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Assay Of Triphenylmethane Reductase Enzyme And PCR-based Identification Of Tmr Gene In Enterobacter Asbriae Strain XJUHX - 4TM

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ABSTRACT

In this study, a bacterial isolate, Enterobacter asburiae strain XJUHX-4TM, isolated from dye contaminated waste water of West Bengal (Habra), was assessed for its triphenylmethane reductase activity, the enzyme responsible for reduction based decolorization of the triphenylmethane dyes. The organism was found to demonstrate high decolorizing activity of a recalcitrant triphenylmethane dye, malachite green. The organism showed an increase in enzyme activity as the concentration of the dye was increased. The maximum enzyme activity (3.586 X10^{-3} µmole/min/ml) was found at maximum concentration of the dye (500 µM) used in this study. The enzyme activity in presence of the dye became highly induced which was quite larger than the one produced from the organism in absence of the dye. The gene tmr encoding the enzyme triphenylmethane reductase was also detected which showed prominent dominance in this strain.

Keywords : Enterobacter asburiae strain XJUHX-4TM, triphenylmethane reductase,tmr gene

Introduction

Malachite green (MG), a triphenylmethane dye, is used extensively for dyeing silk, wool, jute, leather, ceramics, cotton and used to treat fungal and protozoal infection (Parshetti et al 2006). The members of triphenylmethane family are animal carcinogens. The Food and Drug Administration nominated MG as a priority chemical for carcinogenicity testing by the National Toxicology Program in 1993 (Parshetti et al 2006). Studies of the biodegradation of triphenylmethane dyes have focused primarily on the decolorization of dyes via reduction reactions (Henderson et al. 1997 & McDonald et al. 1985). Several triphenylmethane dye-decolorizing microorganisms have been reported and their characteristics reviewed. The biochemical mechanism underlying the decolorization of triphenylmethane dyes has been elucidated in fungi (Bumpus et al.1988, Moturi et al. 2010, Shin et al.1998, Vasdev et al. 1995), bacteria (An et al. 2002, Henderson et al.1997, Jang et al.2005, McDonald et al. 1984, and Parshetti et al.2006) and algae (Daneshvar et al.2007). Triphenylmethane dyes are decolorized by lignin peroxidase of Phanerochaete chrysosporium (Bumpus et al. 1988). Laccase from the extracellular fluid of Cyathus bulleri (Vasdev et al.1995) and peroxidase from Pleurotus ostreatus (Shin et al. 1998) also decolorize triphenylmethane dyes.

The decolorization of malachite green and crystal violet by intestinal microflora and several anaerobic bacteria proceeds through enzymatic reduction to their respective leuco derivatives (Mc. Donald et al. 1985 and Henderson et al. 1997). The enzyme which is mainly involved in this reaction is triphenylmethane reductase. Jang et al. in the year 2005 first isolated the enzyme in their pure form and also identified the tmr gene encoding the enzyme. The tmr gene was also isolated from Pseudomonas sp. MDB-1 strain by Li et al. in the year 2009.The enzyme triphenylmethane reductase were also found to be present in Kocuria rosea MTCC 1532 (Parshetti et al.2006) and Pseudomonas sp. strain DY1 (Du et al.2011).

In this study, an attempt has been made to test the triphenylmethane reductase activity in a bacterial isolate, Enterobacter asburieae (GenBank Accession No. HQ830346) strain XJUHX-4TM .The organism was isolated from dye contaminated waste water of a place called Habra, situated in the district 24 parganas (North),West Bengal, and was found to exhibit high decolorizing activity of the triphenylmethane dye, malachite green. A comparative study was also undertaken to compare the enzyme activity both in presence and absence of the dye. The gene tmr which codes for the enzyme was also detected.

Materials and methods

Bacterial strain and media: Enterobacter asburieae was grown on Nutrient agar (5g peptone/L,3g beef extract/L ,15g Agar/L,pH-6.8) as well as on nutrient broth medium (5g peptone/L,3g beef extract/L, pH-6.8) containing MG (100mg/L) and maintained. The bacteria were grown on these media under aerobic condition at 37°C in a shaker with a speed of 150 rotations per min in case of liquid culture and aerobically in an incubator at 37°C in case of semi-solid agar culture.

Harvesting of bacterial cells for enzyme production: To compare the triphenylmethane reductase activity of the organism both in presence and absence of the dye, the organism was grown in nutrient broth both in presence and absence of the dye separately. Cells were centrifuged at 8,000 rpm for 20 minutes. These cells were suspended in sodium phosphate buffer (50 mM, pH 7.4) and the cells were disintegrated in a mortar and pestle and again centrifuged at 4ºC, 8000 rpm for 20 minutes. The clear supernatant was used to measure enzyme activity. Similarly, cells grown on dye containing media was collected after complete decolorization of the dye and harvested in the procedure stated above to determine the enzyme activity.

Enzyme assay: The method of enzyme analysis was performed according to previous reports (Du et al. 2011, Jang et al. 2005 and Parshetti et al. 2006).The standard assay system for TMR comprised of 50 mM sodium phosphate buffer (pH 7.0), 50mM NADH, suitable amount of malachite green and crude extracts of enzyme isolated after harvesting in a total volume of 1 ml.

Each reaction was initiated by the addition of the enzyme, and the initial reaction rate was determined by monitoring the decrease in absorbance at 340 nm (absorption maxima for NADH) in the first 120 sec in a temperature-controlled cuvette in a 1.0-cm light path at 35°C. One unit of enzyme activity was defined as the amount that catalyzed the reduction of 1 µmol of NADH by using a molar extinction coefficient of 6.22X103 lts/mole. To determine the effect of initial dye concentrations on enzyme production, the dye concentrations were varied from 10 to 500 µM and the activity was measured.

Calculation of TMR activity: The calculation of enzyme activity was calculated according to the protocol described by Jang et al. in 2005 and Moturi et al. in 2011.Enzyme activities were expressed in micromoles per min per ml. The molecular coefficient of NADH at 340 nm is 6.22X103lts/mole; hence a solution of 1µmole/ml has absorption of 6.22.

Therefore, the enzyme activity (mole/min/ml) =

$$\frac{\text{Extinction change /min}}{\text{Molecular extinction coefficient of NADH}} \times \frac{\text{Total volume}}{\text{Sample volume (Enzyme)}}$$

For 1 ml reaction mixture used in this assay:

Enzyme activity (µmole/min/ml) =

Extinction change per minute /6.22 X (Total volume/Sample volume)

Total reaction volume used in this assay was 1 ml, in which 200 µl of crude enzyme was added.

Enzyme activity (µmole/min/ml): Extinction change per min/6.22 X 1/0.2.

2.5 PCR based identification of tmr gene : Genomic DNA from the bacterial species were isolated using bacterial and fungal genomic DNA extraction kit from HiMedia and DNA concentrations were estimated by visual examination of ethidium bromide-stained agarose gels as well as by spectrophotometrical examination. Chromosomal DNA isolated, was used as the template for PCR. The primer sets were designed according to the one proposed by Jang et al. PCR was undertaken with a sense P1 primer (5'CTCATATGTCAATTGCGGTTACAGGTGCTAC3') and an antisense P3 primer (5'-CACTCGAGTTACATTTTCAGGGCTTGTTTTACGG-3').Before amplification cycle DNA was denatured for 2 min at 95°C .The cycling parameters consisted of 25 cycles at: denaturation at 94°C for 40S, primer annealing at 55°C for 40S, extension at 72°C for 1 min. After 25 cycles an extension step (7 min at 72ºC) was performed. The samples were held at 4°C until analysis by agarose gel electrophoresis.

2.6 Statistical analyses: Data were analyzed by paired t-test (at 5% significance level) with the help of statext v 1.4.2 software.

Results and discussion

Triphenylmethane reductase analyses: The data of table 1clearly states that the triphenylmethane reductase is an inducible enzyme which gets induced in presence of malachite green, a triphenylmethane dye.The control cells, which were not exposed to Malachite Green showed very nominal amount of enzyme activity. It was previously noted that the bacteria Enterobacter asburiae completely decolorizes the dye containing nutrient broth media within 4 hrs (from the experiment done before and data not shown).Therefore, the cells collected after 4 hrs of bacterial growth in MG containing media were tested for enzyme production, which showed a huge increase in the enzyme activity when compared to control (according to the result of paired T-test, at 5% level of significance). On contrary, cells collected after 12 hrs. exhibited again very nominal amount of enzyme activity, which was not significantly (according to the result of paired T-test, at 5% level of significance) very different from the control.

It was also evident from the table that as dye concentration was increased, the activity of the triphenylmethane reductase increased gradually. Therefore, the significantly increased activity of triphenylmethane reductase confirmed the role of this enzyme in MG decolorization by Enterobacter asburieae strain XJUHX-4TM.

Detection of tmr gene: The PCR amplified product was visualized on the 0.8% agarose gel. Amplification of 800 bp product indicated (Fig.1) the presence of tmr gene with P1 and P3 primers, the specific primer set for tmr gene. Using the same sets of primer Jang et al. determined the presence of tmr gene in Citrobacter sp. Strain KCTC 18061P, Moturi and Charya in 2010 observed its presence in Mucor mucedo. Li et al. in 2009 also obtained amplification of tmr gene with same primer sets in Pseudomonas sp.MDB-1 strain which was capable of decolorizing malachite green till 10mg/L.

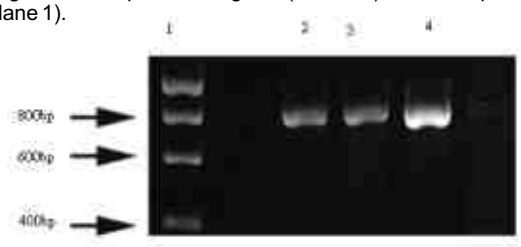
Conclusion

In this study we have successfully traced out the presence of the enzyme triphenylmethane reductase and the gene encoding the enzyme in this naturally occurring microorganism. This organism can be exploited as a promising solution in the field of bioremediation of textile dyes. To the best of our knowledge, Enterobacter asburieae strain XJUHX-4TM was the first strain from the genus of Enterobacter that has been shown to decolorize MG and contain the enzyme for its biotransformation. It can be urged that the organism can be a good potential candidate for the decolorization of not only malachite green and but also other recalcitrant dyes of the same group, hence a green signal for treatment of dye containing waste water of natural bodies.

Table 1: Result of Triphenylmethane reductase activity in the cells collected from MG-containing medium after 4 and 12 h. The data are means of triplicate experiments ± SD.

Conc. of dye (µM)	Enzyme activity (µmole/min/ml) (After 4 hrs)	Enzyme activity (µmole/min/ml) (After 12 hrs)
10	$1.322 X10^{-3} \pm 0.0021$	$0.08 X10^{-3} \pm 0.101$
50	$2.433 X10^{-3} \pm 0.013$	$0.11 X10^{-3} \pm 0.237$
100	$2.556 X10^{-3} \pm 0.0034$	$0.157 X10^{-3} \pm 0.113$
200	$2.879 X10^{-3} \pm 0.111$	$0.213 X10^{-3} \pm 0.6621$
300	$3.125 X10^{-3} \pm 0.00965$	$0.225 X10^{-3} \pm 0.0343$
400	$3.453 X10^{-3} \pm 0.101$	$0.218 X10^{-3} \pm 0.177$
500	$3.586 X10^{-3} \pm 0.03$	$0.254 X10^{-3} \pm 0.265$
Control	$0.110 X10^{-3} \pm 0$	$0.091 X10^{-3} \pm 0.128$

Fig.1: The amplified tmr gene (lane 2-4) and 200bp ladder (lane 1).



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