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Dual Role of Multicopper Oxidase of *Klebsiella pneumoniae* as a Copper Homeostatic Element and a Novel Alkaline Laccase with Potential Application in Green Chemistry

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ARS conceived and designed the study and supervised the work. SZ, KS and ST performed the experimental work. KAAG helped in data analysis. ARS and SZ wrote the article.

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Copper detoxification, Multicopper oxidase, *Klebsiella pneumoniae*, Copper homeostasis, Alkaline laccase, *Cue* gene.

ABSTRACT

Copper though an essential element, poses serious consequences at elevated concentrations. The bacterial cell utilizes a battery of copper detoxification and exclusion mechanisms of which multicopper oxidase (CueO) is an integral component. In addition to acting as copper regulatory elements, CueOs have been shown to possess laccase activity. In this study, we have cloned and over-expressed the CueO of locally isolated *Klebsiella pneumoniae* KW strain. The CueO protein was purified to homogeneity by nickel affinity chromatography. Enzyme assays of CueO protein with phenolic substrates revealed its laccase activity. The kinetic studies showed Km value of 0.2µM, kKcat 0.68 S^{-1} and Kcat/km 1.2S^{-1} µM^{-1} for 2,6-Dimethoxyphenol (DMP) and Km value of 0.25mM, Kcat 300 S^{-1} and Kcat/Km=1200S^{-1}mM^{-1} for Syringaldazine (SGZ). Regulation of *cueO* in response to various concentrations of copper was studied at transcriptional level. Quantitative analysis through real time PCR demonstrated that the mRNA level increased enormously – up to 18.3 times - under copper induction. Time course study revealed a bimodal pattern of expression with two maxima, first at 15 min and second at 90 min exposure time. The role of CueO in copper detoxification as well as its laccase activity makes it suitable for biotechnological applications.

INTRODUCTION

CueO is a multi-copper oxidase and one of the integral components of *E. coli* copper homeostasis machinery controlled by cue R (Grass and Rensing, 2001, 2003). CueO after binding with cytoplasmic copper is translocated to periplasmic space via *Tat* translocation pathway (Bercks *et al*., 2000) which envisages its role of quenching excessive copper in cytosol, albeit it may be marginal. Its presence in periplasmic space is confirmed by a twin-arginine signal sequence and *p*-phenylenediamene oxidation activity in perplasmic fraction of *Campylobacter jejuni* (Hall *et al*., 2008). Together with copper translocating ATPase A (CopA), CueO is found to be primary defense against toxic accumulation of copper in *C. jejuni* and mutant of any of these genes renders the strain hypersensitive to copper. CueOs from various sources have demonstrated ability to oxidize phenolic substrates thus dubbed as "laccase like enzyme". Laccases (EC 1.10.3.2) are oxidoreductases, which catalyze the monoelectronic oxidation of substrates at the expense of molecular oxygen have been shown to play diverse role in various organisms ranging from antibiotic biosynthesis, structural part of spore (Martins *et al*., 2002), development (Endo *et al*., 2002) and oxidation of manganese (Francis and Tebo, 2001).

Roberts *et al*. (2002) studied the crystal structure of CueO of *Escherichia coli*. They proposed a reaction mechanism involving excitation of electron from substrate coupled to reduction of molecular oxygen to water. In addition to its role in copper homeostasis, CueO has also been found to oxidize iron (Grass and Rensing, 2001; Kim *et al*., 2001) which further complicates its exact role in periplasmic space. Outten *et al*. (2001) has demonstrated that CueO converts Cu^{+1} to less toxic Cu^{+2}.

Due to inherent oxidase activity of CueOs, several significant efforts have been undertaken to determine biotechnological and green chemistry applications of laccases (Riva, 2006). Interests in these enzymes have grown significantly in recent years. Their uses span

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from textile to the pulp and paper industry, and from food applications to bioremediation processes. These enzymes have also been used in organic synthesis, where their typical substrates are phenols and amines and their products are dimers and oligomers derived from coupling of reactive radical intermediates. Enzymes can also serve as biomarkers for early detection of pollution during bio monitoring programs (Bano *et al.,* 2017). In the present study, we report the laccase activity of CueO of a local isolate *Klebsiella pneumoniae* KW. *cueO* gene was cloned and over expressed in *E. coli* and purified after refolding. The laccase activity was assayed with model substrates DMP (2, 6-dimethoxyphenol), SGZ (syringaldazine) and ABTS (2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid)).

MATERIALS AND METHODS

Strain and reagents

Klebsiella pneumoniae KW (Zulfiqar and Shakoori, 2012) isolated from industrial effluents of Kot Lakhpat, Lahore was used in this study. All reagents and kits were purchased from Thermoscientific© except ABTS, DMP and SGZ which were purchased from Sigma©. This culture has been deposited in First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences (IAGS), University of the Punjab, Lahore under accession number FCBP-WB-0687.

Cloning of cueO *of* Klebsiella pneumoniae *KW*

cueO gene of *K. pneumoniae* KW was amplified using the following gene specific primers; forward primer, CueO-F: 5'TTGACCTTCCCGTTACGG (T_m=64.0°C) and reverse primer, CueO-R: 5'GTTCCGTCCTTCTTCCC (T_m=63.0°C). The primers were designed using primer 3.0 online software and their properties were checked using 'OligoCheck' program.

The 50µl amplification reaction mixture consisted of 1x PCR buffer (75mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$ and 0.01% tween 20), 0.125mM dNTPs mixture, 1.5mM magnesium chloride, 100 pmoles of each forward and reverse primer, 2.5 units of *Taq* DNA polymerase (Fermentas Cat # EP0402) and 100ng of template (genomic DNA). The PCR thermal cycle comprised initial denaturation at 95°C for 5 min followed by 35 cycles, each of denaturation at 95°C for 1min, annealing at 58°C for 1 min 30 sec and extension at 72°C for 2min with final extension at 72°C for 10 min. The PCR product after sequencing was submitted to NCBI (accession number: AB772008.1) and then inserted into pTZ57R/T vector with 3:1 ratio of pmole ends. The competent *E. coli* DH5α cells were transformed with the Recombinant DNA. Positive transformed colonies were screened on agar medium supplemented with 100 µg/ml ampicillin, 133 µM IPTG and 27 µg/ml X-gal.

Transformation with desired orientation of *cueO* in the vector was confirmed through PCR with M13 Forward primer and gene reverse primer CueO-R and also through restriction analysis with *Kpn* I and *Hin*d III. The cloned DNA fragment of 1.6kb of *cue*O was further confirmed through sequencing using M13 forward and M13 reverse primers. Additional primers (CueO-F2: TACTAACCAGCTGGCGGAAG and CueO-R2: GGCGTTATGGAAATCAAAGC) from the internal region of the gene were also used to obtain full length sequence of *cueO* gene of *K. pneumoniae* KW.

The above recombinant vector pTZ57R-*cueO* was used as template to amplify *cueO* using *cueO* expression primer (Forward) 5'CATATGCAACGTCGAGACTTC3' having *Nde* I site and reverse primer CueO-R. The DH5α cells were transformed with recombinant DNA comprising pTZ57 and 1.6 Kb amplification product. pET28a was used as expression vector, so that the expressed CueO will have a His-tag on its N-terminus. The so far cloned construct *pTZ57-cue*O as well as *pET*28a (expression vector) were digested with *Nde*I and *Eco*RI in order to produce complementary sticky ends. The two digested products (Vector to construct ratio of 1:5 pmole ends) were ligated followed by transformation of DH5α cells with this recombinant DNA. The construct *pET*21-*cue*O was purified from DH5α and BL21 cells (expression host), made competent with ice cold $CaCl_2$, were transformed with this vector for over expression of protein.

Expression and purification of CueO

For expression of CueO, *E. coli* BL21 cells (expression host) were transformed with pET28-cueO. The positive transformants were grown in the presence of selection marker kanamycin (60µg/ml). For overexpression of CueO, inducer was added in mid log culture. The expressed CueO was observed on 12% PAGE. The growth conditions were optimized for inducer concentration, over a range of 0.01-1.5mM IPTG at 37°C. Expression level was also observed under different post-induction incubation periods over a range of 0.01-1.5mM IPTG at 37°C. For soluble expression of CueO, transformed BL21 cells were grown in 200ml LB broth (supplemented with 60µg/ml kanamycin), until OD_{600} 0.6, IPTG (0.85mM) was added and incubated at 20°C overnight. The cells were harvested by centrifugation at 2010xg for 10 min at 4°C and re-suspended in solution A (0.5M NaCl in 20mM Tris-Cl, pH 8.0). The BL21 cells were lysed by sonication and soluble fraction of cell lysate

was collected for purification of His-tagged CueO through nickel affinity chromatography. The Ni-affinity resin was equilibrated with Solution B (5mM imidazol in solution A). The CueO protein was eluted with 250mM imidazol in solution A. The protein estimation was carried out through Bradford assay. Protein purity was measured at every step on 12% SDS-PAGE and activity was checked by staining on gel using various substrates.

Kinetic studies

Laccase activity of CueO protein was measured using SGZ, ABTS and DMP as substrates according to Palmieri *et al*. (1997). Shimadzu UV/Visible (BioSpec 1601) was used to measure absorbance at 470nm, 570nm and 420nm for DMP (ε = 14,800 M^{-1} $cm^{-}1$), SGZ (ε = 65, 000 M^{-1} cm^{-1}) and ABTS (ε = 36, 000 M^{-1} cm^{-1}), respectively. Laccase activity of CueO for each substrate was measured over a range of pH and then at optimized pH different concentrations of the respective substrates were used to characterize the enzyme. Activity assays were performed in Tris-Cl, pH = 4.0 -8.0 for DMP (0.05mM – 0.20mM), Mcllvaine buffer pH = 6.00 – 7.5 for SGZ (1 – 5 µM) and acetate buffer (pH 3-5.5) for ABTS (0.01-0.5mM) with 0.08mM $CuSO_4$ (also optimized) used routinely. Activity is defined as units of enzyme per milliliter of enzyme solution whereas 1U is equal to 1µmol of substrate oxidized per minute. Michaelis-Menten parameters were derived from Lineweaver-Burk Plot reproducibly with 3% deviation. CueO requires a metal ion as co-factor in order to perform its activity. Various ions (Ag^{+1}, Zn^{+2}, Ni^{+2} and Mg^{+2}) were tested as potential co-factors in addition to Cu^{+2} for CueO activity

Native gel assay for CueO activity

Activity of CueO protein towards representative phenolic substrates (DMP) was measured on native PAGE.

Crude extract and purified CueO protein were run on 12% native polyacrylamide gel. The gel was placed on agar (prepared in Tris-Cl, pH 8.0 buffer system) layer supplemented with 1mM DMP and 0.08mM Cu^{+2} ions at 37°C for 10 min. The oxidized product which appeared in form of a colored spot was photographed.

Determinatioin of regulation of cueO *in* K. pneumoniae *at transcriptional level*

Regulation of *cueO* in *K. pneumoniae* KW was studied at transcriptional level. mRNA level was determined in the presence of various concentrations of Cu^{+2}. In two hours old culture, Cu^{+2} was added with the final concentration of 1 to 5mM. In control, cells were grown in the absence of Cu^{+2}. Total RNA was isolated 15min post Cu^{+2} addition through guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Time course study was also performed when 2 h old culture was given a stress of 4mM Cu^{+2} for 30, 45, 60, 90 and 120min followed by RNA isolation. To avoid any traces of DNA, total RNA isolated was treated with DNase I (Fermentas Cat # EN0521).

First strand cDNA was synthesized from 2µg DNase treated total RNA using Thermo Scientific Maxima H Minus First Strand cDNA Synthesis Kit (Cat # K1652) containing M-MuLV Reverse Transcriptase according to manufacturer's instructions.

Real time PCR

Real time PCR was performed for *cueO* along with a gene *Gyrase* subunit A, a constitutively expressing gene used as internal control. PCR reaction mixture (20 µl) contained 1x Maxima™ SYBR Green qPCR Master Mix (Fermentas Cat # K0221), 1 µM each forward (5′ CCCTGAATGCCACTACCTG 3') and reverse primer (5′ TCCGCCAGCTGGTTAGTAAT 3'), 2 µl template (10x diluted cDNA). Thermal cycling consisted of 95ºC for 10 min, followed by 40 cycles, each of 95ºC for 15 sec, 60ºC for 1.0 min and final extension at 72ºC for 1.0 min. To verify the specificity and identity of the PCR product, melt curve analysis was performed between 60ºC and 90ºC with reading after every 0.5 sec.

The relative change in expression (*n*-fold) was calculated as the relative quantity of the *cueO* gene transcripts in the presence of various concentrations of Cu in relation to transcripts under the control condition (0mM Cu^{+2}) using Pfaffle method (Pfaffle, 2001).

Computational study

Prosite Scan applied to protein sequence of CueO produced the highly probity regions found to be conserved throughout all multi-copper oxidases gene sequences isolated from all lines of species. A phylogenetic analysis (CLUSTAL-W) was applied to confirm the above notion. A hydropathic plot prediction was applied to predict the location of the CueO.

RESULTS

Over expressed and purified CueO protein

Cloned gene (1.6kb) of *K. pneumoniae* KW was inserted in pET 28a expression vector at *Nde* I and *Eco* RI sites. CueO protein with a His-tag on its N-terminus was expressed in BL21 cells, transformed with Recombinant pET 28a vector and later grown in LB medium at 37°C after IPTG induction. 60 KDa His-tagged CueO was

found to be expressed as insoluble form. Expression analysis with different IPTG concentrations revealed very good expression of CueO in all the samples induced with 0.05 – 0.15 mM IPTG. Protein expression remained constant after 6 h of induction. Thus 0.05 mM IPTG and 6 h were selected as optimum inducer concentration and induction period to have maximum expression of CueO. Although hydrophobicity analysis predicted the CueO protein to be hydrophilic but over expression in *E. coli* led to the formation of inclusion bodies. This may be due to aggregation of misfolded peptides. However, when transformed BL21 cells were grown overnight at 20 °C, the protein expressed partially in soluble form. The activity of purified CueO protein (Fig. 1A) towards representative phenolic derivative (0.2mM DMP supplemented with 0.08mM $CuSO_4$) measured on native gel showed protein to be active while during purification phases (Fig. 2B). Although hydrophobicity analysis predicted the CueO protein to be hydrophilic but over expression in *E. coli* led to the formation of inclusion bodies. This may be due to aggregation of misfolded peptides. Soluble expression of re-folded protein was needed. Transformed BL21 cells were therefore grown overnight at 19°C after 30 min heat shock (42°C) to achieve protein expression in soluble form The activity of purified CueO protein (Fig. 1A) towards representative phenolic derivative (0.2mM DMP supplemented with 0.08mM $CuSO_4$) measured on native gel showed protein to be active while during purification phases (Fig. 1B).

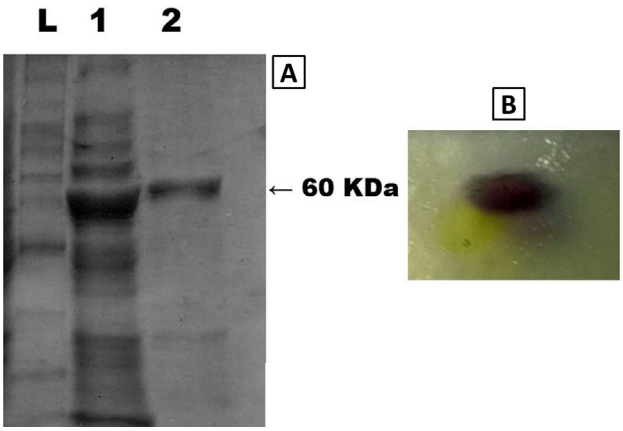

Fig. 1. A, Lane L, Novex unstained protein ladder, (10494-0/2); Lane 1, IPTG induced supernatant; Lane 2, Purified CueO protein. B, Zymogram for activity assay of purified CueO protein.

Enzymatic assays of CueO

CueO of *K. pneumoniae* KW was assayed for its laccase activity towards phenolic substrates (Fig. 2A). For DMP, CueO showed optimum activity in Tris-HCl buffer pH 8 at 37°C at 0.08mM Cu^{++}. The Michaelis-Menton plot for CueO activity using DMP as substrate gave K_m value of 0.2µmol, K_{cat} 0.68 S^{-1} and $K_{cat/km}$ 1.2$S^{-1}µM^{-1}$. The optimum activity of CueO using SGZ as a substrate was found to be in Mcllvaine buffer pH 6.5 at 37°C (Fig. 2B). The K_m value was found to be 0.25mM, K_{cat} 300 S^{-1} and $K_{cat/km}$ 1200S^{-1} mM^{-1}. There was no measurable activity of CueO detected with ABTS as substrate. No detectable activity of CueO was found with ions other than Cu^{+2} (Fig. 2B).

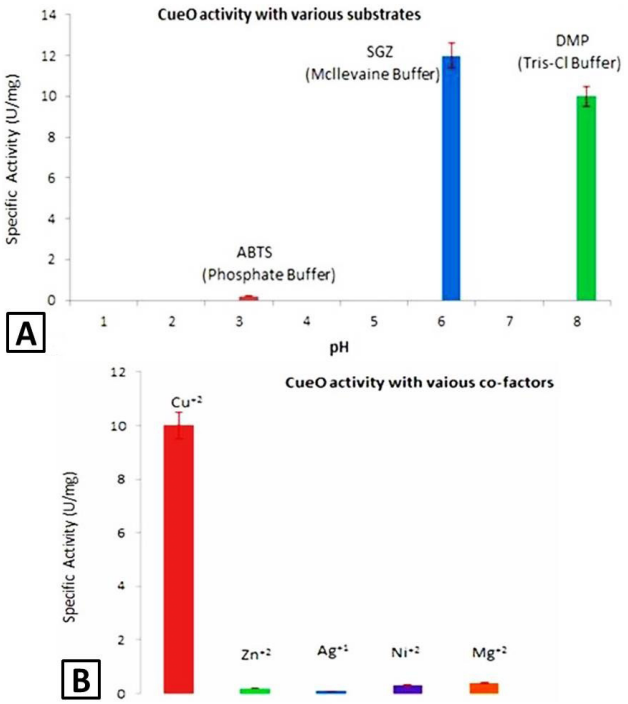

Fig. 2. CueO of *K. pneumoniae* KW is an active enzyme exhibiting laccase like activity. A, the enzyme activity was determined against some phenolic substrates in respective buffer systems across a range of pH. The optimum activity for SGZ and DMP was found at pH 6.5 and 8, respectively; B, CueO activity with various metal ions as co-factors depicted that Cu is the sole cofactor for CueO amongst the ones used in the experiment.

Molecular and physical properties of CueO protein

Theoretical calculations on CueO gave pI value of 6.23 and an average molecular mass of 57.79kDa. The amino acid sequence is rich in Alanine (10%), Glycine (10.3%), Leucine (11.1%), positively charged residues (9.1%) and negatively charged residues (10.5%) with extinction co-efficient of 54555 M^{-1} cm^{-1} and aliphatic index 82.56. Grand average of hydropathicity (GRAVY) -0.151 indicates this protein to be slightly hydrophilic

(Kytte and Dolittle, 1982). Moreover, computational tools could predict two transmembrane regions (residues 6-29, 61-81) (Fig. 3) of cueO with high degree of probity.

There was one transmembrane helical region (59-78) predicted using HMMTOP, Expasy proteomics tools. The PSORT program predicted the location of protein to be periplasmic in bacteria (localization score = 10) and presence of signaling peptide making us to assume that protein is translocated by some pathway to periplasmic space after its translation. This may be considered as additional way to quench copper in cytoplasm and transporting it out to periplasmic space in bacteria.

A multiple sequence alignment study revealed highly conserved regions inside CueO which have been preserved in multi-copper oxidases isolated from other sources. Here top 4 hits are compared to show highly conserved regions with overlapping amino acid residues (Fig. 4).

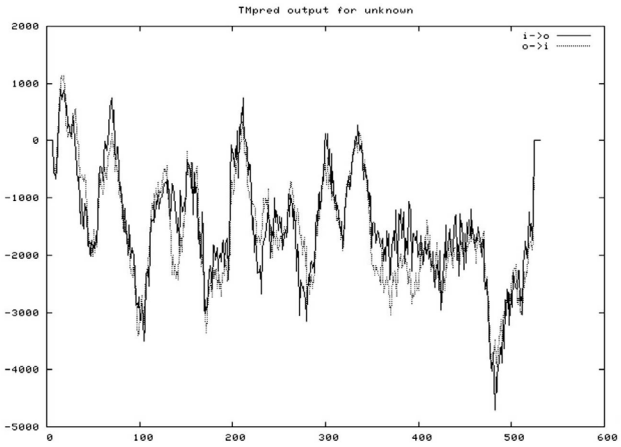

Fig. 3. Two transmembrane helixes predicted in CueO run inside to outside (i-o) and the other way as well.

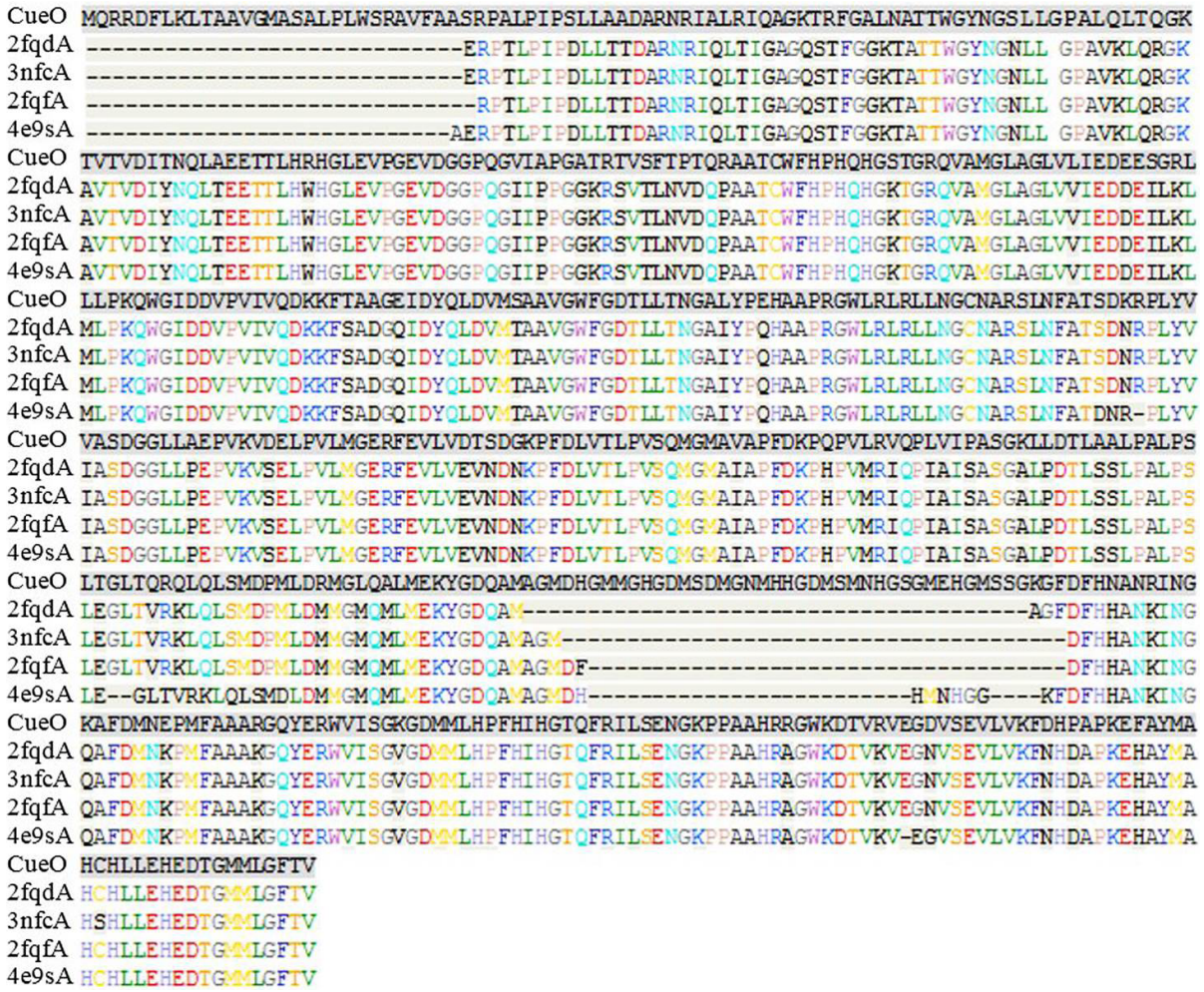

Fig. 4. BLAST search predicted sequence alignment reveals highly conserved regions between CueO and other multi copper oxidases reported in literature.

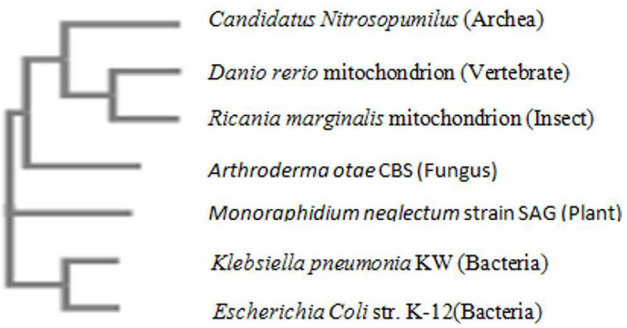

Fig. 5. The CueO core sequence has been conserved in all lines of species in evolutionary discourse which depicts its multiple functionality nature as reported in literature.

Signature patterns (in CueO) conserved in evolutionary tracts

PROSITE scan revealed the two signature patterns in protein sequence of CueO which have been conserved throughout the evolutionary process. A separate prediction tool was used to confine the conserved residues to fine tuned signature patterns prevalent in all multi-copper oxidases. The consensus pattern of multicopper oxidases signature 1 was: G-x-[FYW]-x-[LIVMFYW]-x-[CST]-x-{PR}-{K}-x(2)-{S}-x-{LFH}-G-[LM]-x(3)[LIVMFYW]. HCHLLEHEDTGM amino acid residues constitute copper binding site whereby H is covalently bounded. The consensus pattern of multi-copper oxidases signature 2 was: H-C-H-x(3)-H-x(3)-[AG]-[LM]. The first 2 H's are copper type 3 binding residues. The C, the third H, and L or M are copper type 1 ligands.

In addition to hits found for CueO matching multicopper oxidases/laccases, the hits revealed that the conserved sequence is also prevalent in proteins as blood coagulation factors V and VIII, Yeast FETIII involved in iron uptake, and yeast fission homolog SpAC1f7.08. An even more generic pattern of CueO conserved existence is found across the board in all living organisms groups as shown in the phylogenetic analysis (Fig. 5).

3-D structure of Cue protein

Apart from standard secondary structures in CueO, a large portion is in form of irregular shape peptide which might suggest a need to study its role in proper function of whole protein. I-TASSER program was applied to predict the CueO conformation features. Secondry structure of CueO is predicted to be comprising of helix regions, medium range strands and coils bordering the 3D conformation (Fig. 6A).

Computational studies also predicted the active site residues histidine and cystine which interact with Cu^{2+} trinuclear cluster (Fig. 6B).

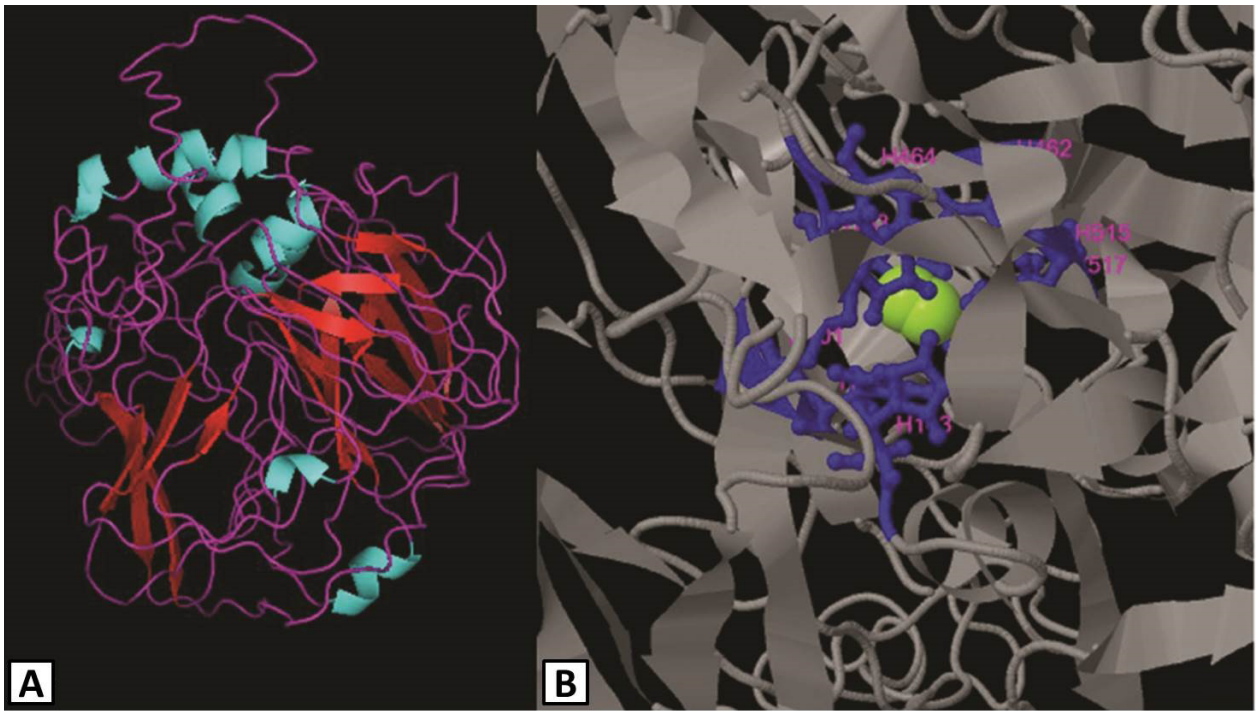

Fig. 6. A, I-TASSER predicted 3D model of CueO KW without copper centers. B, active centre microenvironment prediction of CueO. Green balls, trinuclear copper cluster; Blue labels, histidine residues coordinating with copper ion.

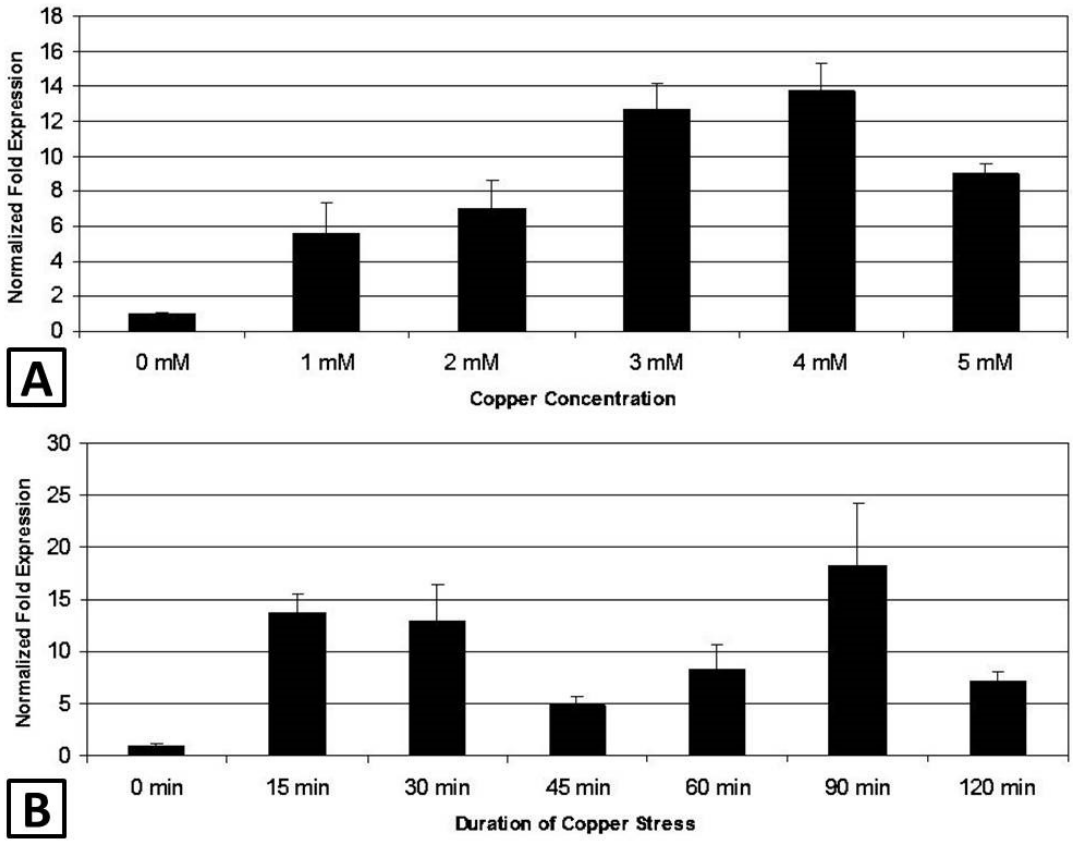

Fig. 7. Relative quantification of *CueO* mRNA level of *K. pneumoniae* KW in response to Cu^{+2}. A, bacterial cultures (20 ml) were grown and Cu^{+2} stress ranging 0 to 5mM was given at early log phase stage. Total RNA isolated was subjected to RT PCR. cDNA synthesized were used for real time PCR. RNA level in each sample normalized to *GyrA* (housekeeping gene) were quantified in relation to that in control (0mM Cu^{+2}). A gradual increase in *CueO* mRNA was observed up to 4 mM Cu^{+2} that decreased with further increase in medium Cu^{+2}; B, RNA level was measured with respect to Cu^{+2} exposure time in the presence of 4 mM Cu^{+2}. A bimodal increase in *CueO* mRNA level was found with two maxima, first at 15 and 30 min and second at 90 min.

Regulation of cueO *in* K. pneumoniae *at transcriptional level*

Quantitative analysis of *cueO* mRNA level through real time PCR revealed that it increases significantly in the presence of Cu^{+2} in the medium (Fig. 7). The *cueO* transcripts increased 5.6 and 7.0 fold in the presence of 1 and 2mM $Cu^{2+}$when the cells grew normal, while in the presence of 3 and 4 mM Cu^{2+} the lag phase is increased and the growth rate is decreased compared to the control culture. The *cueO* mRNA level increased 12.6 and 13.7 fold, which was almost two fold to that previous situation. In the presence of 5mM Cu^{2+} in the medium, the cell growth was drastically affected, and the mRNA level of C*ueO* showed 9.03 fold increase compared to the bacteria growing in the medium without Cu^{+2}. Though this n-fold increase is also very significant however it is lower than those when cells were given lesser Cu^{+2} stress (3 and 4mM).

Figure 7B shows the message level of CueO after exposure of *K. pneumoniae* to 4mM Cu^{2+} after every 15 min up to 2h. The mRNA level showed 13.7 and 13 fold increase, respectively, after 15 and 30 min of metal exposure. This was followed by a fall in mRNA level (4.9 fold increase) during the next 15 min. The message level rose afterwards once again – it showed 18.3 fold increase after 90 min of metal exposure Thus a bimodal distribution of *CueO* mRNA level was found with respect to Cu^{+2} exposure time.

DISCUSSION

CueO gene (NCBI accession No: AB772008.1) has been shown to have laccase activity. Laccases from various sources have been studied for their potential bioremediation applications in green chemistry and other biotechnological applications in industry. However, there have been constraints being faced by researchers in terms

of ease of production of laccase in high amounts and post-translational modifications. Overproduction of laccases in bacteria is thought to circumvent these issues faced with eukaryotic laccases. Therefore, there have been frequent studies reported on laccases of prokaryotic origin. This study aims to characterize CueO of *K. pneumoniae* KW for its laccase activity.

Theoretical studies gave molecular weight of *K. pneumoniae* KW CueO 57.79kDa however, on SDS-PAGE protein band appeared to be at 60kDa which might be due to the presence of additional His Tag attached to C terminus. Overproduction of protein might also contribute to mobility shifts on gel. Overproduction of protein is expected to overload the folding machinery of cell which leads to improper folding of protein. When cells are grown at low temperature, the protein is produced at low rate allowing it to have more time to correctly fold the protein. Growing at low temperature helped us to achieve more than fifty percent expression of CueO in soluble form.

The *K. pneumoniae* KW CueO enzyme is found to be an alkaline laccase in this study. It efficiently oxidizes DMP and SGZ at alkaline pH but there has been no detectable activity towards ABTS. This laccase activity is in coherence with some other studies (Ruijssenaars and Hartmans, 2004). CueO of *K. pneumoniae* KW is active at very narrow range of temperature (37$\pm$ 2^{o}C). However, there is further need to perform studies on different laccase sources (bacteria, fungi, plants) to achieve an enzyme with improved characteristics which is efficient at broad range of pH and temperatures for their potential applications in green chemistry.

In the presence of 1 and 2 mM Cu^{2+} in the medium, no significant deposition of metal ions was observed in the cells (Zulfiqar and Shakoori, 2012), indicating that copper resistance genetic determinants including *cue* and *cus* regulons are sufficient enough to protect cells from toxic effects of the metal. This is also reflected in the normal growth pattern of the bacterium. In the presence of 3 and 4 mM Cu^{+2} the level of *cueO* transcripts increased up to 12.6 and 13.7 fold compared to the control culture. In the presence of these sub lethal copper quantities, cells were found with copper stored in them, indicating that despite increased expression of copper resistant genetic determinants, cells are not fully able to get rid of excessive amounts of the metal, ultimately resulting in reduced growth of the culture. With further increase in Cu^{+2} *i.e.,* 5 mM, *cueO* transcript level was lesser than that in the presence of sub-lethal ones. It can be best explained through culture growth behavior. 5mM Cu^{+2} drastically affected culture growth and cells under severe stress had to shift their transcription machinery to some other side to survive at least.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Statement of conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

Bano, Z., Abdullah, S., Ahmad, W., Zia, M.A. and Hassan, W., 2017. Assessment of heavy metals and antioxidant enzyme in different organs of fish from farm, Hatchery and Indus River of Pakistan. *Pakistan J. Zool.*, **49**: 2227-2233. http://dx.doi.org/10.17582/journal.pjz/2017.49.6.2227.2233

Berks, B.C., Sargent, F. and Palmer, T., 2000, The Tat protein export pathway. *Mol. Microbiol.,* **35**: 260-274. https://doi.org/10.1046/j.1365-2958.2000.01719.x

Chomczynski, P. and Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162**: 156-159. https://doi.org/10.1006/abio.1987.9999

Endo, K., Hosono, K., Beppu, T. and Ueda, K., 2002. A novel extracytoplasmic phenol oxidase of *Streptomyces*: its possible involvement in the onset of morphogenesis. *Microbiology,* **148**: 1767-1776. https://doi.org/10.1099/00221287-148-6-1767

Francis, C.A. and Tebo, B.M., 2001. cumA multi-copper oxidase genes from diverse Mn(II)-oxidizing and non Mn(II)-oxidizing *Pseudomonas* strains. *Appl. environ. Microbiol.,* **67**: 4272-4278. https://doi.org/10.1128/AEM.67.9.4272-4278.2001

Grass, G. and Rensing, C., 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *FEMS Microbiol. Rev.,* **27**: 197-213. https://doi.org/10.1016/S0168-6445(03)00049-4

Grass, G. and Rensing, C., 2001. CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. *Biochem. biophys. Res. Commun*., **286**: 902-908. https://doi.org/10.1006/bbrc.2001.5474

Hall, S.J., Hitchcock, A., Butler, C.S. and Kelly, D.J., 2008. A multicopper oxidase (Cj1516) and a CopA homologue (Cj1161) are major components of the copper homeostasis system of *Campylobacter jejuni*. *J. Bact.,* **190**: 8075-8085. https://doi.org/10.1128/JB.00821-08

Kim, C., Lorenz, W.W., Hoopes, J.T. and Dean, J.F., 2001.Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli* yacK gene. *J. Bact.,* **183**: 4866-4875. https://doi.org/10.1128/JB.183.16.4866-4875.2001

Kataoka, K., Komori, H., Ueki, Y., Konno, Y., Kamitaka, Y., Kurose, S., Tsujimura, S., Higuchi, Y., Kano, K., Seo, D. and Sakurai, T., 2007. Structure and function of the engineered multicopper oxidase CueO from *Escherichia coli*- Deletion of the methionine-rich helical region covering the substrate-binding site. *J. mol. Biol.,* **373**: 141-152. https://doi.org/10.1016/j.jmb.2007.07.041

Kytte, J. and Dolittle, R. F., 1982. A simple method for displaying the hydropathic character of a protein. *J. mol. Biol.,* **157**: 105-132.

Li, Y.D., Gong, Z.J., Li, X., Li, Y. and Wang, X.G., 2011. Engineering *Klebsiella sp*. 601 multicopper oxidase enhances the catalytic efficiency towards phenolic sub-strates. *BMC Biochem.,* **12**: 30-38. https://doi.org/10.1186/1471-2091-12-30

Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones, G.H. and Henriques, A.O., 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *J. biol. Chem.,* **277**: 18849-18859. https://doi.org/10.1074/jbc.M200827200

Outten, F.W., Huffman, D.L., Hale, J.A. and O'Halloran, T.V., 2001. The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J. biol. Chem.,* **276**: 30670-30677. https://doi.org/10.1074/jbc.M104122200

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capasso, A. and Sannia, G., 1997. A novel white laccase from *Pleurotus ostreatus*. *J. biol. Chem*., **272**: 31301-31307. https://doi.org/10.1074/jbc.272.50.31301

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.,* **29**: 2002-2007. https://doi.org/10.1093/nar/29.9.e45

Riva, S., 2006. Laccases: Blue enzymes for green chemistry. *Trends Biotechnol*., **24**: 210-226. https://doi.org/10.1016/j.tibtech.2006.03.006

Roberts, S.A., Weichsel, A., Grass, G., Thakali, K., Hazzard, J.T., Tollin, G., Rensing, C. and Montfort, W.R., 2002. Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*. *Proc. natl. Acad. Sci., USA,* **99**: 2766-2771. https://doi.org/10.1073/pnas.052710499

Ruijssenaars, H.J. and Hartmans, S., 2004. A cloned *Bacillus halodruans* multicopper oxidase exhibiting alkaline laccase activity. *Appl. Microbiol. Biotech.,* **65**: 177-182. https://doi.org/10.1007/s00253-004-1571-0

Trevors, J.T. and Cotter, C.M., 1990. Copper toxicity and uptake in microorganisms. *J. indust. Microbiol.,* **6**: 77-84. https://doi.org/10.1007/BF01576426

Zulfiqar, S. and Shakoori, A.R., 2012. Molecular characterization, metal uptake and copper induced transcriptional activation of efflux determinants in copper resistant isolates of *Klebsiella pneumoniae*. *Gene*, **15**: 32-38. https://doi.org/10.1016/j.gene.2012.08.035