixture nitric acid and water. Working standard were prepared daily from the stock solution by serial dilution (20, 40, 80 and 160 ppb) and stored in polyethylene bottles. Mercury contents were determined by flame less cold vapor adsorption spectroscopy by using a flow injection system which linked to an atomic adsorption spectrophotometer (UNICAM, model 929, UK). To determine soluble mercury contents, 5 mL of samples were routinely oxidized by adding 0.01 volume of 65% HNO_3. Ionic mercury was then reduced with $NaBH_4$ (4 g L^{-1}) to metallic mercury, which was volatilized by the carrier gas argon and detected at 253 nm by the atomic adsorption spectrophotomete[10,11]. If necessary, samples were diluted so that they contained less than 100 μg L^{-1} of Hg. To determine total mercury concentrations, 7 mL samples were pre-treated by oxidizing them with 3 mL of 65% HNO_3 for 2 h at 140°C.

Removal of mercuric chloride by *Pseudomonas putida*: Mercury removal was determined by measuring the mercury loss in the culture medium after 48 h of incubation at 37°C. *Pseudomonas putida* that volatilize $HgCl_2$ at high rate was selected for further study of $HgCl_2$ removal by bacterial cells. The bacterium were suspended in 5 mL of pepton water in various concentrations of mercuric chloride (1-120 mg L^{-1}). The cells were then harvested by centrifugation at 6000 g for 10 min. Removal of $HgCl_2$ by *Pseudomonas putida* was detemined by measuring the mercury contents in the supernatant. In order to determine the mercury absorption, cell suspension was prepared by cultivating the bacterium in 5 mL of pepton water in various concentrations of mercuric chloride (1-120 mg L^{-1}) with shaking (180 rpm) at 37°C. After 24 and 48 h cultivation, the cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C, then washed with sterile 100 mM phosphate buffer saline (2 g of NaCl, 0.5 g KCl, 0.15 g of Na_2HPO_4 and 0.47 g of KH_2PO_4, pH 7) and supernatant was analysed by cold vapor atomic absorption spectrophotometry. Bioaccumulation of $HgCl_2$ was determined by measuring the mercury contents in the cell suspension after treatment of cells with nitric acid. To investigate whether mercury was removed from medium in the absence of cells, the remaining metal in the culture medium after 3 days of incubation was determined under different conditions.

Effect of pH and temperature: The effect of pH (4.0-11) was investigated by adjusting pH of the sample using cells of the bacterium. The removal test was conducted on a shaker (180 rpm) at 37°C for 24 and 48 h. The samples were taken and centrifuged at 6,000 rpm for 15 min. The supernatants were analyzed for the remaining mercury in the solution and the percentage of each metal removal calculate based on its initial concentration. The effect of temperature was studied at various incubation temperatures. The optimum pH, temperature and bacterial strain were selected for further studies.

Effect of mercury concentrations: The removal of mercury at the concentrations of 1-120 mg L^{-1} using the suspension of the selected strain at the optimum pH were compared. The removal test was conducted in an incubator shaker (180 rpm) at 37°C. The samples were taken after 24 and 48 h incubation of the cell suspension. The supernatants of the samples were analyzed and the percentage of mercury removal calculated as mentioned above.

RESULTS

Isolation and identification of mercury resistant bacteria: A total of 8 bacterial strains resistant to mercury were isolated from samples of mercury contaminated sludges. Only one strain was capable to mercury removal, when transferred to pepton water containing high amounts of $HgCl_2$. Preliminary identification indicated that the bacteria was a gram negative, motile, yellow pigmented, oxidase negative, catalase positive, aerobic rod shaped bacterium. Following the criteria of Bergey`s Manual of determinative Bacteriology, the isolated was identified as a *Pseudomonas putida.*

Minimal inhibitory concentrations: The 8 isolated bacteria were divided into three classes according to their growth in pepton water containing 1-120 μg mL^{-1} of $HgCl_2$. Two isolated bacteria could grow in pepton water containing 40 μg mL^{-1} of $HgCl_2$. Five strains were resistant to 60 μg mL^{-1} of $HgCl_2$. The MICs result obtained indicate that isolated *Pseudomonas putida* was strongly resistant to mercury. The MIC of the strain after exposing to $HgCl_2$ was 80 μg mL^{-1}.

Removal of Hg^{2+} from solution: Isolated *pesudomons putida* was found to be highly efficient in removal (nearly 99.3 and 99.8% in concentration 40 ppm after 24, 48 h, respectively). Hg^{+2} when grow in pepton water in condition 37°C with rate shaker 130 rpm. *Pseudomonas putida* was transferred to pepton water containing $HgCl_2$ and incubated to removal Hg^{2+}. Biosorbtion and up take of mercury investigated by varying the initial $HgCl_2$ concentration from 1 to 120 mg L^{-1} (Fig. 1). The amount of Hg^{2+} removed increased as the incubation period increased and also increased in proportion to the initial $HgCl_2$ concentration.

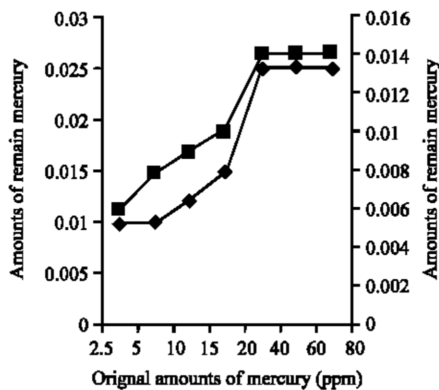

Fig. 1: Biosorption (□) and uptake (♦) of $HgCl_2$ by *Pseudomonas putida* at 37°C, pH 7.0 with shaking

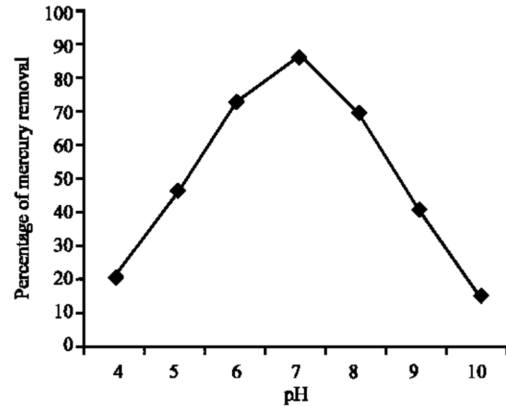

Fig. 2: Effect of pH on mercury removal

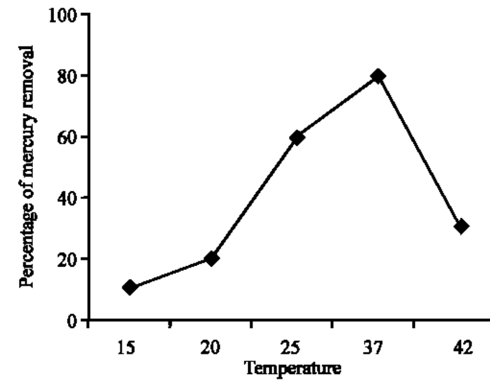

Fig. 3: Effect of temperature on mercury removal

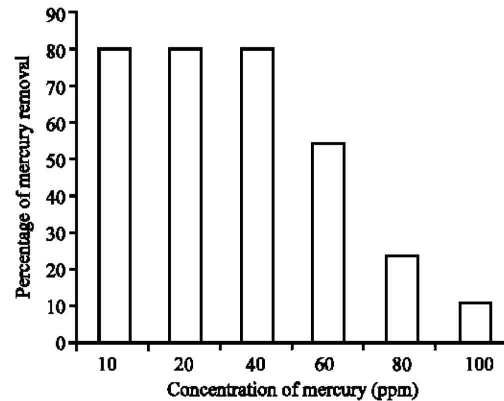

Fig. 4: Effect of mercury concentrations on mercury removal

Effect of pH and temperature on Hg^{2+} removal: Hg^{2+} removal was increased in pH up to 8.0; a further increase in pH, up to 10.5, resulted in a decrease in Hg^{2+} romoval (Fig. 2). The results revealed that the optimum temperature

for the removal was about 37°C and low removal was obtained at 10 and 45°C (Fig. 3), suggesting that Hg^{2+} removal by *Pseudomonas putida* is also dependent on the energy for uptake of Hg^{2+} by the cell, in addition to adsorption on the surface of cells.

Effect of mercury concentration: The effect of mercury concentrations (1-120 mg L^{-1}) at the optimum pH (7.0) on removal of mercury was investigated. It was found that the mercury removal decreased at 40 ppm but above after that they remained stable as the concentrations increased up to 60 ppm and decreased thereafter (Fig. 4).

DISCUSSION

Microbes have been used to solve environmental problems for many years. The use of microorganisms to sequester, precipitate or alter the oxidation state of various heavy metals has been extensively studied[12]. Biological methods for waste treatment, like activated sludge, composting and wetlands, are microbial mediated and have long made major contribution to pollution abatement. Recently, remediation of polluted wastewater and soil by microorganisms, or bioremediation, have been studied extensively and applied with success. Processes by which microorganisms interact with toxic metals are very diverse. However, in practice, there are general categories of biotechnological process for treating wastewater containing toxic metals: biosorption (bioacumulation), extracellular precipitation and up take by purified biopolymers and other specific molecules derived from microbial cells. In the case of biological removal of mercury, mercury resistant bacteria produce the enzyme mercuric reductase, which catalyzes the conversion of Hg^{+2} to volatile Hg^0[13]. In this study, mercury concentration was measured in wastewater. Suitable bacteria that reduce ionic mercury to metallic mercury were isolated from wastewater of chloralkali plant. A total of 8 resistant bacteria that could grow on agar plates containing 40 μg mL^{-1} were isolated from sludge and wastewater. *Pseudomonas putida* isolated from sludge of chloralkali plant has been reported to be most resistant to $HgCl_2$ among isolated bacteria[11]. The bacteria screened with an agar plate containing of $HgCl_2$ in this study are probably special mercury volatilization bacteria, which are selected by the stress of mercury polluted sediments. Among mediums, liquid pepton water medium because lake formation sediment to chloride mercuric distinguished as medium suitable for consider resistance bacteria and removal mercury ion by bacteria. The mercury resistance levels of bacteria strongly depend on the number of cells used in the assay, with much higher resistance levels found at high cell densities and on the buffering and complexing capacity of the test medium. Moreover, much higher resistance is generally observed on plates than in liquid cultures, because on plates the growth of the test strains can locally reduce mercury concentrations. The ability of a single cell to form a colony on an agar plate of a given mercury concentration was used here to determine in a rapid and reproducible way the resistance levels of our strains. The agitation of mercury solution with the bacterium was effective for removal of mercury. The results of biosorption and up take in varying concentrations are presented in Fig. 1, with increase in biomass concentration, the percentage removal increases as the number of possible binding sites are increased. These observations can be explained by the fact that at very low concentrations of metal ions, the ratio of sorptive surface area to the total metal ions available is high and thus, there is a greater chance for metal removal. Thus, until 40 ppm of mercury, the removal capacity is more than higher concentrations. When mercury concentrations are increased, binding sites become more quickly saturated as the amount of biomass concentration remained constant. Under the concentrations tested, the maximum removal of mercury by the *Pseudomonas putida* cells of was 40 ppm. This result was similar to the maximum adsorption of Chromium (II) ion[14]. The results consider resistance represented that resistance bacteria under condition shaking is more of case without shake, which this can because creation dashing and subsequent cause became more oxygenation in the incubation period. In this study also demonstrated the potential of bacteria biomass for the removal, adsorption and uptake of Hg^{+2} from pepton water medium. Experimental results showing adsorption of Hg by *Pseudomonas putida* at varying pH are presented in Fig. 2. The reduction in metal removal with increasing pH beyond its optimum values has been attributed to reduced solubility and precipitation.

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