World Applied Sciences Journal 10 (4): 462-465, 2010
ISSN 1818-4952

Triphenyl Methane Reductase Enzyme Assay and PCR-Based Detection of *tmr* Gene in *Mucor mucedo*

[1]*Bhargavi Moturi and* [2]*M.A. Singara Charya*

[1]Centre for Biotechnology, Jawaharlal Nehru Technological University,
Hyderabad - 5000085, AP, India
[2]Department of Microbiology, Kakatiya University, Warangal - 506009, AP, India

Abstract: *Mucor mucedo* was isolated from dye effluent amended soils and used for decolourisation of triphenyl methane dyes. Intraspecific protoplast fusion of the mutants of the wild strain resulted in a fusant which showed the best decolourisation of triphenyl methane dyes. The triphenyl methane reductase enzyme was quantified and the responsible *tmr* gene detection was done which showed the prominent dominance of *tmr* gene in these strains. The enzyme activity of the wild strain and the fusant increased with increase in the concentration of the two dyes used. The wild strain and the fusant expressed the highest activity at 300 μM concentration of crystal violet and at 80 μM concentration of malachite green. The protoplast fusant expressed the highest enzyme activity in the decolourisation of 300 μM concentration of crystal violet (0.00504 units) and in the decolourisation of 80 μM concentration of malachite green (0.00430 units). Between the two dyes selected, malachite green was resistant to degradation by both the strains when compared to crystal violet.

Key words: *Mucor mucedo* · Protoplast fusant · Triphenyl methane reductase enzyme · *Tmr* gene

INTRODUCTION

Triphenyl methane dyes are aromatic xenobiotic compounds that are used extensively in many industrial processes, such as textile dyeing, paper printing, food and cosmetic manufacturing [1]. Several triphenyl methane dye-decolourising microorganisms have been reported and their characteristics were reviewed [2]. The biochemical mechanism underlying the decolourisation of triphenyl methane dyes has been explained in fungi [3]. Triphenyl methane dyes are decolourised by lignin peroxidase of *Phanerochaete chrysosporium* [4]. Laccase from the extracellular fluid of *Cyathus bulleri* [5] and peroxidase from *Pleurotus ostreatus* [6] also decolourise triphenylmethane dyes. The structural genes encoding lignin peroxidase and laccase have been cloned and characterized [7]. The decolourisation of malachite green and crystal violet by intestinal microflora and several anaerobic bacteria proceeds through enzymatic reduction to their respective leuco derivatives [8]. However, the enzymes involved in this reduction have not yet been isolated or characterized in their purified forms.

A new bacterium *Citrobacter* sp strain KCTC 18061 P, was isolated, that has a higher decolourisation capability than any microorganism reported to date, even at high concentrations of triphenyl methane dyes. An enzyme was biochemically purified from *Citrobacter* sp and characterized that it was capable of decolourising triphenyl methane dyes and that enzyme was designated as triphenyl methane reductase (tmr). The gene encoding this enzyme was cloned and it was heterologously expressed in *Escherichia* coli [9].

Biological methods of treatment for colour removal are immensely useful and cost effective. It is therefore imperative to study the metabolic and enzymatic systems of fungi in order to gain insight into their degradation metabolisms. Various soil samples were collected around different textile dye industries in Mangalagiri andhra Pradesh and the fungal organisms were isolated by standard serial dilution plate technique. A total of 20 fungal isolates were cultured and maintained on Potato Dextrose Agar slants. Of these, one predominant fungus *Mucor mucedo* was selected on the basis of dye agar plate assay and used for study.

Corresponding Author: Bhargavi Moturi, Centre for Biotechnology, Jawaharlal Nehru Technological University, Hyderabad - 5000085, AP, India. Mob: +91-9663479559, E-mail: moturi.bhargavi@yahoo.co.in.

The isolated soil fungus, *Mucor mucedo* was considered to be the resistant, well – adopted, highly acclimatized to dye contaminated soils and also regarded as natural mutant to survive in the dye amended soils. As *Mucor mucedo* was very effective in decolourisation of crystal violet and malachite green, attempts have been made to improve the decolourisation efficiency of the wild fungal strain *Mucor mucedo* by subjecting it to mutagenic changes by treating with physical (ultraviolet radiation for different period of times such as 3, 6, 9, 12 and 15 minutes) and chemical mutagens (ethyl methyl sulfonate, diethyl sulfonate and colchicine at 1, 10, 50, 100 and 150 mg concentrations).

The creation of new genetic combinations by protoplast fusion are valuable for applications of practical importance. The protoplasts were isolated from developed mutants of *Mucor mucedo* (MMM_1, that was exposed to 9 min UV radiation and MMM_2 that was treated with 50 mg of EMS) and the intra-strain protoplast fusion was carried out with the objective of investigating the possible enhancement of the decolourisation activity in the fusant progenies. Novozyme alone at the concentration of 10 mg/ml was the most effective with the protoplast yield of 15.75×10^6/ml and 16.00×10^6/ml respectively in MMM_1 and MMM_2. The yield of the released protoplasts reached the maximum after 6 hours of incubation.

The average frequency of protoplast formation in the two series of experiments was 3.3% at 45% PEG 4000 and 4.8% at 40% PEG 6000. PEG 6000 gave higher frequencies on average. The number of colonies isolated after protoplast fusion of MMM_1 and MMM_2 were 385. Among these, only three of them were chosen as fusants and named as $MMFu_1$, $MMFu_2$ and $MMFu_3$. The fusant $MMFu_3$ showed the maximum decolourisation efficiency in both crystal violet and malachite green proving that the decolourisation activity was increased by intraspecific protoplast fusion of two mutant strains of *Mucor mucedo*.

In the present study, an attempt has been made to check the presence of triphenyl methane reductase gene in the protoplast fusant *Mucor mucedo* obtained which was already found to be decolourising triphenylmethane dyes. Two triphenyl methane group dyes, crystal violet and malachite green were used for the present investigation.

MATERIALS AND METHODS

Triphenyl Methane Reductase Enzyme Assay: The standard assay system for TMR enzyme was followed as per the method suggested by Jang, *et al.* [9]. The reaction mixture comprises 20 mM sodium phosphate buffer (pH-7), 20 μM dye (either crystal violet or malachite green, absorption coefficients of crystal violet and malachite green were 590 and 616 nm), 0.1 mM NADH and a suitable amount of culture broth in a total volume of about 1 ml. To 250 μl of 20 mM sodium phosphate buffer (pH-7.0), 250 μl of 0.1 mM NADH and 250 μl of 20 μM crystal violet or malachite green were added. Finally, 300 μl of 24 hr old broth culture of *Mucor mucedo* (wild and fusant as two different sets) was added and incubated at room temperature for about 2 minutes and then OD was observed at 600 nm.

Calculation of Enzyme Activity: The calculation of enzyme activity was followed as per the method suggested by Jang, *et al.* [9]. Enzyme activities were expressed most conveniently in micromoles per min per ml of enzyme or micromoles per mg of protein. The molecule extinction coefficient of $NADH_2$ at 340 nm is 6.3 x 1000 lts/mole so that a solution of 1 μmole/ml has absorption of 6.3.

In General Terms the

$$\text{Enzyme Activity (mole/min/ml)} = \frac{\text{Extinction change per min}}{\text{Molecule extinction coefficient of NADH x volume in cuvette}} \times \frac{1.0}{\text{Volume used for assay}}$$

For 3 Ml Reaction Mixture Used in the Assay:

$$\text{Enzyme activity (umole/min/ml)} = \frac{\text{Extinction change per min}}{6.3x3}$$

Since 0.3 Ml of Enzyme Was Used in the Assay:

Enzyme activity (umole/min/ml)

= Extinction change per min /6.3x3x1.0/0.3

= Extinction change per min x 1.61

Multiplying by any dilution factor for μmoles/min/ml and dividing by protein concentration in mg/ml for μmoles/min/mg, the final activity was obtained.

PCR Based Detection of Triphenyl Methane Reductase Gene: The DNA of wild strain and the protoplast fusant was isolated [10]. The DNA isolated was quantified using standard diphenyl amine method. One ml of isolated DNA solution has concentration of 0.12 mg / ml. The DNA was used for amplification of TMR gene by standard PCR method. Chromosomal DNA isolated was used as template for PCR.

Requirements: 2X HotStart PCR Master Mix, 50 reactions, 10µl/Reaction 2X HotStart PCR Master Mix is a premixed 2X concentrated solution of HoTaq DNA Polymerase, reaction buffer, $MgCl_2$ and dNTPs. The DNA template and primers are simply added for PCR reactions. The consistency and efficiency of routine PCR amplifications are optimized.

Instructions for each PCR reaction: 20 µl final volume was prepared at room temperature.

DNA template and primers are mixed and made total of 10 µl by adding water. 10 µl of 2X HotStart Taq PCR Master Mix was added and the first PCR step was set to 95°C for 10 minutes to active the HoTaq. Then, the PCR was continued as usual. After PCR amplification, the sample was run on 0.8% agarose gel electrophoresis [11]. The sample was run till half the distance and the bands were observed and photographed under ultraviolet light.

RESULTS AND DISCUSSION

Triphenyl Methane Reductase Enzyme Assay: The results from Table 1 showed that in terms of enzyme activity, the wild strain expressed the highest activity at 300 µM concentration of crystal violet (0.00477 µmoles). The enzyme activity increased with increase in the concentration of the dye up to 300 µM. The enzyme activity was 0.00297 µmoles at 100 µM conc. of dye and was 0.00369 µmoles at 200 µM conc. of dye. The results already proved that the protoplast fusant of the mutants of the wild strain *Mucor mucedo* was very efficient in decolourising both crystal violet and malachite green. The organism expressed the highest enzyme activity in the decolourisation of 300 µM concentration of crystal violet (0.00504 µmoles). The enzyme activity increased with increase in the concentration of the dye used. The enzyme activity was 0.00405 µmoles at 200 µM concentration and it was 0.00396 µmoles in 100 µM concentration of the dye.

The wild strain expressed the enzyme activity (0.00116 µmoles) in the decolourisation of 20 µM concentration of malachite green. The enzyme activity increased with increase in the concentration of the dye used. The enzyme activity was expressed as 0.00240 µmoles at 50 µM conc. of the dye and was 0.00328 µmoles at 80 µM concentration. The results from Table 2 showed that the protoplast fusant expressed the highest enzyme activity (0.00430 µmoles) in the decolourisation of 80 µM concentration of malachite green. The enzyme activity was 0.00182 µmoles in 20 µM concentration and was 0.00313 µmoles in 50 µM concentration of the dye.

Detection of Triphenyl Methane Gene: The amplified product was visualized on the 0.8% agarose gel (Figure 1). The protoplast fusant DNA loaded in the wells showed amplification indicating the presence of *tmr* gene. PCR assay using *tmr* gene specific primers yielded 522 bp amplified product in all the five replicates of fusant DNA. The objectives of the study were to find the occurrence of *tmr* gene in naturally available dye decolourising organisms. The protoplast fusant obtained from the wild strain yielded amplified product using primers specific for *tmr* gene.

Cloning of the gene encoding the *tmr* enzyme and its heterologous expression was reported in *E.* coli [9]. The native form of the enzyme was identified as a homodimer with a subunit molecular mass of about 31 kDa. It catalyzes the NADH-dependent reduction of triphenyl methane dyes, with remarkable substrate specificity. Enzyme activity was found to be maximum at pH 9.0 and 60°C. The enzymatic reaction product of the triphenyl methane dye crystal violet was recognized as its leuco form by UV-visible spectral changes and thin-layer chromatography. The nucleotide sequence of the gene has a single open reading frame encoding 287 amino acids with a predicted molecular mass of 30,954 Da.

Table 1: Triphenyl methane reductase enzyme assay during the decolourisation of crystal violet by wild strain and fusant of *Mucor mucedo*

Conc. of the dye	Wild strain enzyme activity (µmoles)	Fusant strain enzyme activity (µmoles)
100 µM	0.00297	0.00396
200 µM	0.00369	0.00405
300 µM	0.00477	0.00504

Table 2: Triphenyl methane reductase enzyme assay during the decolourisation of malachite green by wild strain and fusant of *Mucor mucedo*

Conc. of the dye	Wild strain enzyme activity (µmoles)	Fusant strain enzyme activity (µmoles)
100 µM	0.00116	0.00182
200 µM	0.00240	0.00313
300 µM	0.00328	0.00430

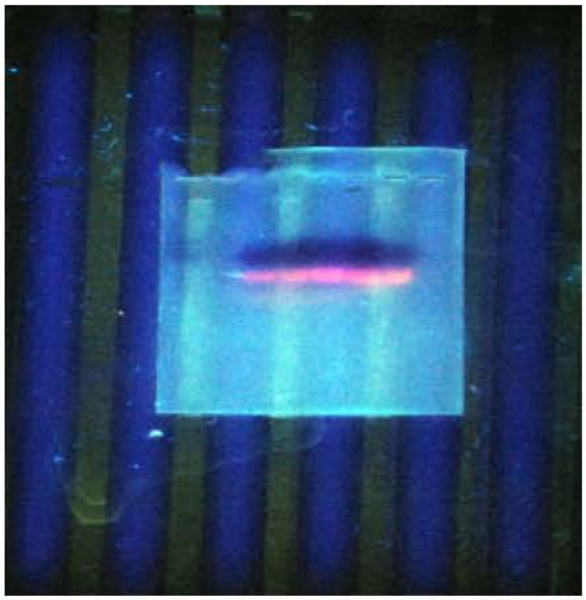

Fig. 1: PCR based detection of triphenyl methane gene

A complete sequence analysis of 16s RNA gene and the gyrase subunit β gene of triphenyl methane dye decolourising strain has been reported [12]. A broad-spectrum dye-decolourising bacterium, strain *Aeromonas hydrophila* was isolated from activated sludge of a textile printing waste water treatment plant that decolourised a variety of synthetic dyes, including trihenyl methane, azo and anthraquinone dyes. Triphenyl methane dyes such as crystal violet and malachite green were decolorized more than 90%. The colour removal of triphenyl methane dyes was due to the enzyme NADH/NADPH-dependent oxygenase.

On the basis of simulated molecular docking using the substrate malachite green and the TMR/$NADP^+$ crystal structure, together with site-directed mutagenesis, a potential molecular mechanism for triphenyl methane dye reduction was elucidated [13]. In this study, we successfully traced out the presence of triphenyl methane reductase gene in the dye decolourising protoplast fusant of *Mucor mucedo*.

REFERENCES

1. Gregory, P., 1993. Dyes and dyes intermediates: In: J.I. Kroschwitz (eds.), Encyclopedia of chemical technology: John Wiley & Sons, New York, N.Y., 8: 544-545.
2. Azmi, W., R.K. Sani and U.C. Banerjee, 1998. Biodegradation of triphenylmethane dyes. Enzyme and Microbial Technol., 22: 185-191.
3. Vasdev, K., R.C. Kuhad and R.K. Saxena, 1995. Decolorization of triphenylmethane dyes by the birds nest fungus Cyathus bulleri. Current Microbiol., 30: 269-272.
4. Bumpus, J.A. and B.J. Brock, 1988. Biodegradation of crystal violet by the white rot fungus Phanerochaete chrysosporium. Applied and Environmental Microbiol., 54: 1143-1150.
5. Suzuki, Y., T. Yoda, A. Ruhul and W. Sugiura, 2001. Molecular cloning and characterization of the gene coding for azoreductase from Bacillus sp. OY1-2 isolated from soil. The Journal of Biological Chemistry, 276: 9059-9065.
6. Shin, K.S., I.K. Oh and C.J. Kim, 1997. Production and purification of remazol brilliant blue - R, decolourising peroxidase from the culture filtrate of *Pleurotus ostreatus*. Applied and Environmental Microbiol., 63: 1774-1748.
7. Cullen, D., 1997. Recent advances on the molecular genetics of ligninolytic fungi. J. Biotechnol., 53: 273-289.
8. McDonald, J.J. and C.E. Cermgha, 1984. Biotransformation of gentian violet to leucogentian violet by human, rat and chicken intestinal microflora. Drug Metabolism and Disposition, 12: 330-336.
9. Jang, M.S., Y.M. Lee, C.H. Kim, J.H. Lee, D.W. Kang, S.J. Kim and Y.C. Lee, 2005. Triphenylmethane Reductase from *Citrobacter* sp. Strain KCTC 18061P: Purification, Characterization, Gene Cloning and Overexpression of a Functional protein in *Eschericia coli*. Applied and Environmental Microbiol., 71: 7955-7960.
10. Lee, C.C., X. Wu, R.A. Gibbs, R.G. Cook, D.M. Muzny and C.T.Caskey, 1988. Generation of cDNA Probes Directed by Amino Acid Sequence: Cloning of Urate Oxidase. Sci., 239: 1288-1291.
11. Cruickshank, R.H. and G.C. Wade, 1980. Detection of pectic enzymes in pectin-acrylamide gels. Analytical Biochemistry, 107: 177-181.
12. Ren, S., J. Guo, G. Zeng and G. Sun, 2006. Decolorization of triphenylmethane, azo and anthraquinone dyes by a newly isolated *Aeromonas hydrophila* strain. Applied microbiology and Biotechnol., 72: 1316-1321.
13. Kim, M.H., Y. Kim, H.J. Park, J.S. Lee, S.N. Kwak, W.H. Jung, S. Lee, D. Kim, Y. Lee and T.K. Oh, 2008. Stuctural Insight into Bioremediation of Triphenylmethane Dyes by *Citrobacter* Sp. Triphenylmethane Reductase. The J. Biological Chemistry, 283: 31981-31990.