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

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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 Hg2+ contaminated soil and water bodies exhibit resistance to Hg2+ by altering their biochemical and molecular characteristics. Microbial Hg2+ tolerance is proposed to be a bio-indicator of environmental Hg2+ toxicity [9]. Some important biochemical alteration in microbes exposed to elevated level of Hg2+ include over-expression of oxidative stress regulatory enzymes such as catalase and mercury reducing enzyme MerA [10,11]. However, Hg2+ is known to inhibit function of multiple proteins and enzymes. Binding of Hg2+ to sulphydryl (-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 Hg2+-induced inhibition of enzymes [12]. However, mercury-tolerant bacteria exhibit multiple mechanisms to tolerate high concentration of Hg2+ in growth medium. Common Hg2+ resistance mechanisms involve over-expression of MerA, Hg2+-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 Hg2+ 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 Hg2+ 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 HgCl2 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 Hg2+ tolerant bacteria by growing them in LB liquid medium containing increasing concentration of HgCl2 up to 250 μM. A single strain was isolated that possessed a minimum inhibitory concentration (MIC) of 250 μM HgCl2 and was identified by 16S rDNA sequencing using forward and reverse primers AGAGTTGATCCTGGCTCAG and TACGTTACCTTGTAGCAGT respectively at AgriGenome Labs Pvt. Ltd, Kerala, India. The sequence was matched to the existing bacterial 16S rDNA sequences in NCBI database 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 Hg2+ 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 spectrophotometer (Model 2202, Japan). Lipase 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 depletions of 6.6 mM H2O2 in 3 ml assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl).

**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 lysate containing 1 mg protein was quantitated by monitoring the time dependent depletions of 6.6 mM H2O2 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 HgCl₂ [20]. Briefly, MerA activity was quantitated by measuring Hg²⁺-dependent oxidation of NAD(P)H to NADP⁺ resulting in depletion of absorbance of NADPH (A₄₅₀). Initial rate of NAD(P)H oxidation was determined in the first 10s, when A₄₅₀ 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 PO₄ and para-nitrophenol (PN) that has absorption maxima at 405 nm [21]. Briefly, time-dependent increase in absorption of PN (A₄₀₀) 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 OD₆₀₀=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 x g for 5 min and A₄₉₀ of the supernatant was determined by measuring the absorbance of the residual CR in the resuspension buffer.

Determinant 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 HgCl₂ were uniformly plated at OD₆₀₀ 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 in the 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 HgCl₂ 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 HgCl₂ up to 250 μM, prolonged the lag phase up to 32 h, leading to
saturation phase of more than 48 h. However, no growth was observed at 300 μM HgCl₂ (Figure 2). Incubating the cells till 72 h in 300 μM HgCl₂ didn’t result in growth of cells, making the minimum inhibitory concentration (MIC) > 250 μM and < 300 μM.

Hg²⁺ 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 Hg²⁺ 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 Hg²⁺ 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 Hg²⁺ in growth medium of the *K. pneumoniae* isolate towards its sensitivity to 250 μg/ml ampicillin. The results show that increasing doses of HgCl₂ 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 HgCl₂ in growth medium. These results indicate that HgCl₂ 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 HgCl₂. This concentration is 5-10 times the concentration normally used to inhibit wild type *K. pneumoniae*. These data show that HgCl₂ increases ampicillin tolerance of *K. pneumoniae* isolate.

Hg²⁺ 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 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 HgCl₂ in growth medium on EPS production by the *K. pneumoniae* isolate. Hg²⁺ 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 Hg²⁺, showing that EPS secretion is saturable around 200 μM HgCl₂ in growth medium. These results indicate the probable involvement of an enzymatic mechanism that is activated by HgCl₂ 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 HgCl₂ in growth medium. This finding shows that the bacterium is adapted to secrete elevated level of catalase woobing
MerA was increased to 3 folds in presence of 200 µM HgCl₂. This indicates the elevated concentration of Hg in its environment. However, inducibility of MerA activity shows that exogenous HgCl₂ is required in bacteria for phosphate assimilation. We observed 22 units of ALKP activity/mg of total protein in cytosol of the K. pneumoniae isolate. MerA is the primary enzyme expressed in response to Hg toxicity that converts ionic mercury (Hg²⁺) to the less toxic form, metallic mercury (Hg⁰). Our results show that the strain exhibits high HgCl₂ tolerance by over expressing MerA that accelerates the conversion of more toxic ionic mercury (Hg²⁺) in the growth medium to less toxic metallic mercury (Hg⁰) 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 HgCl₂ in the growth medium up to 200 µM. However, a 13% depletion of ALKP activity was observed at 250 µM HgCl₂. 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-amylase 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 Hg²⁺ up to 250 µM. However, no significant alteration in lipid degrading activity was observed in presence of exogenous HgCl₂. These results indicate that the Hg²⁺ 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 Hg⁰⁺, we measured their activity in cell-free lysate of the K. pneumoniae isolate grown in LB in presence of Hg⁰⁺ 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 HgCl₂ up to 250 µM. These results show that the bacterium exhibits acute Hg²⁺ regulatory mechanism to reduce cytosolic Hg²⁺ 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 Hg²⁺ 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 Hg⁰⁺ 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 Hg⁰⁺ induced biochemical adaptation of the K. pneumoniae isolate by determining its Hg²⁺-dependent antibiotic tolerance and production of common enzymes. Our study shows that the bacterium exhibits a minimum inhibitory concentration of >250 µM HgCl₂ in its growth medium. However, at 300 µM HgCl₂ 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 HgCl₂ 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 Hg⁰⁺ 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 Hg²⁺-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 Hg²⁺-mediated covalent modification of transpeptidases and transglycosylases that are the targets of ampicillin. Ampicillin-targeting sites of above proteins are proposed to be periplasmic transglycosylases that are the targets of ampicillin. Ampicillin-assay buffer activity of all five enzymes in presence of 250 µM HgCl₂ lysates. Our results show that there was a 30-40% depletion in the activity of all enzymes in presence of 250 µM HgCl₂ observed when the bacterium was grown in presence of 200-250 µM HgCl₂. 40% increase in EPS content was observed when the bacterium was grown in presence of 200-250 µM HgCl₂ (Figure 4). EPS is negatively charged and acts as a trap to immobilize cationic Hg²⁺ 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.

Alternatively, formation of EPS in K. pneumoniae reduces diffusion of ampicillin into the cells, hence, reducing its effective concentration in bacterial cells [14]. Hg²⁺ 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 Hg²⁺ 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 HgCl₂ (Figure 4). EPS is negatively charged and acts as a trap to immobilize cationic Hg²⁺ 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 Hg²⁺-tolerant K. pneumoniae strain, we quantitated production of five common industrially significant enzymes in the bacterium and investigated the effect of exogenous Hg²⁺ 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 Hg²⁺ had no effect on activities of catalase, ALKP and lipase, indicating that the bacterium is adapted to high concentration of Hg²⁺ in its natural habitat and elevated Hg²⁺ 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 HgCl₂ in growth medium, indicating that the bacterium produces more MerA to accelerate reduction of more toxic Hg²⁺ to Hg⁰, that is much less toxic to the cell [31]. Hg⁰ 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 Hg²⁺ only marginally affects its production or activity.

In order to evaluate the sensitivity of above enzymes to Hg²⁺, we measured the effect of Hg²⁺ 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 HgCl₂ in the assay buffer (Figure 5A and 5B). These results indicate that Hg²⁺ inhibits the activities of above enzymes, possibly, by covalent modification of sulfhydryl (-SH) groups of enzymes [12]. Further, in metalloenzymes, Hg²⁺ is known to displace the metal cofactor, leading to their inhibition [32]. Our results show that the enzymes exhibit sensitivity towards Hg²⁺, however, the bacterium possesses biochemical adaptation for acute regulation of Hg²⁺ in its cytoplasm.

**Conclusion**

In conclusion, we have isolated and biochemically characterized a novel K. pneumoniae strain that tolerates 250 µM HgCl₂. Hg²⁺ 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 Hg²⁺ 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 Hg²⁺ as well as production of industrially important enzymes.

**Acknowledgement**

We gratefully acknowledge Department of Science and Technology, Govt. of Odisha for funding the present work. H.G. Behuria acknowledges DST, Govt of India for providing Inspire fellowship.
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