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PCR Analysis of Genes from *Aeromonas punctata* for Reactive Black 5 Dye Degradation

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ABSTRACT

In this present investigation, the culture Aeromonas punctata was subjected to plasmid curing with 300 µg/ml of Ethidium bromide as a curing agent. This experiment demonstrated that genes responsible for degradation of Reactive black 5 dye are plasmid borne. The enzymes namely azoreductases, peroxidases, laccases and monoxygenase played a major role in the degradation of the azo dye Reactive Black 5 into nontoxic products.PCR analysis was carried out to confirm the presence of genes responsible for production of degradative enzymes by using a set of primers corresponding to the consensus sequences. The PCR amplification of laccase gene fragment was close to 800 bp, azoreductase gene fragment was close to 700 bp, peroxidase gene fragment was nearby to 600 bp and monoxygenase gene fragment lies between 500 bp and 600 bp.

Key words: *Reactive Black 5 dye, Azoreductase, Peroxidase, Laccase, Monoxygenase, PCR.*

INTRODUCTION

Azo dyes are synthetic organic components that are characterized by great structural variety. These are largely used in textile industries due to inefficiency of textile dying process. 10-15% of dyes are lost in textile effluents. These industrial effluents containing recalcitrant azo dyes are proved to be highly carcinogenic both to human and aquatic life[1]. These recalcitrant are metabolized via different enzyme system from various microorganisms.

The mechanism of azoreductase dye degradation involves the electron transport-linked reduction of azo dyes in the extra-cellular environment. To achieve this, the bacteria must establish a link between their intracellular electron transport systems and the high molecular weight, azo dye molecules. For such a link to be established, the electron transport components must be localized in the outer membrane of the bacterial cells (in the case of gram-negative bacteria), where they can make direct contact with either the azo dye substrate or a redox mediator at the cell surface[2].

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The capacity of organisms to reduce azo dyes is related to the formation of exo enzymes such as peroxidases and phenol oxidases. Peroxidases are hemoproteins that catalyze reactions in the presence of hydrogen peroxide.[3] Lignin and manganese peroxidases (MnP) have a similar reaction mechanism that starts with the enzyme oxidation by H_2O_2 to an oxidized state during their catalytic cycle.

Laccases have been extensively studied for their degradation of azo dyes.[4] These enzymes are multicopper phenol oxidases that decolourize azo dyes through a highlynonspecific free radical mechanism forming phenolic compounds, thereby avoiding theformation of toxic aromatic amines.[5]

Monooxygenases incorporate one atom of oxygen of O_2 into the substrate, the second atom is reduced toH_2O. Dioxygenases incorporate both atoms into the substrate.

Several reports have confirmed the role of this enzymes in the degradation of azo dyes by PCR analysis. A 660 bp transcript of azoA was detected in S*taphylococcous aureus* according to Chen *et al.*[6] Several white rot fungi, including *Ganoderma lucidum* 10356, *Gloeophyllu trabeum* Mad-617-R, *Grifola frondosa*, *Lentinula edodes* and *Lentinus tigrinus*, each gave a smaller ~150 bp PCR product for laccase.[7] cheong *et al* amplified Laccase gene fragment (1.3 kb) by PCR with primers 1 and 2 which were complementary to the conserved copper-binding regions I and III.[8] According to Young the expected size of the PCR product is approximately 500 bp for monoxygenase in *E.coli*.[9]

MATERIALS AND METHODS

Culture

Aeromonas punctata with the accession number JN561149 was used for plasmid isolation, plasmid curing and PCR analysis.

Plasmid isolation

The overnight culture of *Aeromonas punctata* was used for plasmid isolation. Plasmid DNA was extracted using alkaline lysis method from cell pellets of culture.[10] The pellet from 2.0 ml of overnight culture was resuspended in 100 µl of ice-cold alkaline lysis solution l followed by treating with freshly prepared 200 µl alkaline lysis solution ll. 150 µl of ice-cold potassium acetate solution was added and the tube was kept for 3-5 minutes. The contents were subjected to centrifugation and the supernatant was collected. To this 1 ml of ice-cold 70% ethanol was added and allowed to stand for about 30 mins which resulted in precipitating the DNA. The supernatant was drained off and the tube was allowed to dry for ~5 minutes. The extracted plasmid DNA bands were observed by agarose gel electrophoresis. Agarose gel (1.2 %) was prepared in 1X TAE buffer. Ethidium bromide was added in the agarose gel in the concentration 0.1 mg/100 ml. The wells were loaded with the samples and run initially for 45 min at 50 V. Followed by this, the samples were further run for 100 V, till the tracking dye reached ¾ of the gel. The bands were then observed under the UV transilluminator and the image was photographed.

Plasmid curing

Aeromonas punctata capable of degrading Reactive Black 5 dye was inoculated into 100 ml of nutrient broth medium and incubated for 24 h under shaking speed of 150 rpm. After 24 h of incubation 1 ml of broth was inoculated into fresh nutrient broth with 300 µg/ml of ethidium bromide. Plasmid DNA was extracted and subjected to 1.2 % agarose gel electrophoresis.[10] The procedure was repeated upto 6 days. Plasmid cured cultures were subjected to check their efficiencies in degrading dye by plating 1 ml of culture onto mineral salts medium. [11]

PCR analysis

The plasmid DNA isolated from *Aeromonas punctata* using alkaline lysis method was subjected to PCR analysis.[10]

Polymerase Chain Reaction (PCR)

The PCR analysis for the genes responsible for four enzymes namely azoreductase, peroxidase, laccase and monooxygenase was carried out using following pair of primers in a total volume of 50 µl in a 0.2 ml thin walled PCR tube (Table 1). The Basic Local Alignment Search Tool (BLAST) network

service provided on the National Center for Biotechnology Information (NCBI) website was used to ensure that the proposed primer sequences did not bind non-specifically. The primers were reconstituted in sterile water at a concentration of 100 μM and PCR reaction was performed to confirm presence of genes responsible for production of enzymes capable of degrading Reactive Black 5 dye.

Amplification of the genes for azoreductase enzyme

Forward Primer: 5'- CATGCCATGGCTAT GAAAGGATTAATTATTATTGGC -3'

Reverse Primer: 5' – GCGGATCCGC TTACTATTGGTGCATTAG -3'

The amplification was carried out in a Master cycler® Thermocycler using the following program. The amplification conditions were one cycle at 95°Cfor 3 min, 30 cycles with each cycle including 30 s of melting at 95°C, 30 s of annealing at 50°C and 60 s of extension at 72°C, and one final extension cycle at 72°C for 5 min. The PCR products were examined by 1% agarose gel electrophoresis.[6]

Amplification of the genes for peroxidise enzyme

Forward Primer: 5'- GGAAGTAAAAGTC GTAACAAGG -3'

Reverse Primer: 5'- TCCTCCGCTTATTG ATATGC -3'

Polymerase chain reaction was performed by denaturation for 5 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C, with a final extension of 10 min at 72°C. The PCR products were examined by 1% agarose gel electrophoresis.[12]

Amplification of the genes for laccase enzyme

Forward Primer: 5'- TCCGTAGGTGAACCT GCG -3'

Reverse Primer: 5'- TCCTCGGCTTATTGAT ATTGATATGC – 3'[13]

The PCR conditions were initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 45 s, extension at 72°C for 3 min, final extension: 72°C for 10 min[14].

Amplification of the genes for monoxygenase enzyme

Forward Primer: 5' – GGAATTCTTTT GGCGGCAGA -3'

Reverse primer: 5' – TGGAATTCGCT TGGTCAACC -3'[9]

Cycling was performed with initial denaturation for 5 min at 90°C, 35 cycles with 30 s at 90°C, 30 s at 55°C, 60 s at 72°C and final elongation for 5 min at 72°C. PCR products were separated in 1.2% agarose gel[15].

RESULTS

Plasmid isolation

On plasmid isolation and gel electrophoresis it was observed that *Aeromonas punctata* has single band of plasmid.

Plasmid curing

Curing experiment on *Aeromonas punctata* was carried out to determine that the Reactive Black 5 dye degrading genes are plasmid borne or on chromosome. The plasmid band on agarose gel reduced and disappeared gradually by the end of 6th day (Fig.1).

Plasmid DNA cured cells of *Aeromonas punctata* was not able to grow on mineral salts medium supplemented with the Reactive Black 5 dye as the sole source of carbon. This indicated that the genes responsible for the degradation of Reactive Black 5 dye are present on the plasmid and not on the chromosome.

PCR Analysis

PCR analysis resulted in a band close to 700 bp for gene against azoreductase, almost close to 600 bp product for peroxidase, a band between 500 bp and 600 bp for monoxygenase and a band close to 800 bp for laccase gene fragment was obtained in *Aeromonas punctata* (Fig. 2).

Table 1: Components for PCR amplification

Components	Quantity (µl)
Nuclease free water	18.0
Plasmid DNA (0.2µg/µl)	5.0
Forward Primer(10 µM)	1.0
Reverse Primer (10 µM)	1.0
Taq 2X Master Mix	25
Total Volume	50

Note: The Taq 2X master mix contains ampliconTaq DNA polymerase, Ammonium buffer system, dNTPs and magnesium chloride (25 mM)

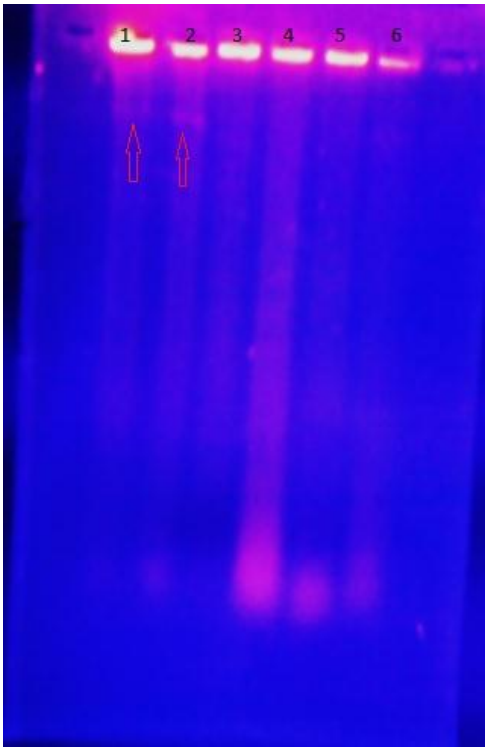

Fig. 1:

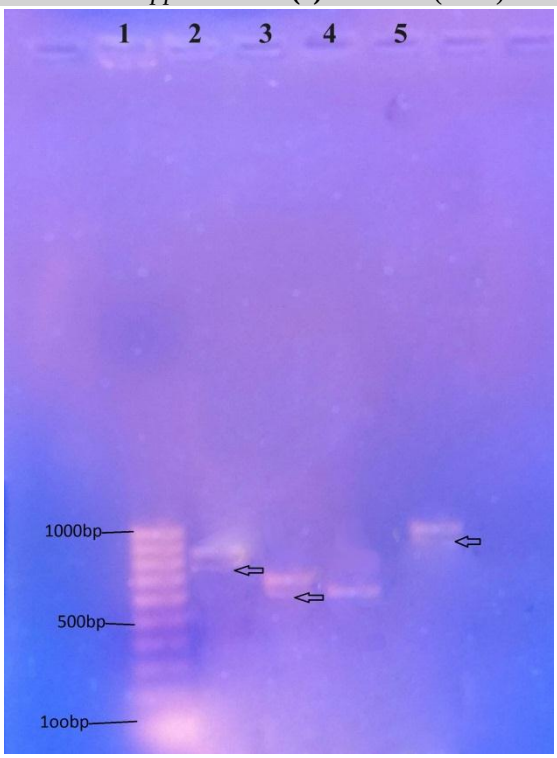

Fig. 2:

DISCUSSION

The present PCR studies demonstrated that *Aeromonas punctata* was able to grow and reduce azo dye Reactive Black 5, indicating that genes for azoreductase, peroxidases, laccases and monoxygenases was functionally expressed in the bacterium on plasmid. Khaled *et al.* reported the presence of plasmid of 3 kb size having azo dye degrading gene in lactic acid bacterial isolates[16]. Kloos *et al.* amplified the expected band of 550 bp from genomic DNA of the strains *Acinetobacter* sp. ADP1 and *P. putida* GPo1 containing alkM or alkB, for monoxygenase respectively[15]. Young observed a faint band approximately 500 bp in size in *E. coli* strains after PCR reaction for monoxygenase[9]. According to Chen *et al*., a genomic DNA fragment of *S. aureus* ATCC 25923 yielded a DNA band of about 700 bp on agarose gel[6]. An expected size of 589 bp DNA fragment was obtained from the genomic DNA of Ascomycete GHJ-4 strain by PCR amplification for peroxidase[12]. Dhakar and Pandey obtained a PCR amplified sequence of laccase gene fragment (corresponding to copper binding domain) with size of approximately 200 bp from a strain of *Trametes hirsute* (MTCC 11397)[14]. The azoreductase gene was amplified by PCR from the genomic DNA of strain KF46F using a set of primers which created new *Nde*I and *Bam*HI restriction sites and was functionally expressed in *E.coli*[17].

Conflict of Interest: Authors have no conflict of interest

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