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Isolation and Identification of Heavy Metal Resistant Bacteria from Industrial Wastewaters in Guilan Province

Khosro Issazadeh*, Hamidreza Savaheli, Naeem Momeni

Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran.

ABSTRACT

Heavy metal pollution by natural factors is a world-wide phenomenon. Release of large quantities of heavy metals without handling proper processes that could decrease the concentration of such a material is a hassle that makes strains resistant to these heavy metals apart from entering into human food chain. In this research, wastewater of four firms in Guilan province such as Foolad, Risandegi, Chooka and Ganje tannery were analyzed according to standard methods. These methods were included culturing the strains on the specified media such as Luria-Bertani agar and Kings' B. In the wastewater of these factories, there were kinds of heavy metals such as Chromium, Nickel, Cobalt, Mercury and etc. However existence of these heavy metals is fatal to keep the life progressive, but a kind of creatures like some bacteria could adapt themselves to the condition. Here we mentioned two genera of bacteria, *Bacillus* sp. and *Pseudomonas* sp., which were more renowned in the category of resistance to heavy metals. First of all, we used Nutrient Agar medium in order to distinction of Gram positive bacteria from Gram negative ones. After that, by dilution preparation from sample and inoculation the bacterial suspension into medium, the range of resistance to heavy metal concentration for *Bacillus* sp. in Luria-Bertani Agar and for *Pseudomonas* sp. in Kings' B medium were determined. This estimation was validated by adding salt of heavy metal by the concentration from 0 µg/ml through 100 µg/ml and pH from 5 to 9. Results showed that both of two isolates had the most accumulation rate in pH=7 and concentration of 50 µg/ml heavy metal.

Keywords: Guilan, Heavy metal resistance, Kings' B, Wastewater

INTRODUCTION

Heavy metal contamination due to natural and anthropogenic sources is a global environmental concern. Release of heavy metal without proper treatment poses a serious threat to public health because of its persistence, biomagnifications and accumulation in food chain. Most of the heavy metals like chromium,

Corresponding Author E-mail: Issa_kaam@yahoo.com

cadmium, lead, mercury and copper are highly toxic for almost all the living organisms. The health of people living near the dumping grounds is also being constantly affected by the metal contamination of food and drinking water. A number of studies have elaborated the effects of heavy metals on animals, plants and human health (Chipasa, 2003 and Cisti, 2004). Mercury is one such metal which has been reported to produce metabolic disorders in variety of animals (Company et al. 2004, Reinhardt and Pelli 1986). Various health problems such as pneumonitis, abnormal cramps, bloody diarrhea and suppression of urine, cancer, and hyper secretion of sweat glands are caused by mercurial and mercuric forms of mercury [Miwa, 1987 and Shakoori et al. 2002). Romero *et al*. (2004) studied the toxic effects of mercury chloride in two cell lines of renal origin (Romero, 2004). The most notable findings in treated cells were the presence of intracytoplasmic inclusion bodies and apoptotic bodies. Recently, microbial bioremediation has emerged as an alternative technique to such traditional chemical treatments (Brierley, 1990). Mercury resistant bacteria have been reported by several authors. Mercury is also efficiently removed by algae. One of the objectives of this study was to evaluate the minimum inhibitory concentration (MIC) of Hg^{2+} against the bacterial isolates and to determine their ability to uptake mercury (Chang, 1998, Davis, 2003 and Zeroual et al. 2003).

MATERIALS AND METHODS

Sample collection

Wastewater samples were collected in screw capped sterilized bottles from five different ponds in industrial area of Rasht in Guilan province and carried in cold temperature to laboratory. Some physicochemical parameters of wastewater such as temperature (ºC), pH and heavy metal concentration (µg/mL) by Atomic Absorption Spectroscopy (AAS) were measured. In order to differentiate gram negative from gram positive bacteria we did Gram staining method. By the means of gram staining, we observed two kinds of colored colonies. After biochemical and systematic observations, red colonies were referred to *Pseudomonas* sp. and blue ones were belonged to *Bacillus* Sp. After that we prepare a series of dilution in order to isolate *Bacillus* sp. and *Pseudomonas* sp. The series of dilution prepared were 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} . Then 0.1 ml of the sample was spread on Luria-Bertani Agar in order to have clean culture of *Bacillus* sp. and 0.1 ml on Kings' medium for culturing *Pseudomonas* sp.. Plates were incubated in 37 ºC incubator for 24 to 48 hours.

Determination of optimum growth conditions

For optimum growth of the bacterial isolates, two parameters such as temperature and pH were considered. For determination of optimum temperature, 5 mL Luria-Bertani broth was added in four sets for *Bacillus* sp. And Kings' for *Pseudomonas* sp., autoclaved and inoculated with 20 mL of freshly prepared culture of isolates. The four sets of tubes were incubated at 25ºC, 30ºC, 37ºC and 42ºC. After an incubation of 12 hours, their absorbance was taken at 600 nm. For determination of optimum pH, test tubes having 5 mL LB and Kings'B for both isolates were prepared in 9 sets, for each isolate and their pH was adjusted at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 then autoclaved. These tubes were inoculated with 20 mL freshly prepared culture of the isolates. After incubation period of 12 hours, their absorbance was taken at 600 nm.

Estimation of heavy metal processing ability of the isolates

We prepared a microbial suspension in nutrient broth. Then 0.1 ml of the suspension was added to Luria-Bertani and Kings'B with the concentration of heavy metal from 0µg/ml to 100µg/ml in order to estimate the optimum concentration of metal that can be tolerated. Salt of heavy metal was added to medium when

it cooled enough. Observations were performed by five plates each having the pH of 5 to 9. Incubation was carried out for 24 hours interval times.

RESULTS

Physicochemical characteristics of industrial wastewater

Table I shows physicochemical characteristics of industrial wastewater of three different samples, from where mercury tolerant bacteria were isolated. The temperature of different samples ranged between 22ºC to 24.66ºC, pH ranged between 8.47 and 8.6, and Hg^{2+} ranging between 1.10 ±0.08 and 1.50±0.08 µg/mL (Table 1).

Table 1. Physicochemical parameters of wastewater collected from Foolad-e-Guilan Firm effluents in Rasht industrial area, Iran**.**

Parameters	**Pond1**	**Pond2**	**Pond3**
Temperature(ºC)	23.66±0.47	24.66±0.47	22.00±0.8
pH	8.47 ±0.04	8.52 ±0.12	8.648±0.4
Mercury(µg/mL)	1.10 ±0.08	1.50 ±0.08	1.30±0.04

Identification of bacterial isolates

Identification of isolates was achieved by biochemical characteristics and according to Bergey's Manual of Systematic Bacteriology (Table2).

Table 2. Morphological and biochemical characteristics of bacterial isolates.

Characters	*Pseudomonas* sp.	*Bacillus* sp.
Gram-reaction	-	+
Morphology	Rods	Rods
Color	Off-white	White-cream
Urease production	-	-
Citrate utilization	-	+
Oxidase reaction	+	-
Triple sugar iron reaction	+	+
Indole reaction	-	-
Motility	+	+

+: positive; -: negative.

Optimum growth conditions

The most suitable temperature for both heavy metal resistant bacterial isolates was found to be 37^{o}C. Maximum growth for *Pseudomonas* sp.was observed at pH 7.5, while *Bacillus* sp.showed maximum growth at pH 7.3. The most tolerable concentration of *Bacillus* isolates for heavy metal was 50 µg/mL and 40µg/mL for *Pseudomonas* sp.

Metal processing ability

Heavy metal processing capability of both the bacterial isolates was checked by adding Hg^{2+} at 100µg/mL in the culture medium. *Pseudomonas* sp.could reduces 93% of mercury from the medium after 40 hours.

The *Pseudomonas* sp.was also capable to remove Hg^{2+} (100 µg/mL) 35%, 55% 70% and 85% from the medium after 8, 16, 24 and 32 hours, respectively. *Bacillus* sp.could also efficiently process mercury from the medium, 80% mercury was removed from the medium after 40 hours. The organism removed 20%, 40%, 50%, and 65% Hg^{2+} from the medium after 8, 16, 24 and 32 hours, respectively.

DISCUSSION

A number of microorganisms have evolved resistance mechanisms to deal with heavy metal compounds. Mercury resistance was first reported in *Streptomyces aureus*(Moore, 1960) and since then has been described in a number of bacterial species. One of the best defined mercury resistance determinants is the *mer* operon encoded by transposon Tn*501,* found in Gram-negative bacteria. The functions of the minimal number of proteins required to confer full resistance are as follows (Hoban, et al. 1997): Mar R is the *mar* regulatory protein which controls the expression of all the other proteins in the operand in response to the presence of Hg^{2+}. Mere, the periplasmic Hg binding protein, transfers Hg^{2+} to the MerT transport protein located in the cytoplasmic membrane. This passes mercuric ions to the cytoplasmic mercuric reductase, which reduces Hg^{2+} to Hg° using as NADPH as the reductant. Hg° is then lost from the cell in the gas phase. In the present study growth rate of both bacterial isolates in the presence of Hg^{2+} was slightly slower as compared with that of non-treated (control) bacterial culture (Brown, et al. 2003 and ESSA, et al. 2002). Chang and Law (1998) described that specific mercury detoxification rate was dependent on cell growth phases as well as the initial mercury concentrations (Brown, et al. 2002 and Wanger 2003). This happened because of higher concentration of metals that probably poisoned essential biochemical reactions (Perego, et al. 1997). Growth period was delayed when concentration of heavy metal was increased (Brady, et al. 1994). In the present investigation Gram-negative bacteria, *Pseudomonas* and *Bacillus* were found to be resistant to mercury at a concentration of 50 and 40µg Hg^{2+}/mol, respectively (Mindlin, et al. 2005), Yureiva, et al. 1997). Glendinning *et al.* (2005) reported that *Bacillus* sp. and *Bacillus cereus* RC607 were resistant to Hg^{2+} at 60µg/mL.Thiomersal biodegrading mercury resistant *P. putida* strain have also been isolated and characterized from other laboratories (Fortunato, et al. 2005). Mercuric reductase reduces Hg^{2+} into Hg° in the presence of NADPH and a sulphydryl compound. Hg° volatilizes out of the system due to its high vapour pressure. Many bacteria belonging to the genera *Pseudomonas, Bacillus* and *Staphylococcus* have been reported to reduce Hg^{2+} to Hg° (Glendinning, et al. 2005, Gupta, et al. 2005, and Shakori et al. 2002). Microorganisms have a high surface area-to-volume ratio because of their small size and therefore provide a large contact area that can interact with metals in the surrounding environment.

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