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BIOREMEDIATION OF MERCURY USING MERCURY RESISTANT BACTERIA

D. R. Kotwal, N. B. Shewale, U. S. Tambat, M. J. Thakare, A. D. Bholay*

P.G.Dept. of Microbiology, K.T.H.M.College, Nasik, SP Pune University, MS, India

ABSTRACT: The present research investigated that the role of mercury resistant bacteria and incorporates them into remediation design. To evaluate this contribution, the present work was aimed to isolate mercury resistant bacteria and determine the optimization of physicochemical parameters. Explore the mer operon and estimate removal of mercury by selected isolates and to detect and characterize the mercury reductase enzyme activity produced by a strain. Twenty eight mercury resistant bacterial strains were isolated from metal contaminated and pesticide areas. Among them two highly potent isolates were screened which were resistant to high mercury concentration and capable of removing mercury. On the basis of morphological and biochemical characters isolated strains HgS-II and HgS-III belongs to *Pseudomonas aeruginosa and Bacillus licheniformis.* these organisms showed optimization of physical condition at pH 6 and at temperature 35ºC. The bacterial isolate *P. aeruginosa* showed highest mercury bioremediating capacity for Hg (II) i.e 62% more than *B.licheniformis* under laboratory conditions. Results of this study demonstrate the occurrence of diverse groups of bacteria capable of high tolerance to mercury. The mercury reducing ability shown by isolates indicated their potential to develop bioremediation technologies and application in clean up the environment and waste contaminated with mercury.

KEYWORDS: Heavy metals, Bioremediation, Mercury resistant bacteria, mer operon, mercury reductase

***Corresponding Author: Dr. A. D. Bholay** Ph. D.

P. G. Dept. of Microbiology, K.T.H.M. College, Nasik, SP Pune University, MS, India

* Email Address: adbholay@gmail.com.

1.INTRODUCTION

In environment mercury (Hg) exists naturally in diverse chemical varieties with distinct solubility, toxicity and reactivity resulting in different effects on ecosystem and human health [1]. Mercury occur in certain forms of organic mercury and inorganic mercury, which involves metallic mercury, mercury vapor (Hg0) and mercurous ($Hg2^{2+}$) or mercuric (Hg^{2+}) salts [2]. Many natural and anthropogenic provenience releases mercury in the environment as a consequence of complex combinations containing physical, chemical and biological reactions [3]. Metal contaminated environments pose serious threat to health and ecosystems. Metals such as arsenic, cadmium, lead, mercury, silver etc. evoke conditions like hypophosphatemia, heart disease, liver damage, cancer, neurological and cardiovascular diseases, central nervous system damage and sensory disturbances. Anthropogenic and geological activities has lead to toxic mercury compounds to be globally distributed, as a result of these microorganism acquired a novel array of resistance system to withstand the poisonous environment. The clustered genes in mer operon helps bacteria to enzymatically reduced and detoxify Hg^{2+} in volatile and less toxic (Hg0) metallic mercury [4,5]. Mercury transport, mercury transformations and regulatory genes are part of the mer operon is an effectively controlled genetic system [6]. Generally most of mercury resistant bacteria exhibit mer a, mer P, mer R genes that encodes proteins for mercuric (II) reductase, extracellular binding, transport and regulatory functions respectively [7]. In various bacteria the organomercurial detoxification system is raise where flavoprotein mercuric reductase is a key component. In every cases the mercury resistance operon is plasmid-encoded as well as inducible on a transposable element. The mer operon is found in few cases, the activities of the enzyme mercuric reductase (MR) and NAD(P)H dependent flavin oxidoreductase which regulate the resistance to inorganic Hg [Hg(II)] [8]. The merA gene is component of the Hg resistance (mer) operon, which is all over among both Bacteria and Archaea, endure these organisms to survive in the latency of elevated Hg concentrations [9]. Antibiotic resistance is enhanced with contribution of mercury pollution. In conjugative plasmids and transposons the Mercury resistance operons are oftenally found which accommodate an applicable model system for the study of horizontal gene transfer in natural populations of bacteria [10]. Bioremediation is sustainable strategy available to clean up contaminated environments by microorganism which degrade toxic organic pollutant to safe level and is an acceptable natural process. To precipitate effectively immobilize inorganic pollutants such as heavy metals, now a days biological remediation technology is used. Removal of the metals

from the contaminated sites or convert into less toxic form is the aim of microbial remediation of heavy metals as compared to other technologies. The bioremediation is less expensive and required less energy. In wide variety of contaminants this technique is useful for the complete destruction or transfer of hazardous to harmless products [11]. To evaluate this contribution, the present work was aimed to isolate mercury resistant bacteria and determine the optimal growth conditions with respect to nutrient, pH and temperature, and to detect and characterize the mercury reductase enzyme activity produced by isolated strain.

2. MATERIALS AND METHODS

Soil sampling

Soil samples were collected from Ambad MIDC area of Nasik and Krishna automobile and metallic works industrial area of Navi Mumbai. Soil samples were obtained from a depth of 2ft from soil contaminated with metal [12].

Isolation and screening of mercury resistant bacteria

For enrichment of the organisms 1gm of soil sample was suspended in 10ml saline water, for enrichment 5ml of soil suspension was inoculated in 100ml nutrient broth media supplemented with 25ppm of mercury chloride at 30ºC for 7 days. An aliquot 0.1 ml of culture from the enriched broth was spreaded on LB agar plates containing 0.1g of mercury and incubated at 30ºC for 72 hrs to isolate the colonies of mercury resistant bacteria [13]. For screening Luria Bertani broth was employed supplemented with different concentrations of mercury as 20, 40, 60, 80 and 100 ppm.

Morphological and biochemical characterization of isolates

The screened most potent mercury resistant bacteria were then characterized by performing Gram staining, endospore staining, motility, starch hydrolysis (amylase), VP, citrate, glucose fermentation, catalase, oxidase, fluroscent diffusible yellow pigment and non-fluroscent diffusible blue pigment test. The bacterial species were further confirmed by VITEK system [14].

Bioremediation of Mercury by isolates

LB media was supplemented with 25 ppm of mercury chloride and inoculated with loopful of mercury resistant bacterial growth and incubated for 72 hrs.After incubation every 24 hrs, 2ml of media was withdrawn and centrifuged at 10,000 rpm for 10 minute to 1.4 ml of supernatant from media was taken in separate test tubes and 0.2 ml of dithiazone reagent(0.25gm of dithiazone powder dissolved in 10ml of acetone) was added, 0.2 ml of sulphuric acid (to maintain pH) and 0.2 ml of Dioxane was added in test tube and checked for reduction of mercury by UV/visible spectrophotometer [15].

Determination of growth curve

The isolated bacteria were separately grown in 50 ml of sterile LB media containing 25, 50, 75 and 100 ppm concentration of mercury then inoculated with 100 µl of the freshly prepared inoculum and incubated at 30ºC in an shaking incubator at 120 rpm for 12 days. An aliquots of culture were taken at regular 24 hrs intervals to measure optical density at 530 nm for determination of growth response of bacteria. Growth curves of bacteria were determined atdifferent concentrations of mercury [12,13].

Optimization of physicochemical parameters

The optimum pH (5,6,7,8 and 9), temperature (20,28,35 and 40ºC) and media requirements (Nutrient broth and Luria bertani broth) with respect to growth was determined in triplicate [13].

Determination and characterization of mercury reductase

Extraction of enzymes

Organism which was most efficient in bioremediation was selected for reductase activity. After 24 hours of incubation the mother culture was harvested by centrifugation at 15000 rpm for 20 minute at 4°C. The cell pellet was re-suspended in 30 ml Phosphate Buffer Saline (PBS), pH (7.0). Cells were then disrupted by sonication with ultrasonic processor at 600 watt and 50% amplitude for 60 seconds and the resultant homogenate was centrifuged at 15000 rpm for 30 minute at 4°C.The supernatant was used as crude enzyme source.

Purification of enzyme

After crude enzyme separation, the extraction of enzyme by organic solvent precipitation purification was carried out. In crude extract of enzyme 1:1 cold acetone (drop by drop) was added with continuous stirring and incubated overnight at 4°c and then centrifuged at 5000 rpm for 10 min at 4ºC. The precipitate was dissolved in a suitable phosphate buffer.

Mercury reductase assay system (MRAS)

Mercury reductase activity was carried out in a MRAS solution in dark tube containing 9980 µl of 50 mM PBS (pH 7.0), 5 µl of 0.5 mM EDTA, 2 µl of 200 µM MgSO4, 10 µl of 0.1% (v/v) β-merchaptoethanol, 1000 µl of 200 µM NADH2 and 2 µl of 25 mg/L HgCl2. One volume of partially purified enzyme extract was added into MRAS and incubated at 28ºC for various time intervals of 30, 60, 90, 120 and 150 minutes. Mercury reductase activity was measured spectrophotometrically at 340 nm. A one unit of mercury reductase activity was defined as one molar of oxidized NADH2 produced per total cell per minute in the assay condition [16].

3. RESULTS AND DISCUSSION

Isolation and screening of mercury resistant bacteria

From various soil samples after enrichment twenty eight bacterial isolates were obtained and they were screened on the basis of growth in mercury containing media and from that two organisms which were tolerating concentration above 60 ppm were selected for bioremediation activity.

Morphological and biochemical characterization of isolates

Microbial characterization and identification was done based on morphological, physiological and bio-chemical tests and results were compared with Bergy's manual of determinative bacteriology. Bacterial strains HgS-II was Gram negative, motile, non-spore forming, glucosefermentating, pigmented organism identified as *Pseudomonas spp* and HgS-III was Gram positive, motile, spore forming, starch hydrolyzing organism identified as *Bacillus spp.*The isolates were identified and confirmed with VITEK 2 system version 05.02 as *Pseudomonas aeruginosa and Bacillus licheniformis* respectively (table 2 and 3).

Table 2: VITEK report of *Pseudomonas aeruginosa*

Selected Organism	**Bionumber:** 1043041003500000 93 % Probability: *Pseudomonas aeroginosa*	**Confidence :** very good identification

Biochemical details

2	APPA	+	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCLE	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	(-)	18	dMAL	-	19	dMAN	-	20	dMNE	(-)	21	BXYL	-	22	BALap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	(-)
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	-	59	GGAA	(-)	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Table 3: VITEK report of *Bacillus licheniformis*

Selected Organism	**Bionumber:** 1773261715476271 93% Probability: *Bacillus licheniformis*	**Confidence**: very good identification

Biochemical details:

1	BXYL	+	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	+
9	BGAL	+	10	PyrA	+	11	AGAL	(+)	12	AlaA	+	13	TyrA	+	14	BNAG	-
15	APPA	-	18	CDEX	+	19	dGAL	-	21	GLYG	(-)	22	INO	+	24	MdG	+
25	ELLM	+	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	(+)	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	(-)	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	-	45	PVATE	+	46	AGLU	+	47	dTAG	+	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	55	PSCNa	-	58	NaCl 6.5%	+	59	KAN	-
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_R	+						

Bioremediation of Mercury by isolates

Remediation of mercury by mercury resistant bacteria after 24, 48 and 72 hrs. by *P. aeruginsa* and *B. licheniformis* are shown in table 1.The results indicated that *P.aeruginosa* was 62% more efficient than *B.licheniformis* in remediation activity.

Table 1: Remediation of mercury(ppm) by mercury resistant bacteria.

Isolates	After 24 hrs.	After 48 hrs.	After 72 hrs.
Control	60 ± 1.48	60 ± 1.48	60 ± 1.48
P.aeruginosa	55 ± 1.32	24 ± 2.13	10 ± 1.73
B.licheniformis	56 ± 1.61	32 ± 1.45	16 ± 2.26

Determination of growth curve

As shown in fig 1 and 2, the growth of *P. aeruginosa and B. licheniformis* was determined at mercury concentration of 50, 75 and 100mg/ml for both the organism the optimum growth was observed on 7th day and after 7th day the growth was declined. The result indicated that both the species could tolerate up to 100mg/ml concentration of mercury efficiently. *P.aeruginosa* showed the maximum growth at mercury concentration of 50 mg/ml than the *B.licheniformis*.

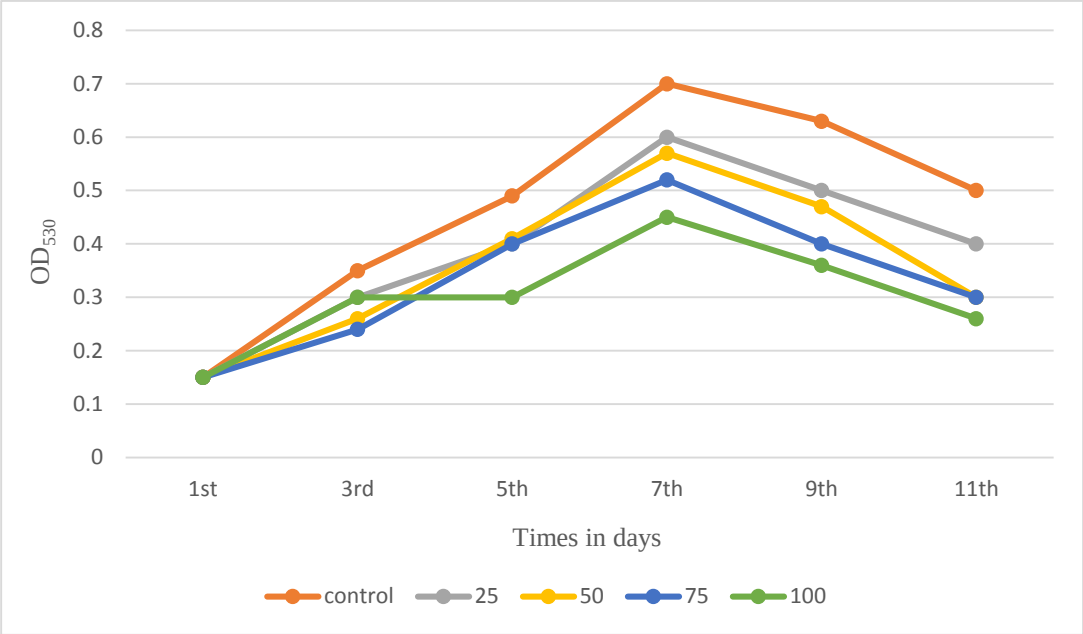

Fig 1: Growth curve of *P.aeruginosa* with and without mercury stress.

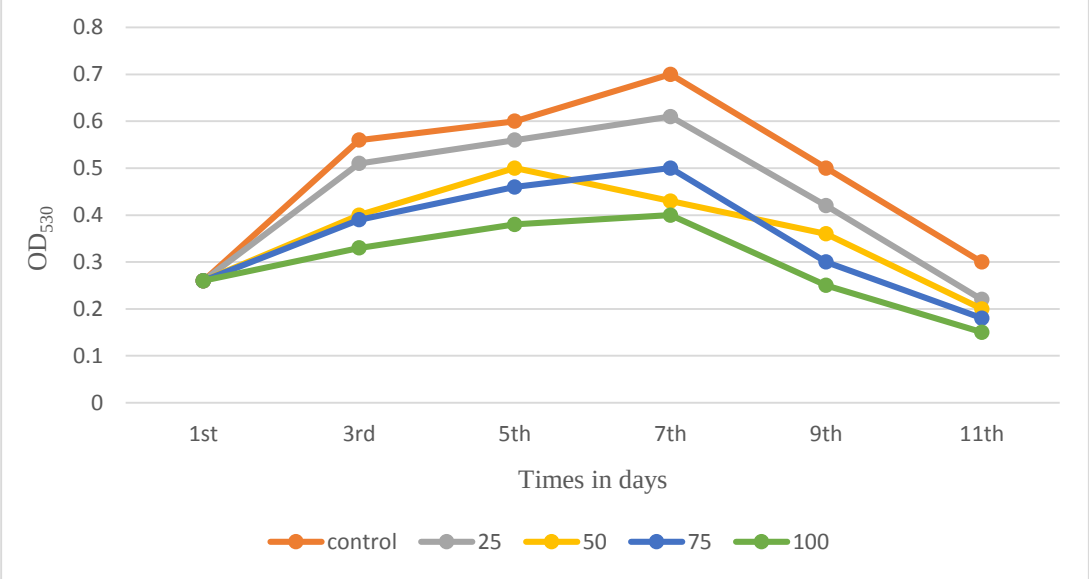

Fig 2: Growth curve of *B.licheniformis* with and without mercury stress

Optimization of physicochemical parameters

Optimization of physical conditions

Optimization of pH for growth was determined at pH 5,6,7,8 and 9. The optimum pH for growth of *P. aeruginosa* and *B. licheniformis* were found to be 6 and 7 respectively(Fig 3). The optimum temperature for growth, was determined at 20, 28 , 35 and 40°C. The optimum temperature required for growth *P. aeruginosa* and *B. licheniformis* were found to be 28°C and 35°C (Fig 4). The result indicated that the organism *P.aeruginosa* showed maximum absorbance at optimum temperature 28ºC while *B. licheniformis* showed maximum absorbance at optimum temperature 35ºC and at optimum pH 6 and 7 the *P. aeruginosa* showed maximum absorbance than *B. licheniformis*.

Optimization of chemical condition

Nutrient Broth and Luria Bertani Broth were used for optimization of media. Luria Bertani broth was found to be most effective in growth promotion of both *P.aeruginosa* and *B.licheniformis*(fig 5). The result indicated that the *P.aeruginosa* showed maximum absorbance in Luria Bertani broth than the *B. licheniformis*.

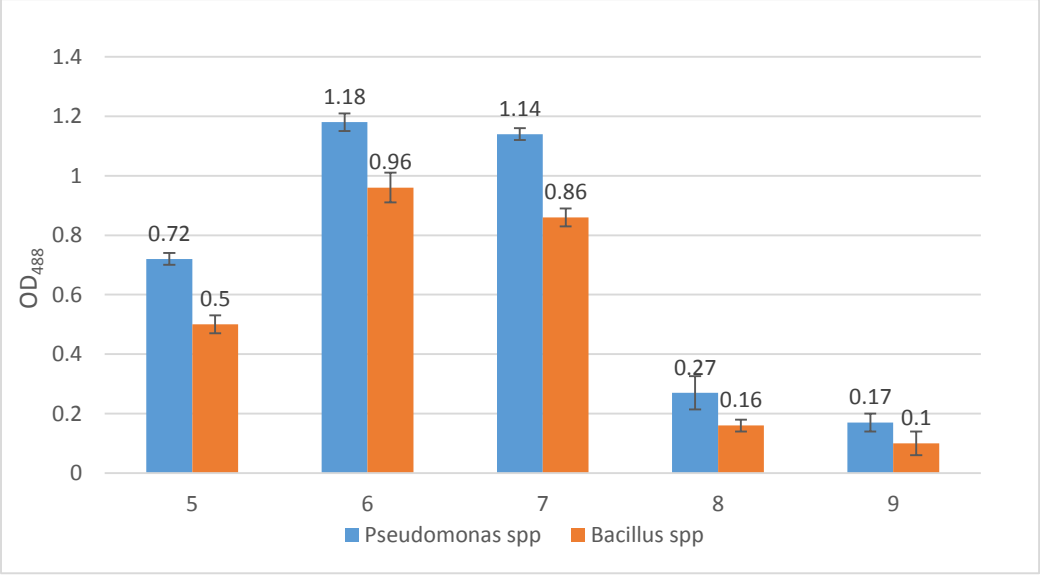

Fig 3: Optimization of pH for *P. aeruginosa* and *B. licheniformis.*

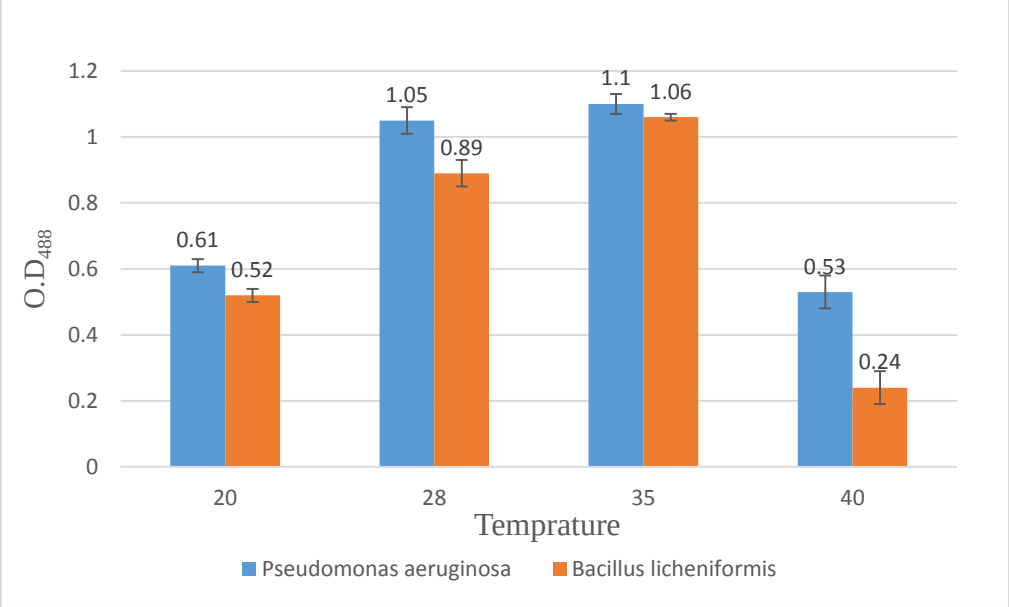

Fig 4: Optimization of temperature for *P. aeruginosa* and *B. licheniformis*

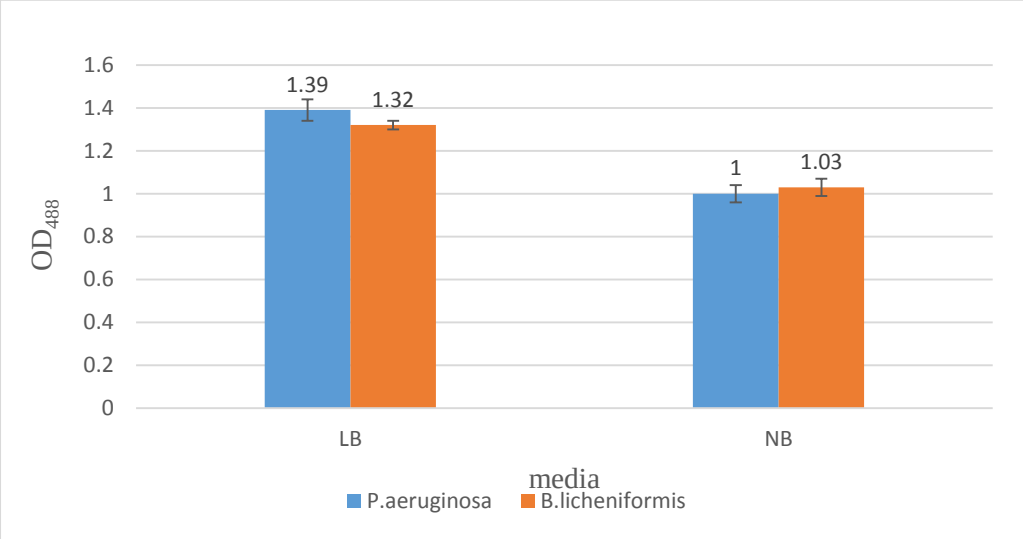

Fig 5: Optimization of media for *P.aeruginosa and B.licheniformis*.

Detection and characterization of mercury reductase

The determination of mercury reducing ability of *P.aeruginosa* was successfully carried out by mercury reductase assay system the details are given in Table 4. The result indicated that the highest mercury reductase activity observed was at first 30 min incubation and gradually decreased as prolonged time. Similarly the percent reduction of Hg^{2+} was also maximum during the first 30 min.

Table 4 : Mercury reductase enzyme activity by *P. aeruginosa*

Time (minutes)	**Oxidized NADH2 (µM/10^8cells/min)**	**Enzyme activity (unit)**	**Reduction of Hg^{2+} (mg/L/min)**	**% Reduction Of Hg^{2+}/min**
30	0.0042	0.28 ±0.06	0.22 ±0.07	1.10
60	0.0031	0.21 ±0.03	0.13 ±0.05	0.65
90	0.0024	0.16 ±0.05	0.11 ±0.03	0.55
120	0.0017	0.11 ±0.08	0.06 ±0.04	0.30
150	0.0014	0.09 ±0.02	0.03 ±0.02	0.15

4. CONCLUSION

The results accomplish in the current investigation arrays frequencies of mercury resistant bacteria in the contaminated areas are high and thus a potential threat to the nearby population.The study successfully isolated twenty eight bacterial isolates from which two potent mercury resistant strains were identified as *Pseudomonas aeruginosa* and *Bacillus licheniformis*. The result indicated that *P. aeruginosa* was 62% more efficient than *B. licheniformis* in remediation activity. Both the species could tolerate up to 100mg/ml concentration of mercury efficiently. Mercury resistant bacteria have the mercury reductase activity and mer operon which converts toxic form of mercury to the non-toxic form which help it to thrive in the existence of mercury. The optimum pH and temperature required for growth of *P. aeruginosa* was pH 6 and Temprature 28°C and for *B.licheniformis* was found to be pH 7 and Temprature35°C. Luria Bertani broth was found to be most effective in growth promotion of both *P.aeruginosa* and *B.licheniformis*. mercury reducing ability of *P.aeruginosa* was determined and successfully carried out by mercury reductase assay system. The result indicated that the highest mercury reductase activity was observed at first 30 min incubation and gradually decreases as prolonged time. These organisms can be used in future in regards of enhancing our capability to explore the methods for the mercury contaminated waste remediation.

5.ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

Declare here if any financial interest or any conflict of interest exists.

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