IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Expression and characterization of LlMTII protein from *Leucaena leucocephala* and metal stress tolerance in *E. coli* BL21 strain.

[1]Anjana Wahengbam, [2]Prashanth Bollempally, [3]Prashant Singam*

[1]Research Scholar, [2] Research Scholar, [3]Assistant Professor (Corresponding Author)

[1]Department of Genetics and Biotechnology,

[1] Osmania University, Hyderabad, India.

Abstract: A metallothionein Type II gene was previously isolated from *Leucaena leucocephala*, a leguminous species and cloned into pET28a(+) vector and moved into *E. coli* BL21 strain for protein expression. 0.1mM IPTG was used for induction of enhanced expression and total protein content was loaded onto 12% SDS gel. Purification was done using NI-NTA Affinity Column Chromatography and loaded onto 18% SDS gel. In the former, slight increase in band intensity was seen between 5-10 kDa whereas in the purified samples, bands were observed at 7-8 kDa which is congruent to the fact that the predicted molecular weight of the *Leucaena* MTII protein is 7.6 kDa. Different concentrations of Cu, Cd and As were used to analyze the efficiency of recombinant BL21 cells in combating heavy metal toxicity. OD values taken at intervals of 3, 5 and 7 hours and overnight shows marked decrease in growth rate of control non-recombinant cells whereas growth rate in the recombinant cells are consistently same for all. These findings can lead to the conclusion that heterologous expression of MTII gene from *Leucaena* in other living systems is highly effective in protection against heavy metal toxicity.

Index Terms **– Metallothionein, heavy metal toxicity, cloning, protein expression.**

I. INTRODUCTION

Metallothioneins (MTs) are a group of cysteine-rich proteins with approximately 65-80 amino acids and have a low molecular weight of only about 7-10 kDa. Metallothioneins has thiol residues which can bind a variety of metals. All the cysteine residues are confined to two domains- α and β domains separated by a linker. The major physiological function of metallothioneins is homeostatic regulation of essential metal ions like Cu and Zn in absence of stress conditions. Metallothionein also plays an important role in detoxification of toxic heavy metals (Loebus et.al., 2013). Plant metallothioneins belong to family 15 in the metallothionein classification which is again are of four types – type I, II, III and IV. The different types are categorized according to the arrangement of cysteine residues and expression levels in different tissues (Cobbett & Goldsbrough, 2002).

Heavy metal ions are not essential for plant growth and are toxic to living systems even in minute amounts, including cadmium (Cd), lead (Pb), and arsenic (As) among others (Gu et al., 2014). If present in excessive amounts, these heavy metals pose a serious threat to environment and human health. Accumulation of heavy metal ions in food crops will enter the human food chain posing a threat to human health (Cui et al., 2022). Owing to their ability to bind di-cationic metals, MTs play important roles in detoxification and maintaining heavy metal homeostasis (Kägi and Schäffer, 1988, Roesijadi, 1992). Plant MTs play important roles in maintaining the homoeostasis, detoxification of toxic metals, and protection against oxidative stress (Palmiter, 1998; Adams et al., 2011; Coyle et al., 2002; Wong et al., 2004). A type III metallothionein has

been isolated from *Cucumis sativus* and expressed in *E. coli* BL21 cells shows increased tolerance to Cu^{2+} and Cd^{2+} ions, specifically to cadmium (Xu et.al., 2018). Recombinant $PsMT_A$ shows high level of copper accumulation in *E.coli*. *CrMT* from *Canavalia rosea* increased tolerance of yeast to heavy metals (Zou et al., 2022). An MT gene *NtMT2F* from tobacco when overexpressed in *E. coli* and *Arabidopsis thaliana* showed increased tolerance to Cadmium stress (Li et al., 2023). *Quercus suber* type 2 plant MT provides metal tolerance to yeast strains susceptible to yeast strains(Mir et.al., 2004).

Previously, we have successfully isolated Metallothionein type II gene from *Leucaena leucocephala*, a sturdy, invasive species from the Fabaceae family well known for its phytoremedial properties (Wahengbam et.al., 2025). In this study, we have cloned the *LlMTII* gene into expression vector pET28a(+) and transformed *E. coli* BL21 cells in order to study the efficiency of this gene in resisting heavy metal toxicity.

II. RESEARCH METHODOLOGY

1. Preparation of vector and insert: Restriction sites of *BamHI* and *XhoI* were selected for the cloning of *LlMTII* into pET28a (+) expression vector. *BamHI* recognition site was added to MTII forward primer and *XhoI* recognition site to reverse primer. PCR was performed [(94℃,4 min), (94℃,30 sec), [(58℃,30 sec), (72℃, 30 sec), (72℃,4 min); 35 cycles], double restriction digestion of the amplicon was carried out with the above-mentioned enzymes. The digested products were run on 1% Agarose gel and eluted with Thermo Scientific™ GeneJET Gel Extraction Kit. Same digestion was carried out with pET28a (+), run on gel and eluted.

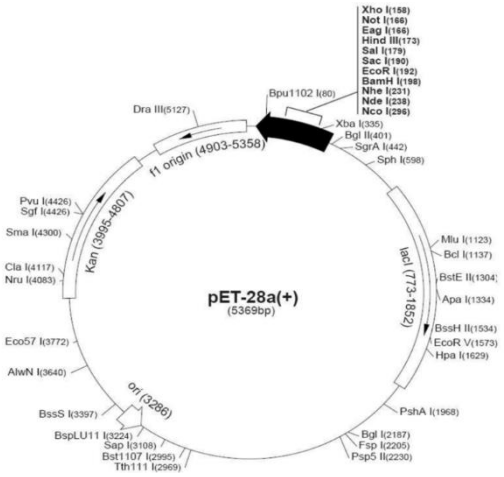


Fig 1: pET28a(+) vector map

2. Cloning of LlMTII gene into pEt28a vector: Ligation of vector and insert was done (10X Ligation buffer Vector-2µL, Vector-2µL, Insert-10µL, T4 DNA Ligase-10 µL) and incubated overnight at 4℃. Competent *E. coli* TOP10 cells were prepared using 100mM $CaCl_2$. The ligated sample was transformed into TOP10 cells via heat shock method (42℃). Transformed culture was spread onto LB plates (Kanamycin 50mg/L).

3. Confirmation of Recombinants: Single colonies that developed on Kanamycin LB plates were inoculated into LB broth**.** Colony PCR was done with MTII primers. Plasmid isolation of positive colonies via alkaline lysis method was done and clones were confirmed with PCR and restriction digestion.

4. Transformation into *E. coli* BL21 cells: The recombinant pET28a(+)*LlMTII* plasmid was transformed into BL21 culture via heat shock method. The transformation was confirmed by PCR and restriction digestion.

5. Total protein isolation and purification of metallothionein recombinant protein using NI-NTA Affinity Column Chromatography: The recombinant cultures were inoculated in LB broth (Kan 50mg/L) for 2.5 hours at 37°C. When the culture reaches 0.6 at OD_{600}, culture was induced with IPTG (final concentration 0.1 mM) for overexpression, carried out at 18°C for 14-16 h. After incubation, cells were harvested by centrifuging at 6000 rpm, 4°C for 30 min. 10 mL of chilled lysis buffer was added to per g wet weight of cell pellet and vortexed for resuspension. To every 10 mL cell suspension 100 µL of lysozyme stock (final concentration 100 µg/mL) and 1 µL of PIC was added and the suspension was incubated at room temperature for 30 min. Every 10 mL suspension was then lysed for 20 min using an ultrasonic probe sonicator. Cell suspension became almost transparent upon complete lysis. Prepared samples from different cell extracts were run on 12% polyacrylamide gel along with 6 µL of 10-250 kDa protein ladder. The gel was visualized using Coomassie Blue staining.

Recombinant protein was purified from supernatant obtained from dissolving insoluble fraction by **Immobilized Metal Affinity Chromatography (IMAC)** using Ni-NTA **(nickel-nitrilotriacetate) resin** in an empty gravity flow column. Ni-ions bind strongly to $(His)_6$ tag present at the N-terminal of the recombinant protein and. Purified recombinant protein was eluted out using 300 mM Imidazole in elution buffer pH 8.0. Four elutions were performed. Elution fractions were run on 18% polyacrylamide gel along with 6 µL of 3.5-245 kDa protein ladder (G2P). The gel was visualized using Coomassie Blue staining.

6. Heavy metal stress analysis of pET28a+: *LlMTII* recombinant *E. coli*: Different concentrations of $CdCl_2$ (0.5mM, 1.0mM, 1.5mm), As_2O_3 (250µM, 350µM, 450µM) and $CuCl_2$ (3mM, 5mM, 7mM) were added to LB broth (Kan 50mg/L) and inoculated with recombinant BL21 cells. 0.1mM IPTG was added for induction of protein expression and shaken at 37°C. Growth was measured in a spectrophotometer at OD600 at 3h, 5h, 7h intervals along with overnight growth.

IV. RESULTS AND DISCUSSION

1. Cloning and confirmation of recombinants: *BamHI* and *XhoI* digestion of pET28a+ was done, along with *LlMTII* (with restriction sites). Eluted samples of digested pet28a vector and insert were observed on agarose gel (Fig 2.). Transformation of *E. coli* TOP10 cells was confirmed (Fig.3). Transformation was confirmed with PCR (Fig 4) and restriction digestion with *BamHI* and *XhoI* (Fig 5).

Fig 2: Eluted samples of restriction digested samples of pET28a(+) vector and MTII gene
Lane 1: 1 kb ladder, Lane 2: Digested sample of pET28a(+), Lane 3&4: Digested samples of MTII

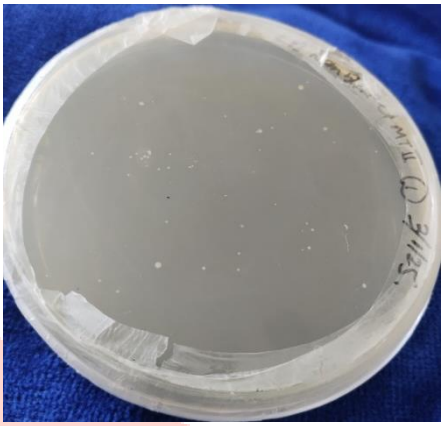


Fig 3: Single colonies on LB (Kan 50mg/L) plates

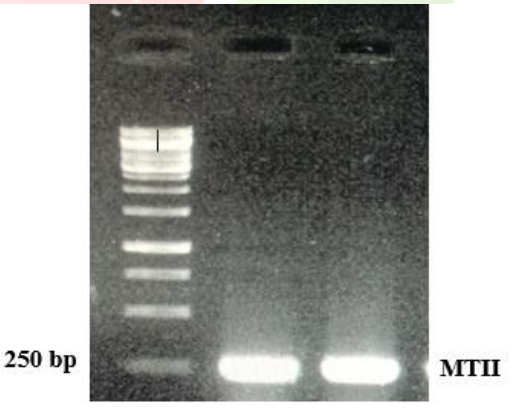


Fig 4: PCR confirmation of MTII with Plasmid isolated from single colonies as template. Lane 1: 1 kb ladder, Lane 2 & 3: MTII amplicons

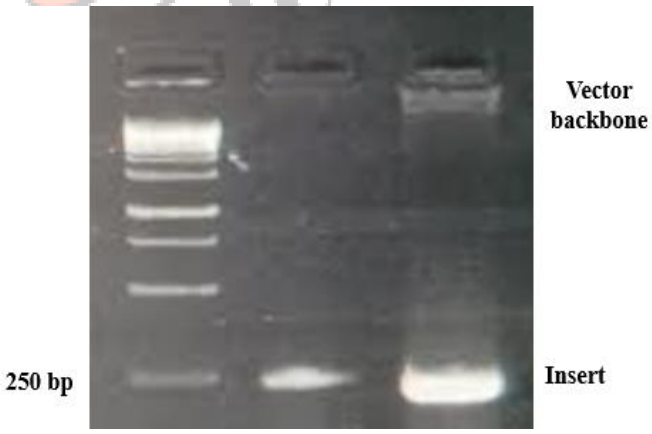
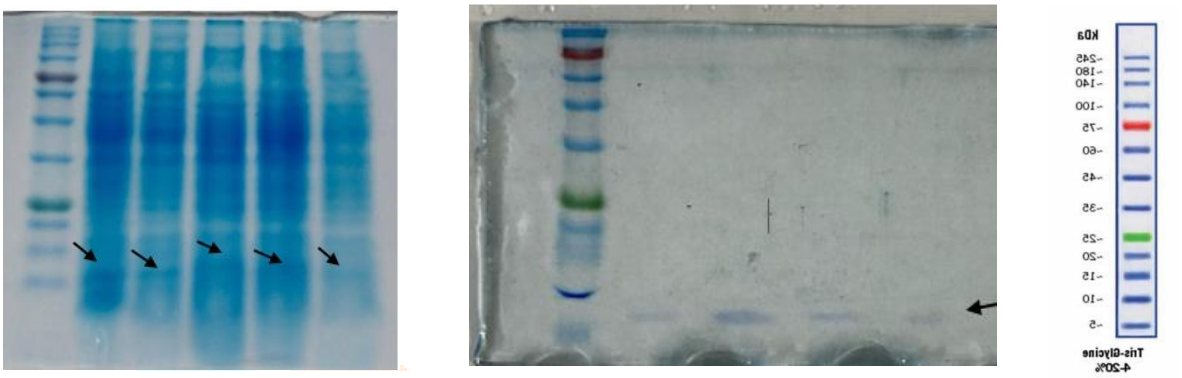


Fig 5: Restriction Digestion Confirmation of the pET28a(+) and MTII recombinant plasmid, Lane 1: 1 Kb Ladder, Lane2 & 3: Double Digestion with *BamHI* and *XhoI*

2. Total Protein isolation and Purification of recombinant metallothionein II protein and SDS PAGE: Total protein content of recombinant BL21 cells were isolated and purified and run on 12% and 18% SDS gel respectively (Fig 6).

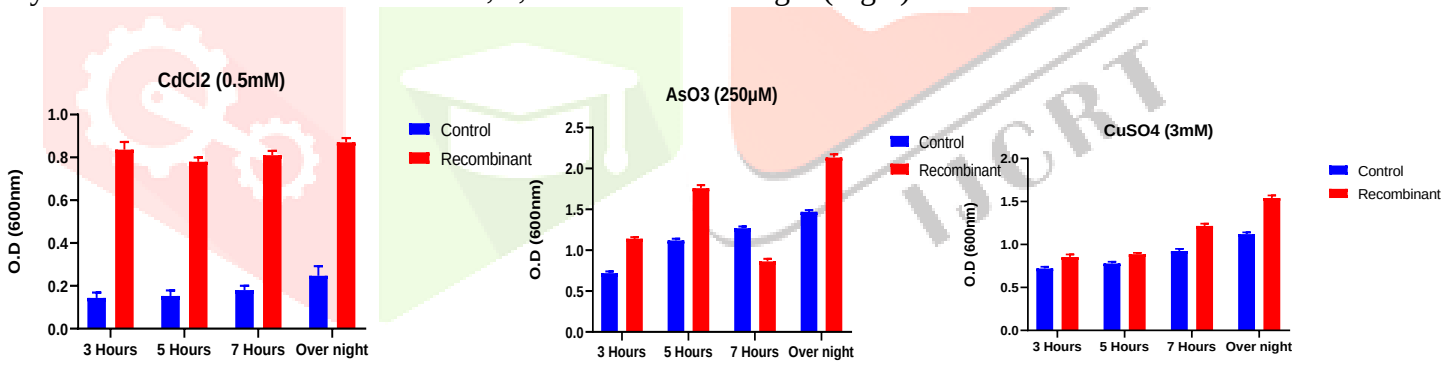
Increased band intensity between 5-10 kDa indicating enhanced expression of *Leucaena* MTII gene and purified samples shows bands around 7-8 kDa which is consistent with our previous analysis that the predicted molecular weight of this MTII protein is 7.6 kDa.



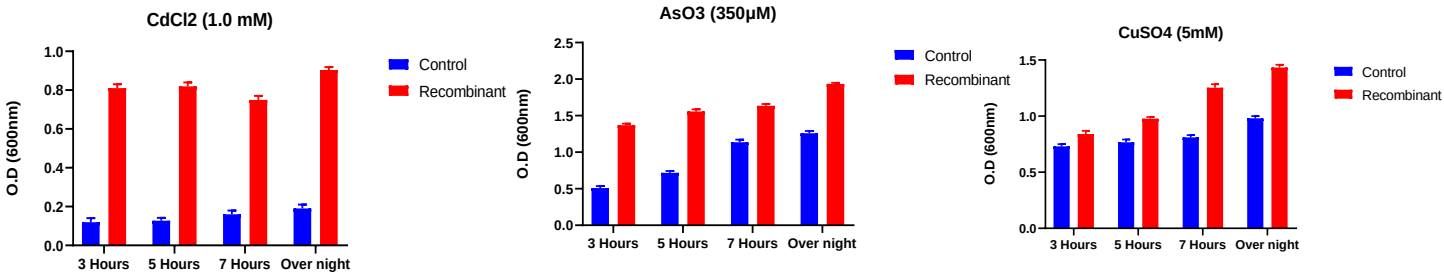
(a) **(b)** **(c)**

Fig 6: SDS PAGE analysis of histidine tagged LlMTII Protein (a) Total Protein content of recombinant BL21 cells harboring *LlMTII* gene after 0.1mM IPTG induction (b) Ni-NTA column-purified samples of MTII proteins (c) Protein ladder 3.5-245 kDa protein ladder (G2P)

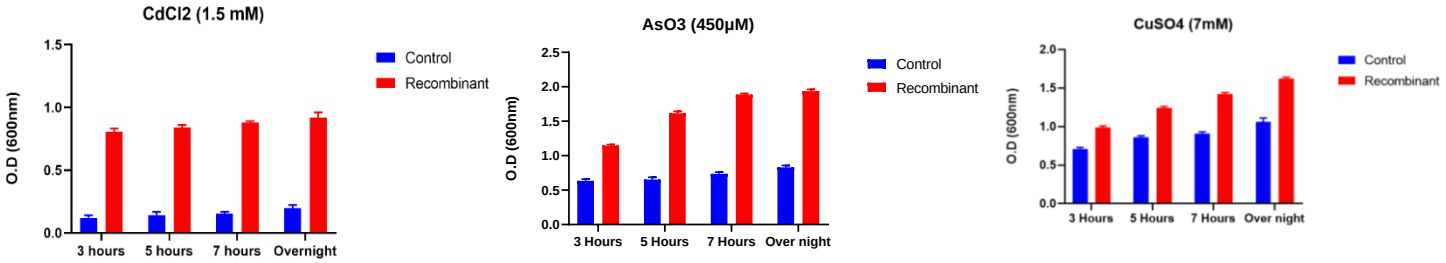
3. Heavy metal stress analysis of recombinant BL21 cells: Different concentrations of $CdCl_2$ (0.5mM, 1.0mM, 1.5mm), As_2O_3 (250µM, 350µM, 450µM) and $CuCl_2$ (3mM, 5mM, 7mM) were taken for this analysis. OD values were measured at 3, 5, 7 hours and overnight (Fig 7).



(a)



(b)



Growth rate and final biomass is significantly higher in the recombinant cells than the control at different time intervals. In all the assays, growth rate in all the recombinants cultures is consistently same. Similar growth rate in Cd and Cu (controls), even though the concentration of Cu is higher shows that metallothionein protein has a role in regulating copper uptake and regulation in cells as it is an essential micronutrient but is toxic in high concentrations. Arsenic concentrations used are very low but growth rate is almost similar to Cu in control cells which can mean As is more toxic compared to cadmium.

V. CONCLUSION

Metallothionein Type II, isolated from an abiotic stress resilient species like *Leucaena leucocephala* is an effective agent against toxic heavy metals. In this study, expression of MTII protein in *E. coli* under different types of heavy metal stress has been studied by testing the growth rate of recombinant *E. coli* and comparing them against non- recombinants as control. Recombinant *E. coli* expressing the *LlMTII* gene exhibits a significantly increased capacity to tolerate heavy metals compared to control strains. After exposure to Cd, As and Cu, the *E. coli* showed increased survival rates and assume normal growth. These results confirm the functional expression of metallothionein type in *E. coli* and its role in sequestering toxic metal ions. These findings suggests that genetically engineered microorganisms can be used in bioremediation of contaminated soil and water. Furthermore, The *Leucaena* MTII gene can be genetically manipulated for improving various important food crops.

V. Acknowledgment

We are thankful to the Department of Genetics and Biotechnology, Osmania University, for providing support for research. We acknowledge the financial support from OU-DBT-BUILDER Program II with grant number BT/INF/22/ SP41415/2021.

References

1. Loebus J, Leitenmaier B, Meissner D, Braha B, Krauss GJ, Dobritzsch D, Freisinger E (2013). The major function of a metallothionein from the aquatic fungus *Heliscus lugdunensis* is cadmium detoxification. *Journal of Inorganic* Biochemistry, 127, 253-260.
2. Cobbett C, Goldsbrough P. (2002). Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*, 53, 159–182.
3. Gu YG, QS Li, Fang JH, He BY, Fu HB, Tong ZJ (2014). Identification of heavy metal sources in the reclaimed farmland soils of the pearl river estuary in China using a multivariate geostatistical approach. *Ecotoxicology and Environmental Safety*, 105, 7-12.
4. Cui Y, Bai L, Li C, He Z, Liu X (2022). Assessment of heavy metal contamination levels and health risks in environmental media in the northeast region. *Sustainable Cities and Society*, 80, 103796.
5. Kaegi J. H. R, Schaeffer, A. (1988). Biochemistry of metallothionein. *Biochemistry*, 27(23), 8509–8515.
6. Roesijadi G (1992). Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquatic Toxicology*, 22, 2, 81-113.
7. Palmiter RD (1998). The elusive function of metallothioneins**.** Proc. Natl. Acad. Sci., 95, 8428–8430.
8. Adams JP, Adeli A, Hsu CY, Harkess RL, Page GP, de Pamphilis CW, Schultz EB, Yuceer C (2011). Poplar maintains zinc homeostasis with heavy metal genes *HMA4* and *PCS1*. *Journal of Experimental Botany*, 62, 11, 3737–3752.

9. Coyle P, Philcox JC, Carey LC, Rofe AM (2002). Metallothionein: the multipurpose protein. *Cellular and Molecular Life Science*, 59, 627–647,

10. Wong JWC, Xiang L, Gu XY, Zhou LX (2004). Bioleaching of heavy metals from anaerobically digested sewage sludge using FeS_2 as an energy source. *Chemosphere*, 55, 1,101-107

11. Xu X, Duan L, Yu J, Su C, Li J, Chen D, Zhang X, Song H, Pan Y (2018). Characterization analysis and heavy metal-binding properties of *CsMTL3* in *Escherichia coli*. *FEBS Open Bio*, 8, 11,1820-1829.

12. Zou T, Pu L, Lin R, Mo H, Wang Z, Jian S, Zhang M (2022). Roles of *Canavalia rosea* metallothioneins in metal tolerance and extreme environmental adaptation to tropical coral reefs. *Journal of Plant Physiology*, *268*, *153559.*

13. Li R, Yang Y, Cao H, Peng X, Yu Q, He L, Chen J, Xian L, Liu W (2023). Heterologous expression of the tobacco metallothionein gene *NtMT2F* confers enhanced tolerance to Cd stress, in *Escherichia coli* and *Arabidopsis thaliana. Plant Physiology and Biochemistry, 195*, 247-255.

14. Mir G, Domènech J, Huguet G, Guo WJ, Goldsbrough P, Atrian S, Molinas M (2004). A plant type 2 metallothionein (MT) from cork tissue responds to oxidative stress. *Journal of Experimental Botany*, 55, 408, 2483–2493.

15. Wahengbam A, Bollempally P, Kanathala AP, Konda AK and Singam P (2025). Molecular cloning & In silico Analysis of Metallothionein Type II Gene from Leucaena leucocephala (a Leguminous Species). *Plant Cell Biotechnology and Molecular Biology*,26, 3-4, 22-38.