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Multi Metal Resistant *Klebsiella pneumoniae* KW is an Efficient Copper Accumulator and Bioremediator of Industrial Waste Water

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ABSTRACT

Increasing concentrations of different essential and non-essential metals in the environment have posed a serious threat to all kinds of life. Bioremediation is considered as the best possible solution among the existing ones to reduce this kind of pollution. In the current study, copper resistant *Klebsiella pneumoniae* strains KW and CC were investigated for their bioremediation potential. Maximum uptake of Cu^{++} by mid log phase cultures of KW and CC was 19.26 and 28.77µg per mg cell dry weight, respectively. The strains also exhibited efflux ability; KW possessed more efficient efflux system as it expelled 94.46% of maximum stored copper within 24 h as compared to 56% by CC. These strains were also found substantially resistant to some other toxic heavy metals generally present in the industrial effluents. These strains exhibited good growth in the wide range of pH (5-10) and temperature (25 -44°C). KW and CC were also investigated for their resistance potential against various antibiotics. KW was found to be resistant to broader range of antibiotics as compared to CC. All these characteristics *viz*., high tolerance against Cu^{++}, excellent Cu^{++} uptake and efflux abilities, remarkable multi metal resistance potential and tolerance against substantial ranges of temperature and pH make *K. pneumoniae* KW an efficient tool for cleaning Cu^{++} rich industrial effluents.

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Key words
***Klebsiella pneumoniae*, Copper uptake and efflux, Bioremediation, Metal resistant bacteria**

INTRODUCTION

Environmental pollution is a serious threat for mankind. Debris from mining and milling, liquid waste containing pesticides, radioactive waste containing fission fragments and mainly effluents from various engineering and chemical industries are major contributors of anthropogenic emissions of heavy metals to the environment (Singh *et al*., 2017). Metals like as, Pb, Mn, Fe, Cd, Hg, Cu, Fe, Zn and Cr are used for number of medical and industrial applications. Copper has been used as an antifungal and antimicrobial agent in agriculture. It has also been used in hospitals to reduce the infections caused by bacteria (Hobman and Crossman, 2015). Whereas Zn^{++} and Cd^{++} are extensively used in batteries, electroplating, paints, pigments, topical creams, rechargeable cells and stabilizers (Hynninen, 2010; Orell *et al*., 2010; Outten and Munson, 2013).

Although heavy metals are an essential constituent of all organisms as they are required for various biological functions but when exceed in concentration, they become a threat for all forms of life including humans, animals, plants and microorganisms (Nies, 2003). Even the ppm level contamination of these metal ions can cause long-standing and incurable diseases (Tadesse *et al*., 2018). Excess of these metals damage cellular processes through generation of reactive oxygen species, reduction of various molecules, replacements of co-factors etc. All these lead to alteration in nucleic acids, proteins, oxidative phosphorylation and osmotic imbalance. They also interfere with the structures and functions of various enzymes by binding to thiol groups, and can even replace the prosthetic groups of those enzymes. They not only disturb the cellular functioning of cells but also accumulate in the cells and may ultimately lead to death of organism.

One way to avoid the toxic effects of such metals is to reduce their amount in the environment. Therefore, industrial effluents, that contain large amounts of metal compounds, must be treated for effective removal of highly toxic metals and/or conversion into a non/less-toxic species. Removal of toxic heavy metal ions from waste water can be performed by various techniques. These methods include chemical precipitation (Fu and Wang, 2011), coagulation and flocculation (López-Maldonado *et*

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al., 2014), electrochemical treatments (Aziz *et al*., 2008), ion exchange filtration (Hamdaoui, 2009), membrane filtration (Trivunac and Stevanovic, 2006), reverse osmosis (Chen and Chen, 2003) and electrodialysis (Kurniawan *et al*., 2006). In contrast to these chemical based methods, bioremediation has proven to be more efficient and cheaper way of removal of heavy metals or their reduction from more toxic to less toxic form (Sivaruban *et al*., 2014).

A number of remediation processes, used by bacteria that have acquired metal resistant genes in their genomes to reduce the toxic effects of metals, have been reported (Günther *et al*., 2012; Das *et al*., 2016; Dash *et al*., 2017). There is a need to explore and characterize the naturally occurring metal resistant bacteria for remediation purpose. In the present study two copper resistant strains of *Klebsiella* sp. were characterized for multi metal resistance, copper uptake and storage abilities, biochemical analysis and antibiotic resistance. Strains were grown in varying temperature and pH to find the optimum growth parameters. Minimum inhibitory concentrations were determined and growth curves were prepared to appreciate the response of bacteria to different concentrations of copper. Metal uptake ability was also measured to access the metal accumulation ability of particular strain. Antibiotic resistance profile and biochemical analysis were done to characterize the isolated strains.

MATERIALS AND METHODS

Stock solutions of metal salts

Stock solutions (50,000ppm) of copper, cadmium, zinc, lead and mercury were prepared in deionized water using their respective salts viz., $CuSO_4$, $CdCl_2$, $ZnCl_2$, $Pb(CH_3COO)_2$ and $HgCl_2$.

Bacterial strains

Two copper resistant *Klebsiella pneumoniae* KW and CC strains are used in this study. *K. pneumoniae* KW was isolated from an industrial source (effluent collected from Kot Lakhpat industrial area) and *K. pneumoniae* CC from a non-industrial source (Campus Canal, Lahore) (Zulfiqar and Shakoori, 2012). MIC of Cu^{++} for both strains was 6mM (380ppm).

Biochemical characterization

Biochemical characterization of *K. pneumoniae* KW and CC was carried out using bacterial miniaturized identification kits QTS-24 (DESTO Laboratories, Karachi, Pakistan) following manufacturer's instructions. The strains were tested for the detection of activitiy of enzymes: β-galactosidase, urease and catalase; decarboxylations of the amino acids arginine, lysine and ornithine; deamination of tryptophan; utilization of citrate and malonate as only carbon source; production of indole and hydrogen sulfide; detection of acetoin through butylene glycol pathway and gelatinase; fermentation of sugars (glucose, mannose, sorbitol, rhamnose, sucrose, raffinose, maltose, melibiose and arabinose); fermentation of inositol and adonitol; and reduction of nitrates to nitrites.

Antibiotics resistance potential

A total of thirteen antibiotic discs (ammoxillin, ampicillin, erythromycin, kanamycin, chloramphenicol, bacitracin, nalidixic acid, oxytetracycline, polymyxin B, streptomycin, tetracycline, vancomycin and neomycin) were used to check the antibiotic resistance potential of metal resistant *K. pneumoniae* strains. For this purpose, overnight cultures (150µl) of *K. pneumoniae* CC and KW were spread on LB agar medium in plates (100mm× 15mm) on which antibiotic discs (Thermo Scientific™ Oxoid™ Antimicrobial Susceptibility Disks), each containing 30µg of the antibiotic, were placed with the help of sterilized forceps. Plates were incubated at 37°C overnight. Diameter of clear zone around each disc was measured and results were recorded.

Multi metal resistance potential

K. pneumoniae strains were allowed to grow on LB agar medium supplemented with different concentrations of Cd^{++}, Zn^{++} and Pb^{++}. Any growth up to 48h was observed to determine resistance potential of the strain against respective metals.

Effect of copper on growth

In order to determine the effect of copper on growth of bacterial isolates, these were allowed to grow on LB medium supplemented with 100, 200 and 300ppm Cu^{++} and without Cu^{++} (Control). Each LB medium (50ml) in 250ml flask was inoculated with overnight culture in triplicates. The cultures were incubated at 37ºC with shaking at 100rpm. An aliquot (1ml) of culture was taken out at regular intervals of 2h (0, 2, 4, 6 and 8h). OD_{600} was measured against respective control (e.g., LB medium supplemented with 100ppm Cu^{++} was used as reference for measuring OD_{600} of *K. pneumoniae* cultures grown in the presence of 100ppm Cu^{++}). Optical density was plotted against the time of incubation of culture.

Copper bioremediation potential

Copper uptake ability of *K. pneumoniae* KW and CC was determined in the presence of various concentrations of Cu^{++}. Overnight culture of each strain was used to inoculate 100ml LB broth prepared in deionized water in 250 ml flasks and supplemented with 100, 200 and

300ppm Cu^{++}. LB medium without metal served as negative control. All cultures were allowed to grow at 37°C with shaking at 100rpm. From each culture, 2ml sample was taken out at the time of metal addition (0h), during log phase (3, 5 and 7h) and at 24h old culture. From each sample, 1ml was used to measure OD_{600} of the culture against respective control and 1ml (Vc) was used to determine Cu^{++} content in the cells. For this, it was centrifuged at 17540x g for 2 min. The pellets were washed with 0.9% saline and digested with 50µl conc. nitric acid. Volume was made up to 1ml (Vs) with deionized water. µg Cu^{++}/ml sample (R) was determined by taking readings through atomic absorption spectrometer (Thermo Unicam-SOLAAR) against normal segmented curve obtained from serially diluted standard Cu^{++} solutions. The instrument parameters used were 324.8nm emission wavelength with 0.5nm band pass with air acetylene flame. Copper uptake potential of *K. pneumoniae* strains was determined by dividing R and Vs with Vc, OD and Wt. per OD. Here Wt. per OD represents weight of dry cell pellet per ml cell culture where OD_{600} of the culture is 1. It was determined for each strain of *K. pneumoniae* under study. The strains were grown in LB medium for 5h. OD_{600} of these mid log phase cultures was measured and 400ml of each culture was centrifuged at 4000x g for 20min. Cell pellet obtained was dried at 65°C overnight. Wt. per OD of each strain was calculated by dividing weight of cell dry pellet (mg) with volume of the culture (400ml) and OD_{600} at the time of cell pellet harvesting.

Effect of temperature and pH on growth

Optimum temperature for the growth of metal resistant bacterial strains was determined by allowing growth of *K. pneumoniae* cells in LB medium (pH 7.0) at temperatures 25, 37 and 44°C. LB broth (100ml) was inoculated with 1ml overnight culture. Each flask was incubated at respective temperature at 100rpm in shaking incubator. For determining the effect of pH on growth of *K. pneumoniae*, the cells were allowed to grow in LB medium at pH 5, 7, 8 and 9. Overnight culture (1ml) was added in flasks each containing 100ml LB broth with varying pH and incubated at 37°C and 100rpm in a shaking incubator. OD_{600} of all the cultures was measured at one-hour regular interval till 9h and plotted against respective time intervals. The duration of lag phase and growth rate in log phase represented the effect of temperature and pH on growth of *K. pneumoniae.*

RESULTS

Biochemical characterization

Table I shows the biochemical characterization of *K. pneumoniae* KW and CC. Both strains exhibited same characteristics including production of urea and H_2S, oxidation of sugars (glucose, mannitol, arabinose and sucrose), reduction of nitrates, utilization of carbon and nitrogen sources, metabolization of various compounds and catalase, oxidase and urease activities. However, KW exhibited lactose-fermentation ability in contrast to CC that was found to be a non-lactose fermenter as revealed by ONPG test.

Table I. Biochemical characterization of *Klebsiella pneumoniae* KW and CC.

Sr. #	Test	KW	CC
1	ONPG	+	-
2	URE	-	-
3	CAT	+	+
4	ADH	-	-
5	LDC	+	+
6	ODC	-	-
7	TDA	-	-
8	CIT	+	+
9	MALO	+	+
10	IND	-	-
11	H_2S	-	-
12	VP	+	+
13	GEL	-	-
14	GLU	+	+
15	MAN	+	+
16	SOR	+	+
17	RHA	+	+
18	SUC	+	+
19	RAF	+	+
20	MAL	+	+
21	MEL	+	+
22	ARA	+	+
23	INO	+	+
24	ADO	+	+
25	N2/NO3	+/-	+/-

+, activity; −, no activity; ONPG, ortho-Nitrophenyl-β-galactoside; URE, urease; CAT, catalase; ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; TDA, Tryptophan deaminase; CIT, citrate; MALO, malonate; IND, indole ; H2S, hydrogen sulfide; VP, Voges-Proskauer test; GEL, gelatinase; GLU, glucose; MAN, mannose; SOR, sorbitol; RHA, rhamnose; SUC, sucrose; RAF, raffinose; MAL, maltose; MEL, melibiose ARA, arabinose; INO, inositol; ADO, adonitol; N2/NO3, Nitrogen/ nitrates.

Antibiotic resistance

Growth of bacterial strains was tested in the presence of 13 antibiotics. Results revealed that KW was more resistant against antibiotics as compared to CC (Table II). KW showed resistance potential against ampicillin, bacitracin, chloramphenicol, erythromycin, nalidixic acid and vancomycin and varying degree of sensitivity against other antibiotics. CC showed resistance against chloramphenicol and vancomycin while it was sensitive to all other antibiotics used in this study.

Multi-metal resistance

K. pneumoniae KW was able to grow in the presence of high concentrations of some other heavy metals used in this study. MICs of Cd^{++}, Zn^{++}, Pb^{++} and Hg^{++} were found to be 450ppm, 650ppm, 3200ppm and 35ppm. MIC of Cd^{++} for *K. pneumoniae* CC was found to be 100ppm. This strain exhibited heavy growth in all concentrations of Zn^{++} (maximum concentration used was 1000ppm).

Table II. Sensitivity and resistance pattern of *Klebsiella pneumoniae* KW and CC in response to various antibiotics.

Antibiotic	*K. pneumoniae* KW	*K. pneumoniae* CC
Amoxicillin	S (11mm)	S (16mm)
Ampicillin	R	S (0.5mm)
Bacitracin	R	S (17mm)
Chloramphenicol	R	S (1mm)
Erythromycin	R	S (15mm)
Kanamycin	S (2mm)	S (15mm)
Nalidixic acid	R	S (14mm)
Neomycin	S (16 mm)	S (14mm)
Oxytetracycline	S (12 mm)	S (14mm)
Polynixin B	S (13 mm)	S (12 mm)
Streptomycin	S (11 mm)	S (15 mm)
Tetracyclin	S (13 mm)	S (15 mm)
Vancomycin	R	R

Growth curves

Growth patterns of *K. pneumoniae* KW and CC in the presence of varying concentrations of Cu^{++} and their comparison with those in the absence of Cu^{++} revealed effect of Cu^{++} on growth of *K. pneumoniae* strains (Fig. 1). It was found out that 100ppm Cu^{++} did not affect the growth of both *K. pneumoniae* strains. Cu^{++} (200ppm) caused a little non-significant decrease in the growth of CC. However, 300ppm Cu^{++} resulted in increased duration of log phase (1h to 2h) and significantly reduced growth rate of CC during log phase. In case of KW, 200ppm Cu^{++} caused visible decreased growth during log phase, though it did not affect duration of lag phase. However, a prolonged lag phase (4h) was observed in the presence of 300ppm Cu^{++} followed by decreased growth rate during log phase.

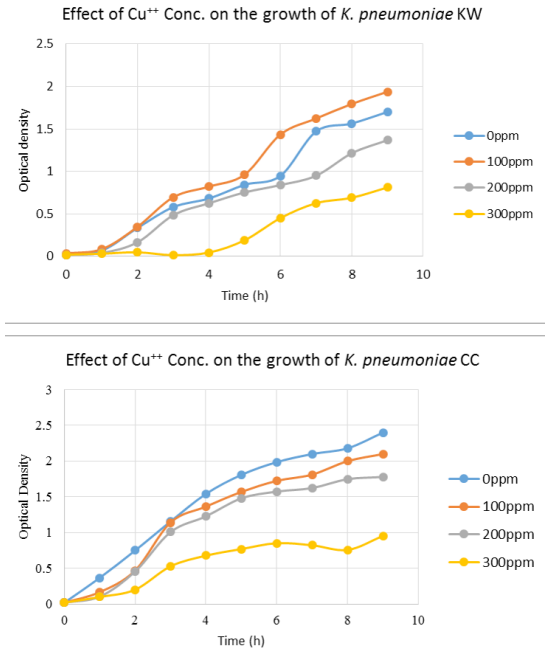

Fig. 1. Effect of presence of different concentrations of Cu^{++} in the medium on the growth pattern of *K. pneumoniae* KW and CC. 300 ppm proved to be detrimental for the growth of both strains.

Figure 2 reveals the effect of temperature on growth of *K. pneumoniae*. Bacteria exhibited maximum growth at 37°C. A prolonged lag phase was observed when bacteria were grown at 25°C after which rapid growth was observed in log phase comparable to that in 37°C. When bacteria were grown at 44°C, there was no delay in the lag phase and cells entered into exponential phase along with those grown at 37°C but a decreased growth rate was observed in log phase as compared to that at 37°C.

Klebsiella were grown in LB medium with varying concentrations of pH, i.e., 5, 7, 8 and 9. Although slight difference was observed in initial hours but as the bacteria grew further, they exhibited same growth pattern in all cases. pH of medium at every time point was also checked and it was found that with the growth of bacterial culture, pH of media also changed. In media with initial pH 5, 7 and 8 a gradual increase in pH occurred and all media attained

pH 8.8 ± 1which was optimum pH for its growth hence this experiment showed that bacteria had the capability to gradually change the pH of the medium (Fig. 2).

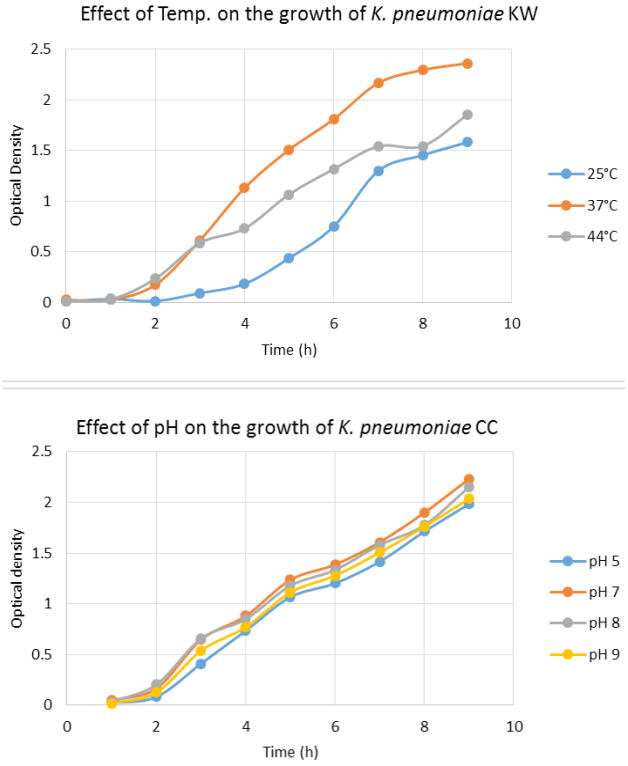

Fig. 2. Effect of temperature and pH on growth pattern of *K. pneumoniae* KW and CC. Optimum temperature for both strains was determined as 37°C. Both strains appeared capable to survive and grow in a wide range of pH.

Uptake of copper

Klebsiella strains grown in the presence of different concentrations of Cu^{++} revealed significant metal uptake potential (Fig. 3). During log phase, maximum Cu^{++} up-taken by KW was 19.26 and 15.62µg per mg cell dry weight in the presence of 200 and 300ppm Cu^{++} in the medium, respectively. At 24h post Cu^{++} addition, 1.07 and 1.20µg Cu^{++} per mg cell dry weight was found in the presence of 200 and 300ppm Cu^{++} in the medium, respectively. This data revealed that, 94.46 and 87.85% of Cu^{++} up-taken was effluxed within 24h. This remarkable efflux potential of KW makes it an efficient bioremediator of industrial waste water laden with high amounts of Cu^{++}.

Cu^{++} uptake study revealed CC a very rapid accumulator of Cu^{++}. At 3-7h post Cu^{++} addition, maximum Cu^{++} stored was 2, 3.4 and 28.77µg per mg cell dry weight in the presence of 100, 200 and 300ppm metal, respectively in the medium. Thus, a positive relation between concentration of Cu^{++} in the medium and amount up-taken by the bacterium was found. At 24h post Cu^{++} addition, 4.15, 4.98 and 12.54µg Cu^{++} was found per mg cell dry weight in the presence of 100, 200 and 300ppm Cu^{++} in the medium, respectively. This data revealed that 56% of Cu^{++} at 3-7h was found to be effluxed at 24h by CC in the presence of 300ppm Cu^{++} in the medium.

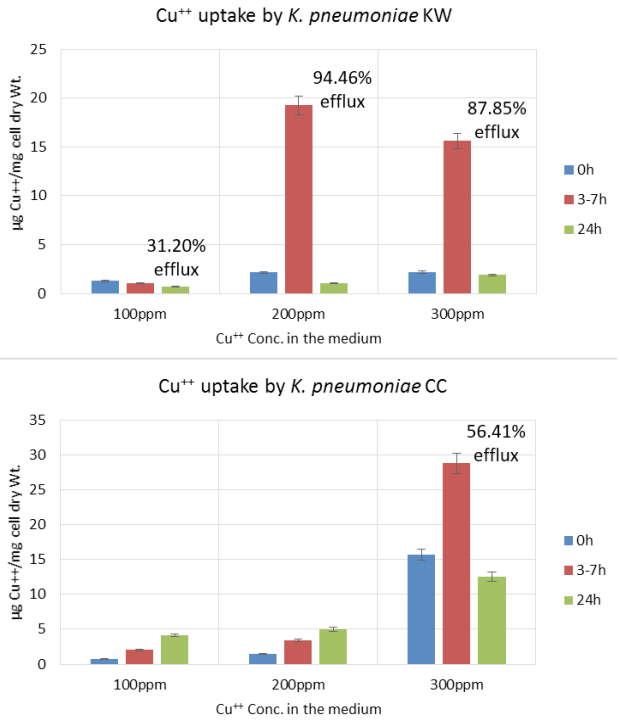

Fig. 3. Cu^{++} uptake by *K. pneumoniae* KW and CC was determined in the presence of 100, 200 and 300ppm Cu^{++} in the medium. For simplicity, only one uptake value (the maximum one) between 3-7h is shown in graphs. CC exhibited more Cu^{++} accumulation ability as compared to KW. However, KW was found to possess more efficient efflux system.

Effect of copper on the protein profile

The protein profiles of *K. pneumoniae* KW grown in the absence and presence of the metal were compared to observe the effect of metal. Cu^{++}, when added in the medium, significantly interfered the protein profile of the bacterium. Figure 4 shows that four types of variations were observed in protein profile of the cells grown in the presence of metal; (1) overexpression of a 45kDa protein, (2) low expression of a 33kDa protein, (3) disappearance or undetectable expression of proteins at 50, 30-35 and around 23kDa and (4) appearance of 150 and 55kDa proteins (otherwise absent in control).

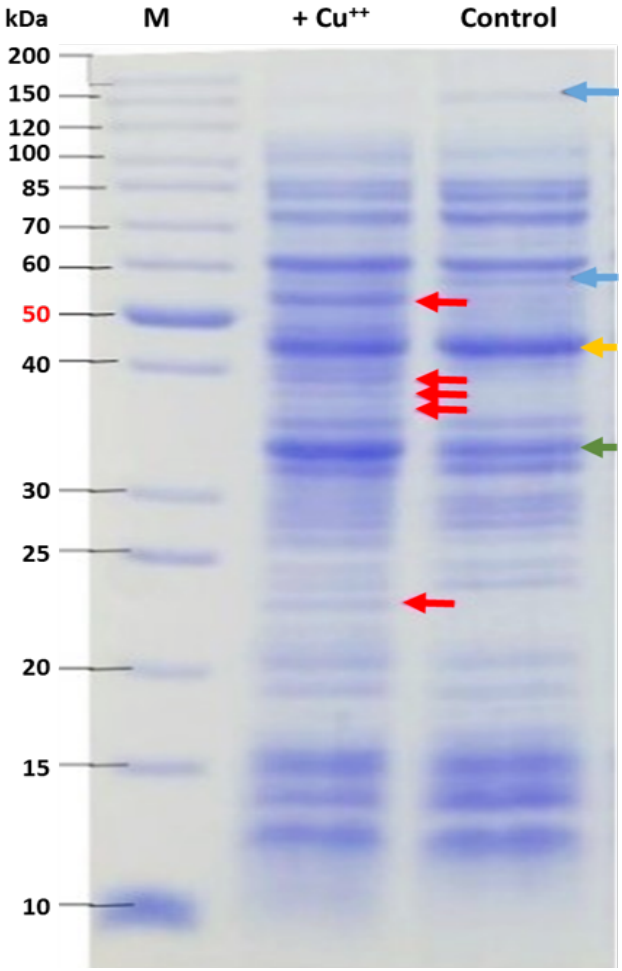

Fig. 4. Protein profiles of *Klebsiella pneumoniae* KW grown in the absence and presence of copper were compared. Overexpression of a 45kDa protein (yellow arrow), low expression of a 33kDa protein (green arrow), disappearance or undetectable expression of proteins at 50, 30-35 and around 23kDa (red arrow) and appearance of 150 and 55kDa proteins (blue arrow) as result of the presence of Cu++ were detected.

DISCUSSION

The industrialization and global mechanization have affected the environment drastically in many ways. Increase in metal-based pollutants is one of such affects. Bacteria and other life forms including algae, fungi, ciliates, mosses and macrophytes have developed many metabolic dependent and independent processes which play significant role in enabling these organisms to tolerate high concentrations of metals. These processes include biosorption, intra- and extra-cellular sequestration, enzymatic detoxification and efflux of metals (Gadd, 2000, 2004; Hynninen, 2010; Orell *et al*., 2010; Gupta and Diwan, 2017). These metal resistant microbes can be used as efficient tools to remove excessive toxic metals from environment through a process termed as bioremediation. Metal resistant naturally occurring bacteria have been isolated from metal rich environmental samples viz., industrial effluents and characterized by several research groups (Zulfiqar and Shakoori, 2012).

In the current study, Cu^{++} resistant *Klebsiella pneumoniae* strains isolated from Kot Lakhpat industrial area (*K. pneumoniae* KW) as well as non-industrial source *i.e*., Campus Canal, Lahore (*K. pneumoniae* CC) were used. MIC of Cu^{++} for both of these strains was 380ppm. Some studies have reported that bacterial spp. belonging to family Enterobacteriaceae are resistant upto different concentrations of Cu^{++}. These include *K. pneumoniae* and *E. coli* W3110 resistant upto 630 and 220ppm Cu^{++}, respectively (Grass and Rensing, 2001; Choudhury and Kumar, 1998). Some *Pseudomonas* spp. have been reported with MICs ranging from 12.6 to >200ppm Cu^{++} (Cooksey and Azad, 1992). It has been reported in several studies that gram negative bacteria are also able to tolerate high concentrations of other heavy metals. Some MICs reported are 40mg/ml Cd^{++}, 1.6mg/ml Cr^{++} and 2mg/ml Zn^{++} (Nies, 1992; Silver and Phung, 1996; Lin and Lin, 2005). In the current study, copper resistant *K. pneumoniae* KW has been proved to be a proficient tolerant against some other heavy metals also including Cd^{++}, Zn^{++}, Pb^{++} and Hg^{++}. *K. pneumoniae* CC exhibited great resistance potential against Zn^{++}. This strain showed substantial resistance against Cd^{++}.

Metal tolerant bacterial strains were also investigated for their antibiotic resistance potential. They showed varying degree of resistance against antibiotics. CC was resistant against chloramphenicol and vancomycin while KW was resistant against ampicillin, bacitracin, chloramphenicol, erythromycin and vancomycin. Thus KW was found to possess more resistance potential against antibiotics as compared to CC. In a study by Shakoori *et al*. (2010), it was reported that arsenic resistant *Citrobacter freundii* and *Bacillus anthracis* were sensitive to erythromycin, kanamycin, nalidixic acid and tetracycline while *Klebsiella oxytoca* was found to be resistant to these antibiotics. Teixeira *et al*. (2016) have also reported that metal resistant strains show higher level of resistance against various antibiotics.

Growth curves data showed that stains KW and CC grew well when Cu^{++} concentration was low. As the concentration increased, a decline was observed in bacterial growth. 300ppm was proved to be detrimental for the growth of both bacterial strains. Copper content in cells was determined in the presence of all these concentrations

i.e., 100, 200 and 300ppm Cu^{++} in the medium. Both CC and KW exhibited remarkable Cu^{++} uptake as well as efflux abilities. Time course study revealed that within 24h, CC expelled 56% of Cu^{++}, it accumulated earlier (28.77µg Cu^{++}/mg cell dry weight). Maximum uptake of Cu^{++} by *K. pneumoniae* KW was 19.26 and 15.62µg per mg cell dry weight in the presence of 200 and 300ppm Cu^{++} in the medium, respectively. Within 24h, *K. pneumoniae* KW cells expelled 94.46 and 87.85% of Cu^{++} back in the medium showing an efficient efflux ability.

High tolerance against Cu^{++}, excellent Cu^{++} uptake and efflux abilities, remarkable multi metal resistance potential and tolerance against substantial ranges of temperature and pH make *K. pneumoniae* KW an efficient tool for cleaning Cu^{++} rich industrial effluents.

Statement of conflict of interest

The authors have declared no conflict of interest.

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