Evaluation of Biotechnological Potential of Novel Mercury Tolerant Strain of Klebsiella Pneumonia

Himadri Gourav Behuria, Rashmi Shee, Sangam Gupta, Bijesh Kumar Biswal and Santosh Kumar Sahu*

Laboratory of Molecular Membrane Biology, Department of Biotechnology, North Orissa University, India

*Corresponding author:
Santosh Kumar Sahu

✉ drsantoshnou@gmail.com

Laboratory of Molecular Membrane Biology, Department of Biotechnology, North Orissa University, Odisha, India -757003.

Tel: +91-9178939281
Fax: 06792-256906

Abstract

In order to investigate the effect of elevated mercury pollution on antibiotic tolerance and enzyme activities of human pathogens, we isolated a novel *K. pneumoniae* strain from the soil of a metallurgical dump yard that was capable of tolerating 250 μM HgCl$_2$. Increasing concentration of Hg^{2+} in growth medium led to increased tolerance of the strain to ampicillin that was accompanied by increased secretion of extracellular polymeric substance (EPS). These results indicate that Hg^{2+}-induced increased synthesis of EPS might be a key factor for increased tolerance of *K. pneumoniae* to ampicillin. The strain exhibited high catalase, alkaline phosphatase, mercuric reductase, amylase and lipase activity. Presence of 250 μM HgCl$_2$ in growth medium increased mercuric reductase activity to 3 fold, however, no significant alteration in catalase, alkaline phosphatase and lipase was observed. The strain retained 86% amylase activity when grown in presence of 250 μM Hg^{2+}. However, cell free lysate of the bacterium exhibited sensitivity towards Hg^{2+} leading to 30 to 40% decrease of these enzyme activities at 250 μM Hg^{2+}. These results show that the bacterium possesses cellular mechanism to protect its enzymes from exogenous HgCl$_2$. Our study reveals that increased heavy metal in the environment is a key factor leading to cross resistance of *K. pneumoniae* against membrane-targeted antibiotics ampicillin. Further, it's a novel mercury-tolerant strain of *K. pneumoniae* isolate that exhibits high biotechnological potential for simultaneous bioremediation of mercury and production of industrially important enzymes.

Keywords: Mercury; Antibiotic; Mercuric reductase; Extracellular polymeric substance; Klebsiella; Alkaline phosphatase; Catalase

List of Abbreviations

ALKP: Alkaline Phosphatase; PSI-BLAST: Position Specific Iterated Basic Local Alignment Search Tool; BSA: Bovine Serum Albumin; CR: Congored; EPS: Extracellular Polymeric Substance; MerA: Mercuric Reductase; PMSF: Phenyl Methyl Sulfonyl Fluoride; PNP: Para-Nitrophenyl Phosphatate; MIC: Minimum Inhibitory Concentration.

Introduction

Anthropogenic activities such as mining, smelting, coal combustion, waste incineration and chlor-alkali plants have increased the load of mercury in water, soil and atmosphere by 3 folds in last 500 years [1]. Most inorganic Hg released into environment is converted into organic mercury by soil microbes. These organomercurials accumulate within the organisms that inhabit Hg-contaminated ecological niches and are subsequently biomagnified as they move along the food chain up to human [2,3]. In India, rapidly expanding industrialization and energy requirement have increased Hg emission at an alarming rate from 920 tons in 2001 to 1450 tons in 2010 [4]. Application of Hg-contaminated sewage sludge in agricultural lands as green manure, a common practice in India has increased Hg accumulation in crops and vegetables [5,6]. Consumption of Hg-contaminated water, aquatic animals (e.g. sea food), plants and their products constitutes the major route of mercury toxicity

in human [7]. In human, mercury is known to affect renal, cardiovascular, reproductive, central nervous system (CNS) and interferes with fetal development [7,8].

Microbes growing in Hg^{2+} contaminated soil and water bodies exhibit resistance to Hg^{2+} by altering their biochemical and molecular characteristics. Microbial Hg^{2+} tolerance is proposed to be a bio-indicator of environmental Hg^{2+} toxicity [9]. Some important biochemical alteration in microbes exposed to elevated level of Hg^{2+} include over-expression of oxidative stress regulatory enzymes such as catalase and mercury reducing enzyme MerA [10,11]. However, Hg^{2+} is known to inhibit function of multiple proteins and enzymes. Binding of Hg^{2+} to sulfhydryl (-SH) groups leading to their covalent modification and displacement of metal ion cofactors (e.g. calcium or iron) from metalloenzymes are amongst important mechanisms of Hg^{2+}-induced inhibition of enzymes [12]. However, mercury-tolerant bacteria exhibit multiple mechanisms to tolerate high concentration of Hg^{2+} in growth medium. Common Hg^{2+} resistance mechanisms involve over-expression of MerA, Hg^{2+}-tolerant enzymes and oxidative stress regulatory enzymes (e.g. catalase). Modification of cell envelope in bacteria such as change in membrane lipid composition, increased secretion of EPS and formation of biofilm on solid surface contribute to their Hg^{2+} tolerance. Many heavy metal tolerant bacteria possess plasmids that harbor genetic information to tolerate high concentration of heavy metal in the growth medium [9].

K. pneumoniae is a common pathogen of human visceral epithelium such as gut, respiratory and urinary epithelium and the causative agent for acute pneumonia. This bacterium exhibits drug resistance against a number of antimicrobials including the cell wall targeted antibiotic ampicillin. Atomic force microscopy of antibiotic resistant *K. pneumoniae* strain shows that these bacteria exhibit morphological alteration in their cell wall [13]. Alteration of membrane lipid composition, increased the synthesis of extracellular polymeric substances (EPS) and biofilm formation are amongst the most common morphological changes that enables resistance of *K. pneumoniae* to Hg^{2+} and other antimicrobials [14,15]. Research by multiple researchers reveals that many bacterial species have coevolved antibiotic tolerance along with heavy metal tolerance [16]. However, the mechanism of heavy metal-induced antibiotic tolerance in *K. pneumoniae* remains unexplored.

Bacteria are otherwise termed as microbial cell factories that produce industrially important enzymes. However, most bacteria are sensitive to heavy metals in their growth medium that significantly inhibit production of enzymes at industrial scale. Heavy metal tolerant bacteria that produce industrially relevant enzymes or metabolites constitute useful bio-resources as they could be utilized for multiple purposes such as heavy metal detoxification as well as production of enzymes. To investigate the effect of heavy metal on antibiotic tolerance and enzyme activities of common human pathogens that inhabit soil, we isolated a novel *Klebsiella pneumoniae* strain from the soil of a metallurgical dump yard. This strain not only tolerates high concentration of $HgCl_2$ in growth medium, but also exhibits

resistance to ampicillin and produces high level of some common industrial enzymes. We evaluated the biotechnological potential of the strain for production of five common industrial enzymes: catalase, ALKP, MerA, amylase and lipase.

Materials and Methods

Microorganism

Soil sample collected from a dump yard of Rourkela steel plant, Odisha, India that was used for dumping industrial waste products for more than 50 years. The sample was screened for the presence of Hg^{2+} tolerant bacteria by growing them in LB liquid medium containing increasing concentration of $HgCl_2$ up to 250 µM. A single strain was isolated that possessed a minimum inhibitory concentration (MIC) of 250 µM $HgCl_2$ and was identified by 16S rDNA sequencing using forward and reverse primers AGAGTTTGATCCTGGCTCAG and TACGGTTACCTTGTTACGACTT respectively at AgriGenome Labs Pvt. Ltd, Kerala, India. The sequence was matched to the existing bacterial 16S rDNA sequences in NCBI data base using position specific iterated basic local alignment search tool (PSI-BLAST) programme [17].

Growth, collection and lysis of cells

The *K. pneumoniae* isolate was maintained in LB Agar (LBA) medium, cultured in LB containing different concentration of Hg^{2+} for 16 h at 25°C and 200 rpm and collected by centrifugation at 13000 × g for 10 min at 4°C. Cell pellets were re-suspended at ~100 mg ml^{-1} in re-suspension buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM BHT and 1 mM PMSF) and used immediately for further experiments. Cells were lysed by sequential incubation with lysozyme (1 mg ml^{-1}) and Triton-X-100 (1% (w/v)) at 37°C for 30min with intermittent mixing. Supernatant was collected after centrifugation of the lysate at 4°C for 20min at 17, 000 × g. Protein was quantitated by Lowry method using a Systronics double beam spectrophotometer (Model Visiscan 167, India) [18].

Evaluation of enzyme activities

Activity of catalase, mercury reductase (MerA), alkaline phosphatase (ALKP) and amylase were measured by spectrophotometric assay using a Systronics double beam spectrophotometer (Model 2202, Japan). Lipase activity was quantitated using plate assay method on freshly collected cell lysate. Data obtained from at least four sets of experiments were analyzed using GraphPad prism (version 6).

Catalase

Catalase activity was quantitated following the methods of Beers and Sizer [19].Briefly, Catalase activity in 0.3 ml lystae containing 1 mg protein was quantitated by monitoring the time dependent depletion of 6.6 mM H_2O_2 at 240 nm in 3 ml assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl).

MerA

MerA assay was performed following procedures of Fox and Walsh in cell lysate containing 1 mg protein in 3 ml assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM β-mercaptoethanol, 200

μM NAD(P)H and 50 μM HgCl$_2$) [20]. Briefly, MerA activity was quantitated by measuring Hg^{2+}-dependent oxidation of NAD(P)H to NADP$^+$ resulting in depletion of absorbance of NADPH (A$_{340}$). Initial rate of NAD(P)H oxidation was determined in the first 10s, when A$_{340}$ decreased linearly with time. One unit MerA activity is defined as the activity that oxidized 1 μmol of NAD(P)H min^{-1} in the above specified condition.

ALKP

ALKP activity was quantitated by monitoring time-dependent hydrolysis of para-nitrophenyl phosphate (PNP) to PO$_4$ and para-nitrophenol (PN) that has absorption maxima at 405 nm [21]. Briefly, time-dependent increase in absorption of PN (A$_{405}$) in the assay mix containing 1 mg protein in 3 ml assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM PNP was monitored for 3 min at 25°C. One unit ALKP activity was calculated as the enzyme activity that produced 1 μmol PN min^{-1} under above assay conditions.

Amylase

Amylase activity in the cell lysate containing 1 mg protein was monitored by time-dependent depletion of 1mg amylose in 3ml assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl) at 25°C [22]. One unit amylase activity was defined as the activity that degraded 1μg starch per min in one ml assay mix in the above assay condition.

Lipase

Lipase activity in the cell lysate was quantitated following the method of Kouker and Jaeger using rhodamine B agar plates [23]. Briefly, cell lysates containing 1 mg protein in 50 μl volume was placed in wells of 5mm diameter on agar plates containing 2.5% (w/v) olive oil and 0.001% (w/v) rhodamine B. The plate was incubated for 48 h at 37°C and the diameter of the orange fluorescent hallos produced around the well was visualized in UV (350 nm) was measured. Lipase activity was calculated from a standard curve obtained by placing known amount lipases in the agar wells in the same condition used for unknown samples.

Quantitation of EPS production

EPS produced by the isolate was quantitated using congo red (CR) binding assay as described previously with modification [24]. Briefly, stationary phase cultures were adjusted to OD$_{600}$=10 in 3 ml resuspension buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM BHT) containing 40 mg L^{-1} CR and incubated for 1 h at 25°C and 200 rpm. Cells were removed by centrifugation at 25°C and 10,000 × g for 5 min and A$_{490}$ of the supernatant was determined by measuring the absorbance of the residual CR in the resuspension buffer.

Determination of ampicillin resistance

Ampicillin resistance of the *K. pneumoniae* isolate was determined by agar-cup assay [25]. Briefly, *K. pneumoniae* grown in LB containing increasing concentration of Hg^{2+} were uniformly plated at OD$_{600}$ of 0.1 on LB-agar plates. Increasing concentration of ampicillin (0, 250 μg/ml, 500 μg/ml, 750 μg/ml and 1000 μg/ml was applied the in wells of 6 mm diameter in 50 μl total

volume and incubated at 37°C for 12 h. Diameter of the clear zone produced around each well shows the sensitivity of the bacterium to ampicillin.

Statistical analysis

Statistical analysis was performed using GraphPad prism (Version 6). Statistical significance of data was evaluated by Student's "t" test for the biochemical studies and One-way ANOVA test was used to compare the results whenever more than two experimental groups were compared. All the data are expressed as Mean ± STDEV (standard deviation) (n=3). Differences were considered to be significant at a probability of $P<0.05$.

Results

Isolation and identification of *K. pneumoniae* industrial isolate

Gradual enrichment of the LB growth medium containing mixed culture of microbial strains collected from soil of industrial dumping area up to 300 μM led to isolation of a single Gram negative bacillus that produced mucoid colonies on LBA plate. Amplification of the 16S rDNA region of its genomic DNA produced a 1415 bp amplicon. Analysis of its sequence at NCBI genomic data base using PSI-BLAST exhibited 99% identity to six highest-scoring 16S rDNA sequences from existing *K. pneumoniae* strains [26].The 16S rDNA from *K. oxytoca* used as a negative control exhibited 98% sequence identity **(Figure 1)**. These results show that the isolate is a novel strain of *K. pneumoniae*.

Determination of mercury tolerance of the *K. pneumoniae* strain

The *K. pneumoniae* was maintained in LBA and grown in LB at 25°C and 200 rpm. In above condition, the culture was saturated at 16h. In presence of increasing concentration of HgCl$_2$, up to 150 μM, lag phase was increased up to 16h, leading to an increase in saturation time up to 30 h. However, further increase of HgCl$_2$ up to 250 μM, prolonged the lag phase up to 32 h, leading to
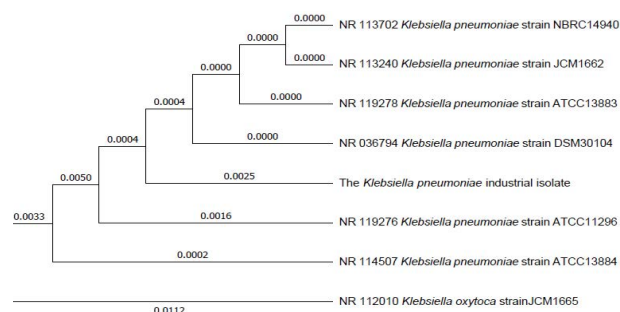

Figure 1 Phylogenetic relationship of the *K. pneumoniae* industrial isolate with six existing strains of *K. pneumoniae* and the negative control *K. oxytoca* as determined by sequence analysis of 16S rDNA using PSI-BLAST. The phylogenetic tree was generated using neighbor-hood joining method [26]. Numbers before and after the strains designate NCBI accession numbers and strain numbers respectively.

saturation phase of more than 48 h. However, no growth was observed at 300 µM HgCl$_2$ **(Figure 2)**. Incubating the cells till 72 h in 300 µM HgCl$_2$ didn't result in growth of cells, making the minimum inhibitory concentration (MIC)>250 µM and <300 µM.

Hg^{2+} increases ampicillin resistance of the *K. pneumoniae* isolate.

K. pneumoniae is known to resist cell wall-targeting antibiotics such as ampicillin. In many bacterial species, heavy metal resistance and antibiotic tolerance have been coevolved. Hence, we determined the effect of Hg^{2+} on the sensitivity of the *K. pneumoniae* to ampicillin, an antibiotic that targets the peptidoglycan layer in bacteria. Our results show that elevated concentration of Hg^{2+} in growth medium increases ampicillin resistance of the bacterium as indicated by a dose-dependent decrease in the size of clear zones around the agar wells. **Figure 3A** shows the result of a representative assay of the effect of 0, 50, 100 and 150 µM Hg^{2+} in growth medium of the *K. pneumoniae* isolate towards its sensitivity to 250 µg/ml ampicillin. The results show that increasing doses of HgCl$_2$ in growth medium gradually decrease size of the zones, leading to its complete abolishment at 150 µM. A gradual dose-dependent decrease in diameter of clear zones was observed up to 1000 µg/ml ampicillin, upon subjecting the cells to increasing doses of HgCl$_2$ in growth medium. These results indicate that HgCl$_2$ in medium induces ampicillin resistance in the *K. pneumoniae* isolate **(Figure 3B)**. Up to 40% decrease in zone size was observed at 500 to 1000 µg/ml ampicillin when the cells were subjected to 200 µM HgCl$_2$. This concentration is 5-10 times the concentration normally used to inhibit wild type *K. pneumoniae*. These data show that HgCl$_2$ increases ampicillin tolerance of *K. pneumniae* isolate.

Hg^{2+} and ampicillin resistance in *K. pneumoniae* isolate is accompanied by enhanced secretion of EPS

Soil bacteria are known to exhibit high biofilm forming abilities, a feat enabling them to survive harsh environmental conditions such as heavy metal toxicity that correlates with increased secretion of EPS. EPS is mostly a negatively charged lipo-polysaccharide
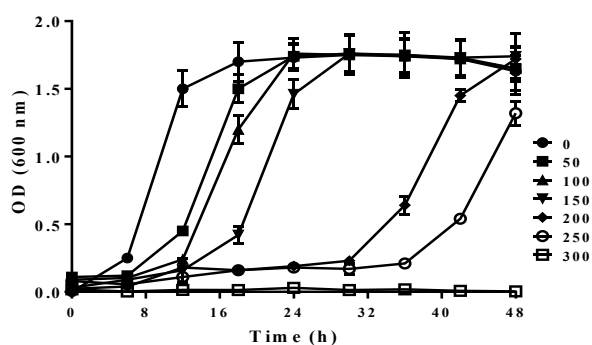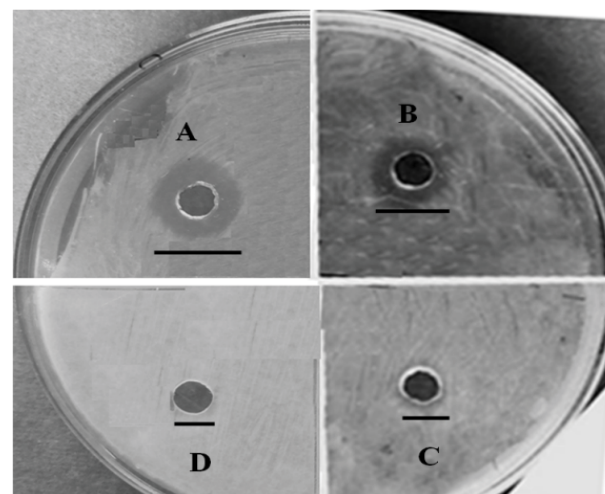
Figure 3 Effect of HgCl$_2$ in growth medium of *K. pneumoniae* on ampicillin resistance. (A) Plate assay showing resistance of the *K. pneumoniae* isolate grown in LB containing increasing concentration of HgCl$_2$ to 250 µg/ml ampicillin. Amount of HgCl$_2$ used in growth medium: clock-wise from upper left are: (A) 0 µM, (B) 50 µM, (C) 100 µM and (D) 150 µM respectively. (B) Dose-dependence of Hg^{2+} in growth medium on resistance to ampicillin. Inset: the numbers (250, 500, 750 and 1000) indicate concentration of ampicillin (in µg/ml) used in the assay to determine ampicillin resistance. Significance of the differences in zone size was determined by one way ANOVA using GraphPad prism. The data in the graph show the mean ± SD.

secretion that serves as a protective barrier in bacteria inhabiting harsh ecological niches. Increased EPS secretion correlates with increased anti-biotic resistance, colonization on respiratory epithelium and pathogenicity in *K. pneumoniae*. Hence, we quantitated the effect of increasing HgCl$_2$ in growth medium on EPS production by the *K. pneumoniae* isolate. Hg^{2+} at 200 µM enhanced EPS secretion in the *K. pneumoniae* isolate by 40% as indicated by increased binding to CR **(Figure 4)**. However, EPS secretion was not enhanced further at 250 µM Hg^{2+}, showing that EPS secretion is saturable around 200 µM HgCl$_2$ in growth medium. These results indicate the probable involvement of an enzymatic mechanism that is activated by HgCl$_2$ in growth medium.

Production of industrially important enzymes from the mercury tolerant *K. pneumoniae* isolate

Evaluation of activities of common industrially relevant enzymes in the strain shows that the bacterium possesses high potential to produce catalase, MerA, ALKP, lipase and amylase **(Table 1)**. Catalase is an important oxidative stress regulatory enzyme that is over expressed in response to the presence of heavy metals that enhances oxidative stress. The *K. pneumoniae* isolate produces 23 units/mg catalase that is not affected by the presence of HgCl$_2$ in growth medium. This finding shows that the bacterium is adapted to secrete elevated level of catalase wooing

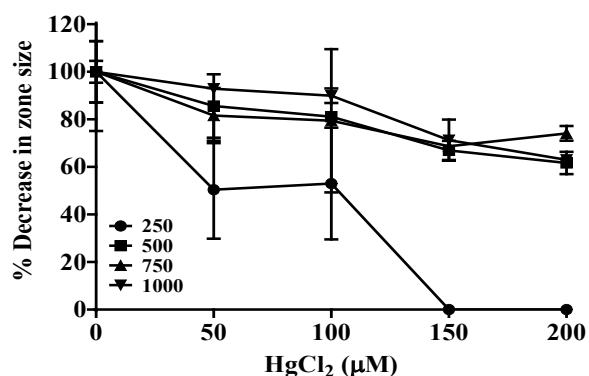**Figure 2** Growth curve of *K. pneumoniae* industrial isolate in the LB medium containing increasing concentration of HgCl$_2$. Concentration of HgCl$_2$ in the growth medium of the bacterium for each curve is mentioned to the right of the figure.

Figure 4 Effect of HgCl$_2$ on the production of extracellular polymeric substances (EPS) produced by *K. pneumonae*. Increase in synthesis of EPS in response to increasing doses of HgCl$_2$ in growth medium was indicated by an increase in binding of congo red (CR) to the bacteria cells. The data in the graph show mean ± SD for four independent sets of experiments.

Table 1 Effect of Hg^{2+} on activity of some industrially important enzymes units (average ± SD).

HgCl$_2$ (μM)	ALKP	MerA	Amylase	Lipase	Catalase
0	22.56 ± 1.3	6.4 ± 0.45	17.25 ± 1.06	12.14 ± 0.87	22.43 ± 2.1
50	22.24 ± 2.1	7.5 ± 0.73	15.36 ± 0.98	12.34 ± 0.93	23.12 ± 2.34
100	21.21 ± 1.23	10.5 ± 0.98	15.56 ± 0.57	12.65 ± 1.4	24.12 ± 2.14
150	20.67 ± 1.12	15.3 ± 1.21	16.24 ± 1.12	12.92 ± 0.79	23.45 ± 1.98
200	20.45 ± 2.1	16.45 ± 1.32	14.89 ± 1.35	13.35 ± 1.2	23.13 ± 1.57
250	17.41 ± 1.22	20.13 ± 1.75	14.8 ± 1.43	13.05 ± 1.3	23.21 ± 2.15

to elevated concentration of Hg in its environment. However, MerA was increased to 3 folds in presence of 200 μM HgCl$_2$. This inducibility of MerA activity shows that exogenous Hg^{2+} increases the cytosolic level of MerA activity in the *K. pneumoniae* isolate. MerA is the primary enzyme expressed in response to Hg toxicity that converts ionic mercury (Hg^{2+}) to the less toxic form, metallic mercury (Hg^0). Our results show that the strain exhibits high HgCl$_2$ tolerance by over expressing MerA that accelerates the conversion of more toxic ionic mercury (Hg^{2+}) in the growth medium to less toxic metallic mercury (Hg^0) that vaporizes to the atmosphere. ALKP is essentially required in bacteria for phosphate assimilation. We observed 22 units of ALKP activity/mg of total protein in cytosol of the *K. pneumoniae* isolate that remained unaffected with increase of HgCl$_2$ in the growth medium up to 200 μM. However, a 13% depletion of ALKP activity was observed at 250 μM HgCl$_2$. Amylase and lipase are two industrially important enzymes that are produced by much soil bacterial for utilization of plant remains and oil in the environment. We evaluated the α-amylose and lipase activities of crude lysate from the *K. pneumoniae* isolate. We observed 17 and 12 units of activities for amylase and lipase in the *K. pneumoniae* isolate respectively when grown in LB. A gradual decrease of amylase activity up to 15% was observed when grown in presence of Hg^{2+} up to 250 μM. However, no significant alteration in lipid degrading activity was observed in presence of exogenous HgCl$_2$. These results indicate

that the Hg^{2+} tolerant *K. pneumoniae* industrial isolate possess high biotechnological potential for production of catalase, MerA, ALKP, amylase and lipase. Further, activity of the above enzymes was not depleted in the cytosol of the *K. pneumoniae* isolate when grown in presence of high concentration of mercury in the growth medium.

To determine the sensitivity of above enzymes to Hg^{2+}, we measured their activity in cell-free lysate of the *K. pneumoniae* isolate grown in LB in presence of Hg^{2+} in their respective assay buffers. However, we observed 30-40% depletion in activities of the above enzymes when the cell free lysate was incubated with increasing HgCl$_2$ up to 250 μM. These results show that the bacterium exhibits acute Hg^{2+} regulatory mechanism to reduce cytosolic Hg^{2+} thereby, preventing its interaction with cellular enzymes. However, the cell free lysate from the *K. pneumoniae* isolate lacks this regulatory mechanism, resulting in binding of Hg^{2+} to the enzymes leading to their inhibition.

Discussion

Indiscriminate use of antibiotics in the sectors of health, food and animal husbandry are the primary sources of antibiotics in the sewage, soil and water bodies. Similar widespread release of heavy metals and their derivatives from different anthropogenic sources lead to their accumulation in the environment that is a leading cause for heavy metal tolerance in soil bacteria. Heavy metal tolerance in many bacteria correlates with their high antibiotic tolerance [27]. However, the cross talk between their heavy metal tolerance and antibiotic tolerance remains elusive. In order to investigate the effect of elevated environmental Hg^{2+} on antibiotic resistance in human pathogenic bacteria, we isolated a *K. pneumoniae* strain from soil samples of an industrial dump yard that was used to dump industrial wastes for more than 50 years. Our analysis led to identification of a novel *K. pneumoniae* strain **(Figure 1)**. We investigated Hg^{2+} induced biochemical adaptation of the *K. pneumoniae* isolate by determining its Hg^{2+}-dependent antibiotic tolerance and production of common enzymes. Our study shows that the bacterium exhibits a minimum inhibitory concentration of >250 μM HgCl$_2$ in its growth medium. However, at 300 μM HgCl$_2$ no growth was observed till 72 h **(Figure 2)**. It is highly probable that our *K. pneumoniae* isolate could also resist other heavy metals as observed for many mercury tolerant bacterial strains.

Interestingly, we observed that increase in HgCl$_2$ in growth medium led to enhanced resistance of the *K. pneumoniae* isolate to the cell wall-targeted antibiotic ampicillin **(Figure 3)**. *K. pneumoniae* is known to exhibit resistance to penicillin antibiotics including ampicillin that inhibits [14]. Ampicillin binds irreversibly to the cell wall cross-linking enzyme transpeptidase, resulting in inhibition of cell wall biosynthesis. Our study is the first report showing that increased Hg^{2+} in growth medium increases tolerance of *K. pneumoniae* to ampicillin. A few reports show that there is a high correlation between heavy metal tolerance and antibiotic resistance in bacteria. This correlation is proposed to arise from sharing of genes between the molecular pathways leading to heavy metal tolerance and antibiotic tolerance in these

bacteria [15,27]. This finding shows that not only indiscriminate use of antibiotics but also wide spread use of heavy metals and their unrestricted disposal into the environment is an important determinant contributing to antibiotic resistance in *K. pneumoniae*. One possible mechanism of Hg^{2+}-induced ampicillin resistance in *K. pneumoniae* is secretion of penicillin degrading enzymes that decreases the effective concentration of ampicillin in the medium [28]. Other probable explanation being Hg^{2+}-mediated covalent modification of transpeptidases and transglycosylases that are the targets of ampicillin.Ampicillin-targeting sites of above proteins are proposed to be periplasmic and hence are easily accessed by exogenous Hg^{2+} [29]. As Hg^{2+} is a covalent modifier of cysteines, it could inhibit the binding of ampicillin to these enzymes leading to the observed ampicillin tolerance in the *K. pneumonae* isolate [30].

Alternatively, formation of EPS in *K. pneumoniae* reduces diffusion of ampicillin into the cells, hence, reducing its effective concentration in bacterial cells [14]. Hg^{2+} in growth medium is known to stimulate secretion of EPS and biofilm formation in many mercury tolerant bacteria as a protective mechanism [24]. Hence, we quantitated the EPS formation in our *K. pneumoniae* isolate in response to increased Hg^{2+} in growth medium. Our results show that the *K. pneumoniae* isolate increases production of EPS by the bacterium. 40% increase in EPS content was observed when the bacterium was grown in presence of 200-250 μM $HgCl_2$ **(Figure 4)**. EPS is negatively charged and acts as a trap to immobilize cationic Hg^{2+} in outside the cell, hence, preventing its entry into the cytoplasm [25]. Increased EPS is proposed to be a key regulator of bacterial resistance against a multitude of antimicrobials.

To evaluate the biotechnological potential of the Hg^{2+}-tolerant *K. pneumoniae* strain, we quantitated production of five common industrially significant enzymes in the bacterium and investigated the effect of exogenous Hg^{2+} in growth medium on the activity of these enzymes. Our results show that the bacterium exhibits high potential to produce catalase, MerA, ALKP, α-amylase and lipase **(Table 1)**. Exogenously added Hg^{2+} had no effect on activities of catalase, ALKP and lipase, indicating that the bacterium is adapted to high concentration of Hg^{2+} in its natural habitat and elevated Hg^{2+} in the growth medium doesn't interfere with production or activity of above enzymes. However, activity of MerA was increased to 3 fold when the strain were subjected to 250 μM $HgCl_2$ in growth medium, indicating that the bacterium produces more MerA to accelerate reduction of more toxic Hg^{2+} to Hg^0, that is much less toxic to the cell [31]. Hg^0 being volatile, evaporates out from the cell without making further damage to the cellular components. α-amylase shows 16% reduction in activity at 250 μM compared to cells grown in LB, showing that Hg^{2+} only marginally affects its production or activity.

In order to evaluate the sensitivity of above enzymes to Hg^{2+}, we measured the effect of Hg^{2+} on their activities using cell free lysates. Our results show that there was a 30-40% depletion in activity of all five enzymes in presence of 250 μM $HgCl_2$ in the assay buffer **(Figure 5A and 5B)**. These results indicate that Hg^{2+} inhibits the activities of above enzymes, possibly, by covalent modification of sulfhydryl (-SH) groups of enzymes [12]. Further, in metalloenzymes, Hg^{2+} is known to displace the metal cofactor,
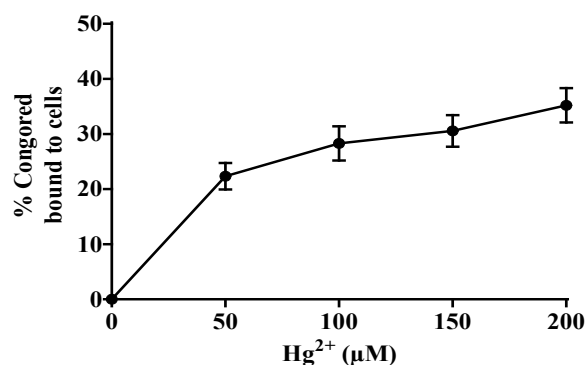

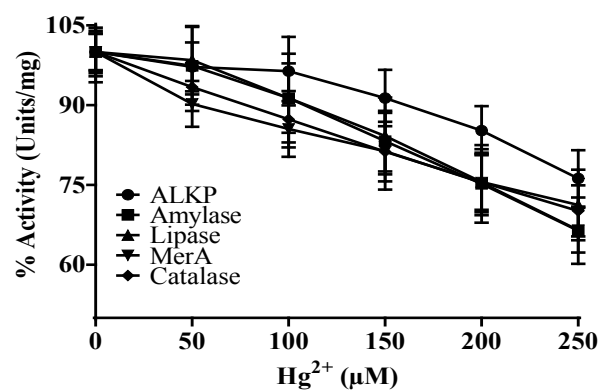**Figure 5a** Effect of $HgCl_2$ on activity of five industrially important enzymes.

Figure 5b Inset indicates the activity for each enzyme. The data in the graph show the mean ± SD for four independent sets of experiments.

leading to their inhibition [32]. Our results show that the enzymes exhibit sensitivity towards Hg^{2+}, however, the bacterium possesses biochemical adaptation for acute regulation of Hg^{2+} in its cytoplasm.

Conclusion

In conclusion, we have isolated and biochemically characterized a novel *K. pneumoniae* strain that tolerates 250 μM $HgCl_2$. Hg^{2+} induces ampicillin resistance in the bacterium that correlates with increased production of EPS in the cell envelope. The strain is an important bio-resource that exhibits high activities of industrially important enzymes such as catalase, MerA, ALKP, α-amylase and lipase. Presence of Hg^{2+} in growth medium doesn't inhibit the activity of these enzymes showing that the novel *K. pneumoniae* isolate could be utilized for efficient and simultaneous bioremediation of environmental Hg^{2+} as well as production of industrially important enzymes.

Acknowledgement

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