------ Raf. J. Sci., Vol. 26, No.1, pp. 32-37, 2017------

Detection the Effect of Cu^{++} on Transcription of *glox* Gene in *Myceliophthora verrucosa* Using RT-PCR

Mohammad I. Khalil
College of Environmental Science and Technology/ University of Mosul
E- mail: mohbioecology@gmail.com

(Received 23 / 3 / 2014 ; Accepted 26 / 5 / 2014)

ABSTRACT

Reverse transcriptase PCR was used to detect the transcription of the *glox* gene in a local isolate of *M. verrucosa*, in two different growth media. The results indicated the presence of gene transcription in this strain with no effect to Cu^{+} on gene expression. This means that this fungus is suitable for use in various fields such as a bioremediation. This method depends on RNA to detect gene expression under various conditions.

الكشف عن تأثير ايونات النحاس على استنساخ جين *glox* في الفطر *Myceliophthora verrucosa* باستخدام طريقة الاستنساخ العكسي للتفاعل التضاعفي المتسلسل

الملخص

استخدمت طريقة الاستنساخ العكسي للتفاعل التضاعفي المتسلسل للكشف عن تأثير ايون النحاس على استنساخ الجين المسؤول عن التشفير لانزيم *glox* في العزلة المحلية، واشارت النتيجة الى ان استنساخ الجين لم يتأثر بوجود او عدم وجود ايون النحاس في وسط النمو، ان قدرة هذا الجين على الاستنساخ في هذه العزلة يعد مؤشرا لامكانية استخدام هذا الفطر في عدة مجالات من المعالجة البايولوجية، كذلك فان هذه الطريقة تعد من الطرق الدقيقة حيث تعتمد على الحامض النووي RNA في التحري عن استنساخ الجينات.

INTRODUCTION

Extracellular peroxidases are important components of the ligninolytic system of *Phanerochaete chrysosporium*. Glyoxal Oxidase (*glox*) is one of the extracellular peroxidase H_2O_2 producing enzyme found in ligninolytic culture of *P. chrysosporium*. The enzyme catalyzes the oxidation of a number of simple aldehydes and α-hydroxy carbonyl compounds (Kersten,1990). *glox* homologous were found in human, plant pathogenic fungi and plants but not in other mammals or yeast. It was reported (Leuthner *et al*. 2005) that *Ustilago maydis* is gene produces H_2O_2 and the membrane bound glo_1 protein is involved in filamentous growth and pathogenicity of *U. maydis.* However the biological role of *glox* in plants remains unclear, (Zhao *et al*., 2012).

In previous study (Khalil *et al.,* 2013) the Iraqi local isolate *Myceliophthora verrucosa* was the only strain able to produce Laccase enzyme and show a high activity of this enzyme within 24 hours compared to the standard strain *P. chrysosporium* which need 7 days to produce the same enzyme. There is a strong relation between laccase and *glox* enzymes; both of them belong to ligninolytic enzymes system (LES) (Maciel *et al.,* 2010). The LES has an important roles in biodegradation and biological function (Singh, 2006). *M. verrucosa* may have other enzymes and can be considered an optimum local strain that has a complete LES (Khalil *et al.,* 2013).

glox gene expression can be detected by using reverse transcriptase polymerase chain reaction (RT-PCR). RNA can also serve as a template for PCR amplification after conversion to cDNA,

RNA- PCR or RT-PCR is a modified PCR procedure designed for analyzing RNA transcript. The RT-PCR is more sensitive than other methods used for DNA analysis especially in response to medium components effect (Bridge *et al*., 1998). Copper has been reported to be a strong enzyme inducer in several species, it is known that Cu induces both lactase transcription and activity and the increase in activity is proportional to the amount of copper added (Levin *et al.,* 2002).

Due to the good ability of the local strain of the fungus *M. verrucosa* to produce laccase enzyme (Khalil *et al.,* 2013), this study was designed to detect another enzyme, the *glox* gene transcript by using the RT-PCR technique and study the effect of the copper in *glox* gene transcription.

MATERIALS AND METHODS

Fungal strain:

M. verrucosa was previously isolated from plastic garbage and identified by using the rDNA method (Khalil *et al*., 2014).

Preparation of the fungal spore inocula:

The spores were harvested in distilled water from a 7 days old fungal plate and then Tween 20 was added to a concentration of 0.01% then mixed by vortex and used to inoculate flasks of liquid media (Probha *et al.,* 2013).

Medium of growth:

Two media differing in the presence and absence of the Cu^{++} ion were used in this study, Submerged cultivation of the fungus was carried out on a rotary shaker at 140 rpm and 30 ° C in 250 ml flasks which contained 200 ml of standard medium g/l : glucose 15 ; pepton 3 ; KH_2PO_4 0.8 ; K_2HPO_4 0.4 ; $MgSO_4.7H_2O$ 0.5 ; $CaSO_4.5H_2O$ 0.125 ; $CuSO_4.5H_2O$ 0.125 ; yeast extract 4. The pH was 5.5 and incubation was for 10 days, (Kenkebashvili *et al*., 2012).

Isolation of RNA

At the end of the incubation period, the fungal mycelium was filtered through sterilized muslin and ground in liquid nitrogen to break open all hyphal constituents. The RNA was extracted by using the Micro RNA isolation kit (miRNA) from Omega, and all other reagents were provided by BioLab, England, and the work was done according to the company protocols. The purity and concentration were measured by using Biodrop. The RNA concentration was adjusted at 300 ng/µl to be equal for all samples and then diluted for RT-PCR reaction.

M-MULV Reverse Transcriptase:

Moloney Murine Leukemia Virus (M-MULV) a Reverse Transcriptase (RT) is an RNA- and DNA-dependent DNA polymerase, the enzyme possesses a ribonuclease H activity specific to RNA in RNA-DNA hybrids, M-MULV RT incorporates modified nucleotides, *E.coli* cells with a cloned fragment of the pol gene encoding Moloney Murine Leukemia Virus reverse transcriptase (Sambrook and Russell, 2001).

PCR Primers:

Three set of primers differ in design methods, the first set designed according to complementary DNA, while the second primers set was designed according to genomic DNA and the third set designed by using National Centre of Biotechnology Information (NCBI) web site. All these Primers were used to amplify cDNA and these were :

1. F: 5'-GCGAATTCATGTTGTCGCTGCTAGCCGTAGT-3 'R: 3'-CGTACGTACTCCAGGGTCGGCGGAGGGT- 5' (Son *et al*., 2012).
2. *glx* F 5`-TCACACCTTCGCTCTACACG-3`
   glx R3` -TATTTACTCCAGGGTCGGCG-5` (Bernard *et al*., 1998)
3. NF: TATCCCCAACGGTGTTCGTG
   NR: TGATGCGCTTTCCAAGAGGT (designed from NCBI by Author)

First strand cDNA synthesis:

The cDNA was synthesized by using the reaction mixture shown in (Table 1) in an eppendorf tube. The RNA denatured for 5 minutes at 70 °C and span briefly then 10 µl of the M-MULV reaction mix and 2 µl of the M-MULV enzyme mix were added, the reaction mixture incubated at 42 °C for one hour and the enzyme inactivated at 80 °C for 5 minutes. The reaction mixture was diluted to 50 µl with 30 µl H_2O for PCR reaction, (Jalali *et al*., 2011).

Table 1: synthesis of cDNA

Content	Volume µl	Conce
Total RNA	3	300 ng/µl
$d(t)_{23}$ VN (50mm)	2	2.5 µM
Nuclease –free H_2O	3	
Total	8	

RT-PCR reaction

cDNA template preparation:

For each cDNA reaction, series (1:5, 1:10, 1:100) dilution of cDNA into RNase-free dH_2O was made,working cDNA dilution depends on abundance of transcript so it may be necessary to make a dilution to determine optimum cDNA input dilution comparing with undiluted cDNA.

Primers were used to set the RT-PCR cDNA template in the reaction made as shown in (Table 2):

Table 2: PCR reaction component

Content	Volume µl	Conc.
Master mix	12.5	1X
Forward	1.5	10 picomole
Reverse	1.5	10 picomole
cDNA template	2.5	Different dilution
ddH_2O	7	

The thermocycler programs consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (95°C for 1 min), and to avoid annealing temperature failure, the gradient primer annealing temperature was used in range 55 ±10 °C (50-60 °C) for 2 min and polymerization (72°C for 2 min). Final amplification was at 72°C for 10 min. (Khalil *et al*., 2014; Alrawi and Alsanjary, 2009).

The PCR products (*glox* gene bands) were separated on 2% agarose gel, stained with ethidium bromide and bands visualized were by gel documentation.

RESULTS AND DISCUSSION

The RNA extraction by miRNA isolation kit showed a high purity and concentration as shown in (Table 3).

Table 3: RNA purity and concentration from the fungus grown in two different medium

Fungal growth medium	Purity	Concentration ng/ µl
standard medium	1.976	314.2
standard medium Without Cu	1.944	281

Pure RNA has an *A*260/*A*280 ratio of 1.9–2.1 (Nielson, 2011). The results showed that the first set of primers only gave pure bands for the most diluted cDNA samples and for both mycelium grown in standard medium or medium lacking Cu^{++} (Fig. 1).

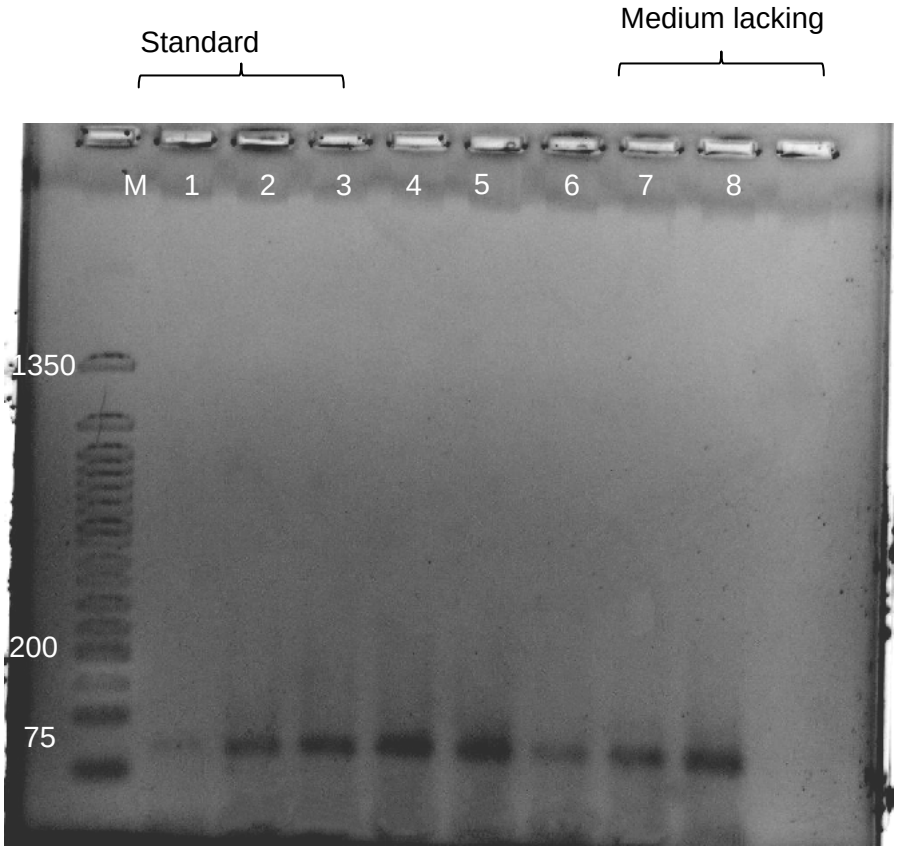

Fig. 1: gel electrophoresis of the PCR products with the first set of primers M : molecular ladder, 1,2,3,4 standard mycelium and at cDNA concs. Undiluted, 1/5,1/10,1/100 respectively; 3 concentration cDNA, 4,5,6 without Cu mycelium in and at cDNA concs. 1/5, undiluted, 1/10,1/100 respectively

The results also showed a pure and strong bands at 75 bp in 2, 3, 4 wells (standard medium) and 5, 7, 8 wells (medium without Cu^{+}), and the bands in 1, 6 were a week due to high concentration of cDNA (because the difference in RNA concentration) which used undiluted cDNA, and the strong bands are from the high dilution ratio. The high concentration of cDNA leads to the reaction component may interact with primers annealing, and react week bands and making a series dilution can avoid the interaction. The pure bands indicates that the local strain *M. verrucosa* contains the *glox* gene. Its expression will not be affected by absence of Cu^{+}. This enzyme is a member in ligninolytic enzyme system in white rot fungi, (Janusze *et al*., 2013).

The results also showed that there were no amplified cDNA by using the second and third primer sets. This may due to the primers design where these primers may contain intron / exon for genomic DNA while the complementary DNA doesn`t contain an Introns just exons and because of this the primers should be designed for exon only as in the first primer set (Fig. 2), (Zhong, 2003)

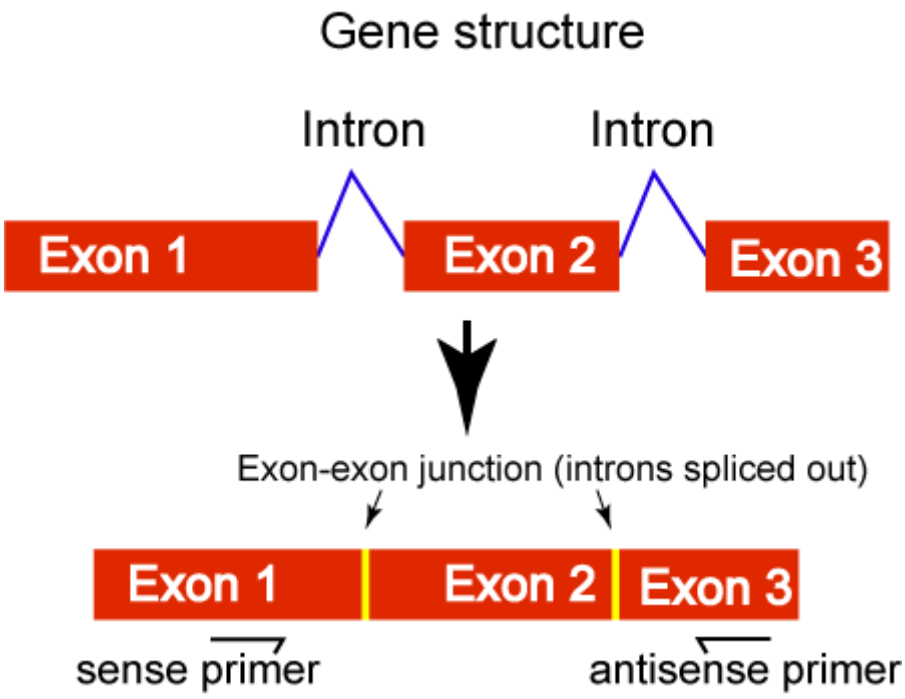

Fig. 2: primers design for reverse transcriptase PCR (Zhong, 2003)

Also the results indicated that the *glox* gene expression didn`t need the Cu^{+}. This ion may be needed for the activation *glox* enzyme. However, how this happens is not clear. The effect of Cu^{+} is it Cu^{+} could be a component of the active site of the enzyme or simply an effector of the enzyme (Kersten, 1990).

The presence of the *glox* gene and enzyme in the local strain *M. verrucosa* point to the possibility of using this strain in bioremediation. The *glox* and Laccase (detected in a previous study) are very important enzymes in bioremediation; they have the ability to break the most strong bond between lignin atoms (Khalil *et al*., 2013).

The RT-PCR has become one of the most widely applied techniques in biomedical research (O`Connell, 2002). The ease with which the technique permits specific mRNA to be detected and quantified has been a major asset in the molecular investigation of disease pathogenesis (Nielson, 2011) . Disease- related imbalances in the expression of specific mRNAs can be sensitive parameter and a very useful tool for detecting variations in the expression of many genes under various condition, (O`Connell, 2002). In many cases the RT-PCR is a very important tool to detect gene expression in various condition especially with these induced during secondary metabolism under nutrient-limiting culture conditions and they cannot be detected by genomic DNA PCR (Singh, 2006).

REFERENCES

Al-Rawi, A.; Al-Sanjary, M. (2009). Comparison of Routine Identification methods for Leptospira with PCR technique. *Raf. J. Sci*., **20**(3), 35-47.

Bernard J.H.; Jill, G.; Masood, A.; Daniel, C. (1998). Glyoxal oxidase in wood peroxidases, manganese peroxidases, and chrysosporium genes encoding lignin expression of *Phanerochaete. Appl. Environ. Microbiol*, **64**(9), 3536-3538.

Bridge, P.; Arora, D.; Reddy, C.; Elander, R. (1998). "Applications of PCR in Mycology". University Press, Cambridge,UK, 373 p.

http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web & PAGE_TYPE=BlastHome

Jalali, S.A.H.; Nikbakht, G.; Mahboobi Soofiani, N.; Jalali, S.M. (2011). Optimization of semi-quantitative RT PCR analysis for CPT I gene expression in Rainbow trout (*Oncorhynchus mykiss*). *Iranian J. Fisheries Sci*. Short Communication, **10**(4), 749-752 .

Janusza, G.; Katarzyna H.K.; Anna, P.; Magdalena, S.; Andrzej, J. (2013). Fungal laccase, manganese peroxidase and lignin peroxidase: Gene expression and regulation. *Enzyme and Microbial Technology*, **52**,1– 12

Kenkebashvili, N.; Vladimir E.; Solomon, P. (2012). Effect of carbon, nitrogen sources, and copper concentration on the ligninolytic enzyme Production by *Coriolopsis gallica*. *J. Waste Conversion, Bioproducts and Biotechnol*. **1**(2), 22–27.

Kersten, P. (1990). Glyoxal oxidase of *Phanerochaete chrysosporium*: Its characterization and activation by lignin peroxidase, *Proc. Nati. Acad. Sci.* **87**, 2936-2940.

Khalil, M.I.; Ramadan, N.A.; Albarhawi, R.K. (2013). Biodegradation of polymers by fungi isolated from plastic garbage and the optimum condition assessment of growth. *Raf. J. Env*., **1**(1), 33-43.

Khalil, M.I.; Ramadan, N.A.; Albarhawi, R.K. (2014). Molecular identification of fungi *Myceliophthora verrucosa* which producing Laccase enzyme. *Raf. J. Sci*., **25** (1), 67-78.

Leuthner, B.; Aichinger, C.; Oehmen, E.; Koopmann, E.; Muller, O.; Muller, P.; Kahmann, R.; Bolker, M.; Schreier, P. (2005). A H_2 O_2 producing glyoxal oxidase is required for filamentous growth and pathogenicity in *Ustilago maydis*, *Mol. Gen. Genomics* , **272**, 639–650

Levin , L.; Forchiassin, F.; Ramos, A. (2002). Copper induction of lignin-modifying enzymes in the white-rot fungus. *Mycologia*, **94**(3), 377-383.

Maciel, M.; Silva, A.; Riberiro, H. (2010). Industrial and biotechnological application of ligninolytic enzyme of the Basidiomycota, areview. *Electronic J. Bioetech*. **13**(6), 1-13.

Nielsen, H. (2011). RNA methods and protocol, methods in molecular biology. *Springer Protocol*, 346.

O`Connell, J. (2002). RT-PCR protocol, methods in molecular biology, volume 193, Humana press Inc, 378.

Probha, T. R.; Revathi, K.; Vinod, M.S.; Shanthakumar, S.P.; Bernard, P.(2013). A simple method for total genomic DNA extraction from water moulds. *Current Sceince*, **104** (3), 247-254.

Sambrook, J.; Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual, the third edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Singh, H. (2006). "Mycoremediation". John Wiley an Sons Inc., 228 p.

Son, Y.; Hyoun-Young, K.; Saravanakumar, T.; Jing Jing, X.; Seung-Moon, P. (2012). Heterologous expression of *Phanerochaete chrysoporium* glyoxal oxidase and its application for the coupled reaction with Manganese Peroxidase to Decolorize Malachite Green. *Mycobiology*, **40**(4), 258-262 .

Zhao, H.; Guan, X.; Xu, Y.; Wong, Y. (2012). Characterization of novel gene expression related to glyoxal oxidase by agro-infiltration of the leaves of *accession Baihe*-35-1 of *Vitis pseudoreticulata* involved in production of H_2O_2 for resistance to *Erysiphe necator*. *Protoplasma*, **250**(3), 765-776.

Zhong, X.(2003). http://www.protocol-online.org/biology-forums-2/posts/6014.html