Global J. Environ. Sci. Manage., 3(4): 427-436, Autumn 2017
DOI: 10.22034/gjesm.2017.03.04.008

ORIGINAL RESEARCH PAPER

Isolation and characterization of mercuric reductase by newly isolated halophilic bacterium, *Bacillus firmus* MN8

M. Noroozi[1], *M.A. Amoozegar*[2], *A.A. Pourbabaee*[3,*], *N.S. Naghavi*[4], *Z. Nourmohammadi*[1]

[1]*Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2]*Department of Microbiology, Faculty of Biology, College of Science, University of Tehran, Tehran, Iran*
[3]*Soil Science Department, Faculty of Agricultural Engineering and Technology, University College of Agriculture and Natural Resources, University of Tehran, Tehran, Iran*
[4]*Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran*

Received 15 March 2017; revised 25 July 2017; accepted 7 August 2017; available online 1 September 2017

ABSTRACT: The current study was aimed at isolating and identifying the halophilic and halotolerant bacteria which can produce mercuric reductase in Gavkhuni wetland in Iran. Moreover, tracking and sequencing *merA* gene and kinetic properties of mercuric reductase in the selected strain were performed in this study. Soil samples were taken from Gavkhuni wetland and cultured in nutrient agar medium with 5% NaCl. To examine the tolerance of purified colonies to mercury, agar dilution method was administered. Similarly, the phylogenetic analysis based on 16SrRNA gene sequencing was conducted. To investigate enzyme activity of kinetic parameters, a spectrophotometer was used to measure the NADPH oxidation decrease at 340 n.m. The results showed that among the 21 halophilic and halotolerant strains isolated from Gavkhuni wetland, 4 were resistant to mercuric chloride. A strain designated MN8 was selected for further studies because it showed the highest resistance to mercury. According to phylogenetic sequencing of 16S rRNA gene and phenotypic characteristics, the strain was categorized in the *Bacillus* genus and nearly related to *Bacillus firmus*. This strain had *merA* gene. The mercuric reductase showed V_{max} and K_m values of 0.106 U/mg and 24.051 µM, respectively. Evaluation of different concentrations of NaCl at 37°C and pH=7.5 in mercuric reductase enzyme activity indicated that the enzyme shows 50% activity in concentration of 1.5 M. Optimum pH and temperature of enzyme activity were 7.5 and 35 °C, respectively. The results suggested that MN8 strain could be a proper candidate for bioremediation of mercury-contaminated environments such as industrial wastewaters.

KEYWORDS: *Halophilic bacteria; merA gene; Mercuric reductase enzyme; Mercury; Polymerase chain reaction (PCR).*

INTRODUCTION

One of the most important environmental concerns is the contamination due to industrial heavy metals that are globally considered as risk factors with various impacts on human health and the environment (Xiong *et al*., 2008; Giovanella *et al*., 2016). There are naturally heavy metals in the hypersaline environmental systems in small amounts and in contaminated environments in high doses because of human activities. A few archaea and many halotolerant and moderately halophilic bacteria are able to tolerate metals through some mechanisms including enzymatic detoxification, metal efflux via specific transporters and extracellular metal sequestration by biopolymers (Voica *et al.,* 2016). Exposure to mercury as a heavy metal is a life-threatening factor to human health (Järup, 2003). Prokaryotes establish a

*Corresponding Author Email: *pourbabaei@ut.ac.ir*
Tel.: +98 26 3223 1787 Fax: +98 26 3223 1787
Note: Discussion period for this manuscript open until December 1, 2017 on GJESM website at the "Show Article".

large part of the atmosphere and are less considerable than other organisms (Ghiasian *et al*., 2017). Moderately halophilic bacteria are in a diverse group of microorganisms which are able to grow in light-salt media and tolerate high salt concentrations. These bacteria are beneficial for the bioremediation of toxic metals from contaminated milieu (Amoozegar *et al.,* 2008). The *mer* operon has two relevant genes: *mer*R and *mer*A genes. The former regulates detoxification enzymes and the latter encodes mercury ion reductase, both of which have associated roles with prokaryotic physiology (Barkay and Irene, 2005). The mercuric reductase (MR) enzyme modifies mercury toxicity and mobility including inorganic Hg [Hg(II)] in nature. Bacteria and archaea have the *mer*A gene to survive against high amount of mercury (Freedman *et al.,* 2012). Mercuric ion detoxification in bacteria is catalyzed through mercuric reductase containing FAD and is dependent on NAD(P)H (EC 1.16.1.1) (Boden and Murrell, 2011). Extreme environments made both naturally or by human activities, e.g. polluted sites and mines, are attracting commercial interests and are suitable bacterial studies on adaptation and evolution. (Héry *et al.,* 2005). This study has been carried out in Gavkhuni wetland in Iran in 2014 in order to isolate and identify the moderately halophilic bacteria which produce mercuric reductase.

MATERIALS AND METHODS

Study area and sample collection

Initially soil samples were taken from Gavkhuni wetland. Gavkhuni wetland with geographical coordinates of 15' 32° - 22' 32° N and 45' 52° - 59' 52°E is located at the southeast of Isfahan, Iran, 30 km away from the nearest town (Varzaneh) and 1470 m above the sea level (Fig. 1). Gavkhuni wetland is an international wetland, registered in Ramsar Convention in 1975. It has maximum width of 50 km and length of 25 km. This wetland has an area of about 470 km^2 depending on water entry to the wetland, as its space reduces in summer because of inflow and higher evaporation rate and expands considerably in humid seasons (Gohari *et al.,*2013).

Isolation of microorganisms, culture media and growth conditions

After preparing serial dilutions, the samples were cultured on nutrient agar medium (peptone 5g, yeast extract 3g, agar 15g, dH_2O 500 mL, and pH=7.5) enriched by 5% (W/V) NaCl and 0.005 mM $HgCl_2$ and finally incubated at 34 °C for 48h with intense shaking. After the growth and appearance of colonies, single colonies were cultured to produce pure strains. Purification of colonies was performed at least in 5 successive cultures of isolates on the nutrient agar medium enriched by 5% (W/V) NaCl. The strains were tested for morphological, physiological and biochemical characteristics using standard microbiological criteria, such as pigmentation, form, colonial elevation and opacity under light microscopy. Gram staining was done and the result was approved by KOH test. The wet-mount method was used to examine the motility of the bacteria (Smibert and Krieg, 1994). The tolerance to a range of mercury ions was determined for the bacteria which had been isolated by agar dilution method. First, various concentrations of mercury (0.005 up to 5 mM) were added to flasks containing the required amount of nutrient agar medium and 5% (W/V) NaCl. Then, contents of the flasks were poured into eight-

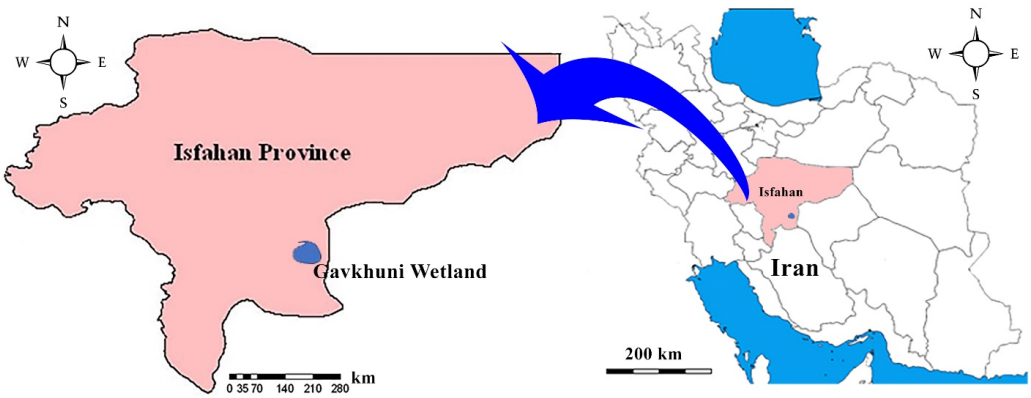

Fig. 1: The study area

centimeter plates. Subsequently, 10 µl of the bacteria suspension (1.5×10^8 cfu /ml) was poured on agar medium using a sampler. The plates were examined after incubation at 34 °C for 72 hours. Based on previous studies on heavy metals-resistant halophilic bacteria, the isolates which can grow in a medium containing 0.1 mM Hg are considered resistant. The lowest concentration of metal compounds that completely inhibited the bacterial growth was named MIC (Minimum Inhibitory Concentration). All experiments were performed twice (Abou-Shanab *et al.,* 2007).

DNA extraction and amplification of 16S rRNA gene

The identification process for mercury-resistant soil bacteria was carried out by 16S rRNA gene sequencing after extracting bacterial genomic DNA from the cultures grown in Luria-Bertani medium using Kit (Sigma). The DNA was amplified by universal 16SrRNA primers of 27F(5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R(5'-ACGGCTACCTTGTTACGA-3') via PCR method. The program of PCR amplification for this gene consisted of 96 °C (5 min) that was followed through 30 cycles of 95°C (15 s), 49 °C (30 s) and 72 °C (1 min) and terminal expanse of 72°C (10 min). Macrogen Co. (South Korea) determined 16S rRNA gene sequencing, and the obtained data were edited on Chromas Pro bioinformatic software (Giri *et al,* 2014).

merA phylogeny and bioinformatic analyses

The *merA* gene of selected strain was amplified by primers F (5′-TTGTACGGGTTGTGAAGAGC-3′) andR(5′-TTATCCTGCACAACAAGATAATTTC-3′) within PCR via the thermal cycler (Eppendroff). Then the amplified segments were ran on 1.5% agarose gel electrophoresis and the resulting products after staining were observed by Gel Doc system (BioRad). The negative control for this reaction was a sensitive strain of the genus *Bacillus*. Macrogen Co. performed sequencing process for the products, and then the sequencing data were checked by Chromas Pro software. Registered strains in data sequence were used to detect similarity of sequences. Multiple sequence alignment was done by ClustalX 2.1 software (Thompson *et al.,* 1997) and, using Mega 6, the neighbor-joining *merA* tree was utilized to establish phylogenetic tree (Saitou and Nei, 1987). At last, the constructed tree topology was drawn through bootstrap assay of neighbor-joining data set according to 100 resampling attempts (Felsenstein, 1985).

Mercuric reductase assay

Luria-Bertani medium with 10 µM $HgCl_2$ was used to dilute (1:20) overnight cultures incubated in shaking incubator at optimal temperatures with optical density at 660 nm of 0.4. Incubation continued for 10 m after adding final concentration of 10 µM $HgCl_2$ to induce bacterial cells. After this period, the obtained suspensions were chilled on ice and then centrifuged in pre-weighed tubes. Supernatants were discarded and the precipitated bacterial cells were re-suspended in PBS, and after weighing were reserved at −20 °C up to testing. Resultant precipitated bacterial cells were poured into a buffer (sodium phosphate 20mM (pH 7.5), 0.5 mM EDTA, and 1 mM β-mercaptoethanol) to prepare suspensions with about 200 mg/ml concentration (fresh weight), and intermittent sonication was utilized to cleave the bacterial cells on ice for 10 m. Broken cells were centrifuged at 14,000 rpm and 4 °C for 30 m. Pellets were delivered on ice after removing the supernatant (Vetriani *et al.,* 2005). The subsequent assessments were conducted in 80 mM of sodium phosphate solution (pH 7.4), 1 mM β-mercaptoethanol, 200 µM NADPH, and 100 µM $HgCl_2$ (Fox and Walsh, 1982). Decreasing trend of A_{340} showed $HgCl_2$-dependent oxidation of NADPH via spectrophotometer. One unit of activity means one µmol of NADPH oxidized per minute. Bradford assay was used to calculate protein concentrations in crude cell extractions. Specific activities at all temperatures were determined using protein concentration in the unheated extraction. In order to assess the effect of NaCl, mercuric reductase enzyme activity was measured in different concentrations of (0.5-2.5M) NaCl using spectrophotometer at A_{340} (Sayed *et al.,* 2014). Moreover, to obtain optimal pH for enzymatic activity, the pH values ranging from 5-10 were adjusted by various buffers. Cell extractions were set in incubator for 10 m. In order to investigate the effect of various temperatures, 20 µl of each extract was mixed with 800 µl of reaction buffer and was separately incubated at 25-70 °C, in triplicate. In order to achieve the kinetic parameters of mercuric (II) reductase, such as K_m and V_{max}, results were set to Michaelis-Menten equation (Zeroual *et al.,* 2003).

RESULTS AND DISCUSSION

Isolation and characterization of the halophilic bacteria moderately resistant to mercury

Among 21 strains of bacteria isolated from Gavkhuni wetland in Iran (saline soil), 4 strains of the halotolerant and halophilic bacteria with intrinsic resistance (≥100 µM) to mercuric chloride were selected. Four strains were resistant to mercury with a resistance range between 125 and 400 µM Hg. None of the strains grew in concentrations over 400 µM. The effects of toxic metal mercury on these bacterial strains enhanced with the increase of concentration. The sequence segment of 16S rRNA gene was obtained from NCBI GenBank (Table 1).

The highest value of MIC was obtained to be 400 µM by MN8 strain. Many studies have been done to determine the minimum inhibitory concentration of microorganisms. In a study conducted on a nickel-polluted soil, the minimum inhibitory concentrations of nickel, chromium, zinc, cadmium, mercury, arsenic, lead, copper and cobalt were 15, 5, 10, 5, 0.5, 20 , 15, 15 and 10 mM, respectively, which are likely to cause high MIC content in bacterial isolates (Abou-Shanab *et al.,* 2007). In another study on the bacteria isolated from an industrial wastewater, the MIC for cadmium was between 4 to 7 mM, for chromium was 0.7 mM, for nickel was 6.75 to 8.8 mM, for lead was 6 mM, for arsenic was between 6.5 to 15 mM and for mercury was 0.75 mM (Raja *et al.,* 2009). In another study, the maximum MICs for the bacteria isolated from pesticide-contaminated soils were 100, 400, 400, 800, 3200 and 400 micrograms per milliliter for mercury,

Table 1: Isolation and characterization of the halophilic bacteria moderately resistant to mercury from Gavkhuni wetland in Iran

Isolate	Phylogenetically related genera	Accession number	Size	MIC* (µM)
MN8	*Bacillus*	LC17 8693	1230 bp	400
MN12	*Halomonas*	LC190732	1290bp	200
MN16	*Halobacillus*	LC128798	1305 bp	125
MN18	*Halomonas*	LC190733	1269 bp	150

*Minimum inhibitory concentration

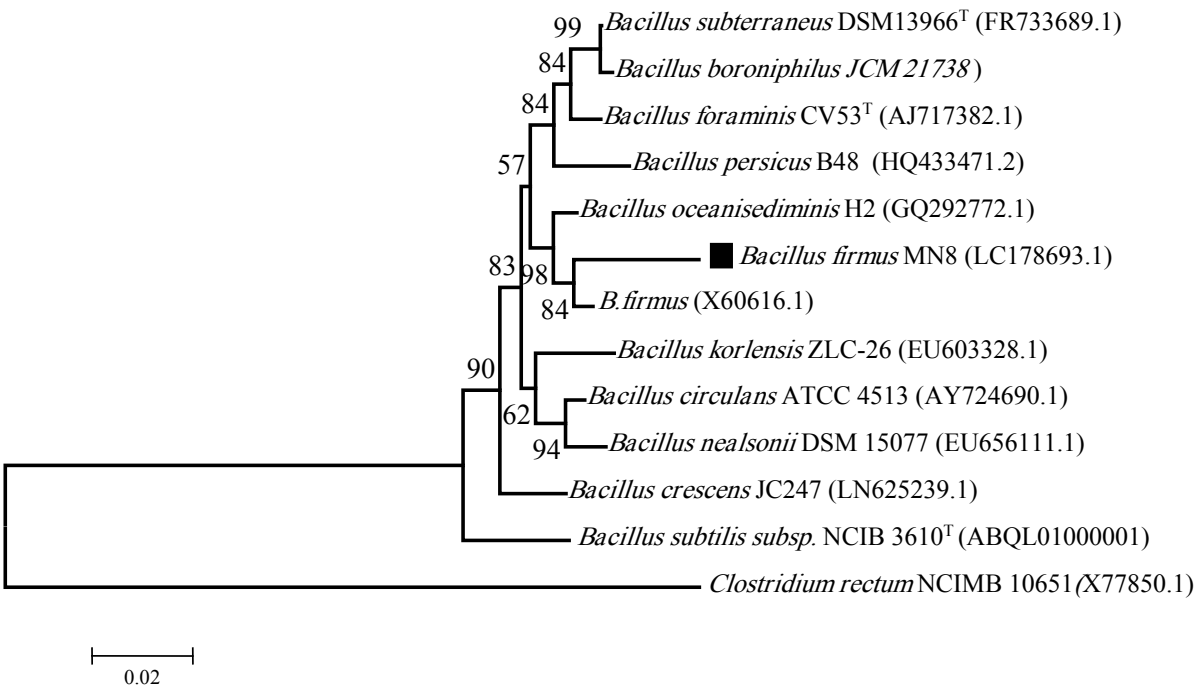

Fig. 2: Neighbor-joining tree based on 16S rRNA gene sequences. Results show the correlation between MN8 strain and its near relatives in *Bacillus* genus. The *Clostridium rectum* NCIMB 10651 sequence is out-group in the tree

nickel, copper, chromium, lead and cadmium, respectively (Anjum *et al.,* 2011). In another research, heavy metals-resistant strains were isolated from the wastewater. Among the isolated species, the minimum inhibitory concentration of 1 mM was found for mercury regarding *Klebsiella* (Akhavan Sepahy *et al.,* 2015).

The results obtained in the present study showed the highest MIC of mercury in a 400 µM by MN8 isolate and this MIC value was significantly relevant to the toxicity of mercury. This difference in MIC can be due to the different structure of the study area and the type of bacteria.

Bioinformatic analyses

Analysis of 16S rRNA gene sequence showed that isolated bacterial strains belong to the *Halobacillus*, *Bacillus* and *Halomonas genera*. The phylogenetic tree for 16S rRNA sequences of MN8 strain is shown in Fig. 2. This strain belongs to *Bacillus* genus and shows the highest sequence similarity to 16S rRNA gene sequence of *Bacillus firmus*.

The MN8 strain, among mercury resistant isolates, was selected for further studies because of higher MIC. It turned out to be a spore-forming rod, Gram-positive, motile, catalase-positive and oxidase-positive with creamy, round and smooth colonies.

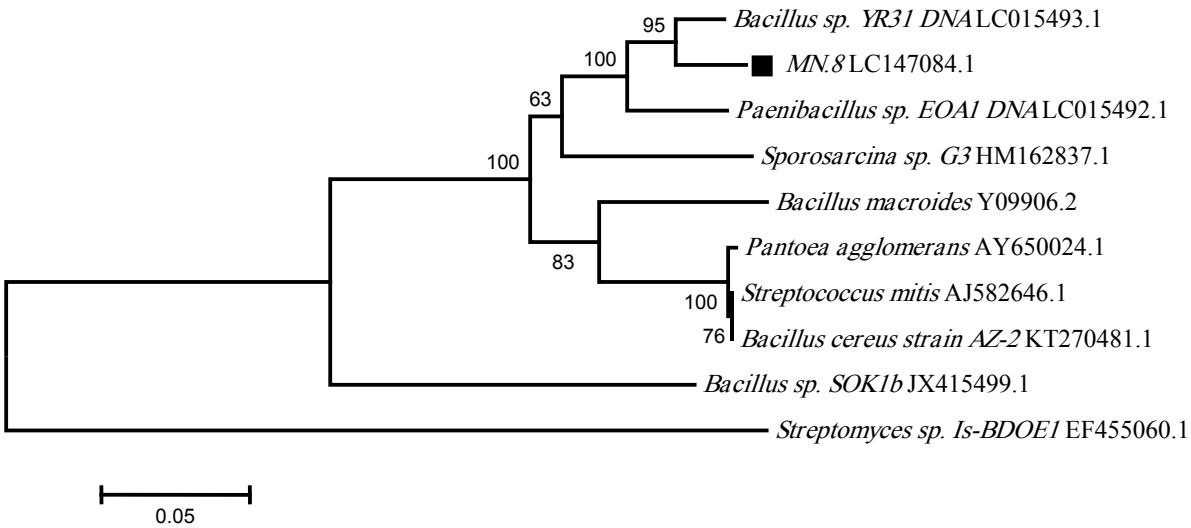

Fig. 3: *merA* amino acid sequence-based neighbor-joining tree

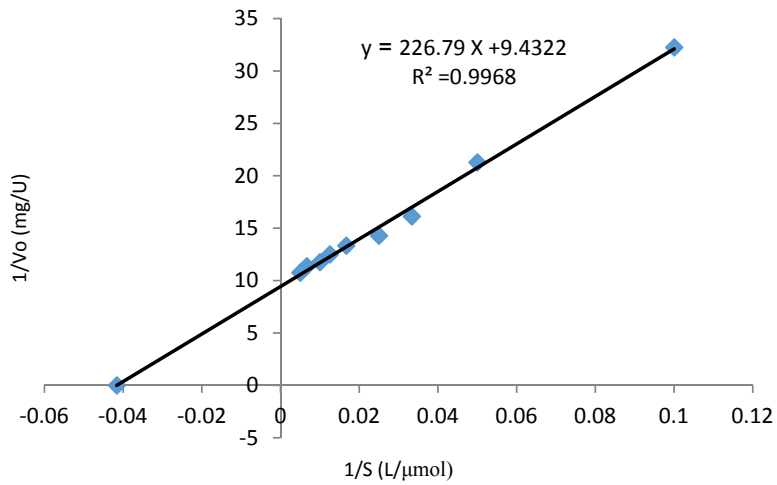

Fig. 4: Determination of Michaelis-Menten constant (Km) and Vmax of the mercuric reductase by Lineweaver-Burk plot

In most of the previous studies, more mercury-resistant Gram-negative bacteria were isolated as compared to Gram-positive bacteria (Nakamura *et al.,* 2001; Petrus *et al.,* 2015; Giovanella *et al.,* 2016). In the present study, Gram-positive and negative bacteria were isolated for their resistance to mercury. However, the maximum value of MIC was found in a Gram-positive bacterium, MN8 strain. Greater MIC presence of Gram-positive bacteria in the media containing mercury may be attributed to their higher capability to absorb and then acquire resistance over time. In addition, these bacteria manifest a naturally higher resistance to toxic metals. In the present study, the large number of Gram-positive bacteria can be ascribed to thicker cell wall of the bacteria and their higher intrinsic resistance compared to Gram negative bacteria. Conserved region of *mer*A gene was amplified for recognizing mercury-resistant genotype of MN8 strain, and a distinct banding pattern at 1600 base pair was obtained. The complete CDS sequence of *mer*A gene for mercuric reductase of *Bacillus firmus* strain MN8 was obtained from NCBI GenBank, and the access number was LC147084.1. The *mer*A amino acid sequence in MN8 strain was analyzed in terms of phylogenetic and results showed that the gene is closely related to *mer*A segments of strains in the *Bacillus* genus. Moreover, the relation between MN8 and reference strains was shown in Fig. 3. Out-group is the sequence of *Streptomyces sp.* Bar, 0.05 substitutions per nucleotide position (Fig. 3).

Mercuric reductase activity by crude cell extract of strain MN8

In a study by Chatziefthimiou *et al.* (2007) on a strain of mercury resistant, thermophilic, thiosulfate-oxidizing bacterium, it was demonstrated that although this bacterium was resistant to high levels of mercury, the presence of *merA* gene was not necessarily the cause of resistance to mercury. However, it was the cause of adaptation to mercury presence during its life span. Data obtained from this research indicate that MN8 strain of Gavkhuni wetland in Iran has a superior resistance to mercury most likely due to the mercuric reductase (MR) enzyme. Unrefined extract kinetic parameters of mercuric reductase were achieved by meriting enzyme activity results in Hg density and through equation of Michaelis-Menten. The data fitted the equation (r2 = 0.9968) with V_{max} and K_m values of 0.106 U/mg and 24.051 µM, respectively (Fig. 4).

The kinetic characteristics of MN8 strain demonstrate that this enzyme has the highest specific activity in concentrations over 40 µM of mercury. Significant differences were observed at concentrations less than 40 µM and over 100 µM. These results suggest that mercury concentrations between 40 µM and 100 µM are sufficient for the maximum specific enzyme activity. This low level of mercuric reductase K_m showed a high enzyme-substrate affinity. Evaluation of the effect of different concentrations of NaCl at 37°C and pH=7.5 on mercuric reductase enzyme activity indicates that

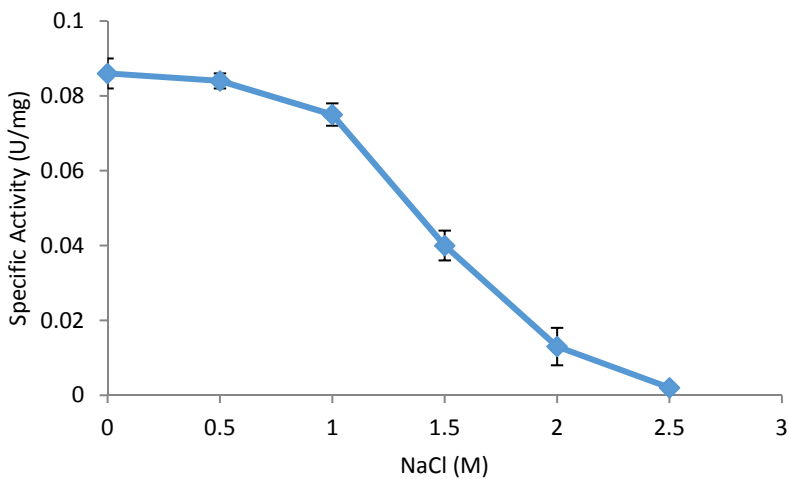

Fig. 5: Effect of concentration of NaCl on mercuric reductase specific activity in crude cell extract by *Bacillus firmus* strain MN8

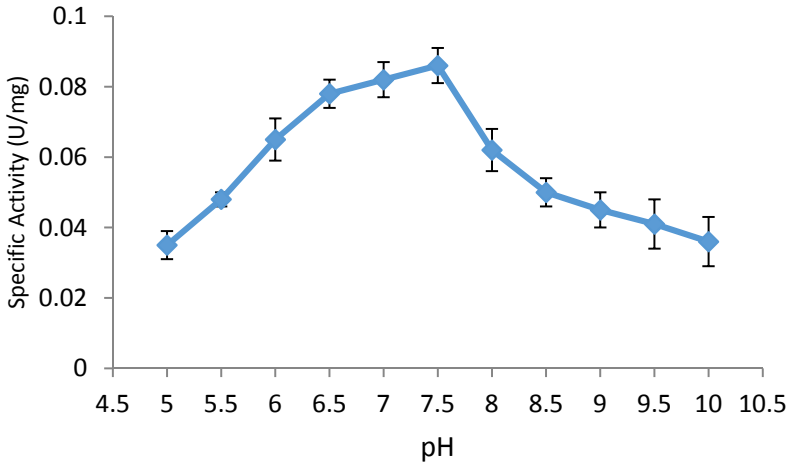

Fig. 6: The pH effect on special activity of mercuric reductase in rude cell extract by *Bacillus firmus* strain MN8 (Results show means of three separated experiments and indicate deviation bars)

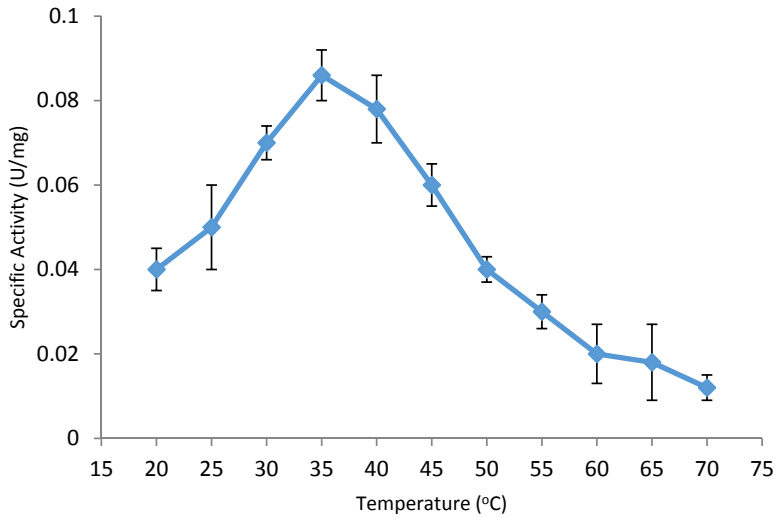

Fig. 7: The temperature effect on specific activity of mercuric reductase in crude cell extract by *Bacillus firmus* strain MN8

although specific activity of the enzyme decreases with the increase of NaCl concentration, the enzyme still shows 50 % activity in concentration of 1.5 M (Fig. 5).

Barkay *et al.* (1997) reported NaCl interference with mercuric reductase activity. In another study by Sayed *et al.* (2016), similar results were demonstrated while the mutants generated by site-directed mutagenesis showed the maximum enzymatic activity at 4 M NaCl by replacing the acidic amino acids at the C-terminal end of *merA* amino acid sequences. In turn, investigation of pH and temperature effects on enzyme acting revealed the enzyme optimum pH and temperature of 7.5 and 35 °C, respectively (Figs. 6-7).

High activity of this enzyme is in the range of pH 6-8 and the maximum activity is in pH 7.5. These results are consistent with the findings of other researchers about the evaluation of Gram-positive and Gram-negative microorganisms (Olson *et al.,* 1982).

An increase in the pH up to 8 enhances the enzyme activity. There is evidence that alkaline pH is suitable for enzyme function. According to the findings of Ledwidge *et al*. (2010), deprotonating of cysteine thiol groups of MR enzyme leads to the stability of negative charges and facilitates the enzyme reaction though enzyme activity decreases largely in pH 9. The pH variation affects enzyme activity because changes in ionization affect the system components. Because of protein nature of enzymes with ionizable groups, various ionization forms of enzymes can be found. In the current study, rise of temperature over 30 ˚C had a positive effect on enzyme activity with the optimal temperature range of 35-50 °C. An increase in enzymatic activity at temperatures over 35 °C can be due to the dependence of the catalytic activity of the enzyme to high temperatures. By increasing temperature, enzyme-substrate kinetic energy and the rate of enzyme-substrate collisions will be upgraded, leading to elevated enzyme activity (Bisswanger, 2014). Enzyme activity decreased at the temperatures higher than 50 ˚C. Enzyme ionization forms may be influenced by the temperatures over optimum level which can reduce the enzyme activity. The translational, vibrational and rotational energies of molecular bonding are enhanced in the presence of increased temperatures, cleaving several bonds. This indicates the 3D form of the proteins and thus can lead to enzyme denaturation (Somero, 1995).

CONCLUSION

The mercury resistant bacterium, *Bacillus firmus* strain MN8, had the *mer*A gene encoding mercuric reductase. For this reason, this bacterium could transform the toxic mercury to nontoxic forms and revive in high concentrations of mercury. The mercuric reductase of this strain can act in wide ranges of temperature and pH. Moreover, this strain can maintain 50 % of its specific activity in NaCl 1.5 M. Results indicate that MN8 strain can be a proper candidate for bioremediation of mercury-contaminated harsh environments, such as industrial wastewaters.

ACKNOWLEDGMENT

The authors would like to appreciate all the Department individuals who assisted and supported the project performance.

CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this manuscript.

ABBRREVIATIONS

16S rRNA	Small subunit ribosomal RNA
A_{340}	Absorbance at 340 nm
bp	Base pair(s)
°C	Celsius
CDS	Coding DNA sequence
CFU /ml	Colony forming units per milliliter
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EC number	Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
g	Gram
Gel Doc	Gel Documentation
h	Hour
Hg	Mercury
$HgCl_2$	Mercuric chloride
K_m	Michaelis-Menten constant
Km	*Kilometer*
Km^2	Square kilometer
KOH	Potassium hydroxide
L	Liter
L/µmol	liter/ micromoles
M	Molar
merA	Mercuric reductase gene
merR	Regulatory mer operon
MIC	Minimum inhibitory concentration
min	Minute
ml	Mililiter
µl	Microliter
µM	Micromolar
mM	Milimolar
µmol	micromoles
MR	Mercuric reductase enzyme
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate

NCBI	National Center for Biotechnology Information
n.m	Nanometer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
R^2	Coefficient of determination
rpm	Revolutions per minute
S	Second
U/mg	Unit per milligram
V_0	Initial velocity
V_{max}	Maximal velocity
W/V	Weight per volume
X	x variable
Y	y variable

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AUTHOR (S) BIOSKETCHES

Noroozi, M., Ph.D. Candidate, Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran. Email: *marznoroozi@yahoo.com*

Amoozegar, M.A., Ph.D., Professor, Department of Microbiology, Faculty of Biology, College of Science, University of Tehran, Tehran, Iran. Email: *amoozegar@ut.ac.ir*

Pourbabaee, A.A., Ph.D., Associate Professor, Soil Science Department, Faculty of Agricultural Engineering and Technology, University College of Agriculture and Natural Resources, University of Tehran, Tehran, Iran. Email: *pourbabaei@ut.ac.ir*

Naghavi, N.S., Ph.D., Assistant Professor, Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran. Email: *naghavi@iaufala.ac.ir*

Nourmohammadi, Z., Ph.D., Assistant Professor, Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran. Email: *z-nouri@srbiau.ac.ir*

HOW TO CITE THIS ARTICLE

Noroozi, M.; Amoozegar, M.A.; Pourbabaee, A.A.; Naghavi, N.S.; Nourmohammadi, Z., (2017). Isolation and characterization of mercuric reductase by newly isolated halophilic bacterium, Bacillus firmus MN8. Global J. Environ. Sci. Manage., 3(4): 427-436.

DOI: 10.22034/gjesm.2017.03.04.008

url: http://gjesm.net/article_26733.html