Research Article

MOLECULAR CHARACTERIZATION AND OPTIMIZATION OF BACTERIA IN AZO DYE DEGRADATION

G. MILIKLI*, C.S.V.RAMACHANDRA RAO
Department of Biotechnology, MIC College of Technology, JNTUK, Kakinada, AP, India.

* Corresponding author email: milikli.bio2312@gmail.com

ABSTRACT

Azo dyes belong to the most important group of synthetic colorants and are used extensively in the textile industries for dying cotton, wool, silk, nylon, etc. They are generally considered as the xenobiotic compounds, which are very recalcitrant to biodegradation. The most affected is the water because both terrestrial and aquatic life will be adversely affected, and causes diseases by the presence of hazardous particles and changes the quality of water by all means like odour, quality and there by increases the economic activity. The organism isolated from contaminated sites of textile industry soil sample, that degrade Bromophenolblue was subjected to 16s rRNA and the organism was characterized as Bacillus subtilis. It was able to decolorize 92% of Bromophenolblue dye (5 mg/l) within 12hr at optimized conditions pH 8, temperature 40ºC, and carbon source (glucose).

KEYWORDS: Xenobiotic, Bromophenolblue, Biodegradation, Bacillus subtilis.

INTRODUCTION

Major classes of synthetic dyes are Azo dyes. Yearly worldwide 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced. Azo dyes, which are aromatic compounds with one or more (−N=N−) groups. The most commonly used synthetic dyes in the textile, food, paper making, printing, leather and cosmetic industries. They are generally considered as the xenobiotic compounds, which are very recalcitrant to biodegradation1,2. During the dyeing processes about 10–90% of the dye stuff do not bind to the fibers and therefore, released into the sewage treatment system or the environment1,3. The inefficiency in dying process has resulted in 10-15% of unused dye stuff entering the wastewater directly. Improper textile dye effluent disposal in aquatic ecosystems leads to the reduction in sunlight penetration which in turn decreases photosynthetic activity, dissolved oxygen concentration, water quality and depicts acute toxic effects on aquatic flora and fauna, causing severe environmental problems worldwide. In addition to their visual effect, their adverse impact in terms of chemical oxygen demand (COD), and their metabolites are toxic, carcinogenic, mutagenic, leading to potential health hazard to humankind. Removal of hazardous industrial effluents is one of the growing needs of the present time4 & 5.

The dye degradation of physical/chemical methods has inherent drawbacks like economically unfeasible (more energy and chemicals), unable to remove the recalcitrant triphenylmethane dyes and their organic metabolites completely from such effluents. Generation of significant amount of sludge during chemical treatment may cause secondary pollution problems and substantially increases the cost of the treatments.

Biological degradation, being inexpensive and ecofriendly, is considered a valuable removal method for many toxic pollutants. Several microorganisms, including a number of bacteria, yeast, and fungi, have been investigated for their ability to biodecolorize triphenylmethane dyes6,7,8,9,10,11,12.
This study aims to investigate the potential of *Bacillus subtilis* isolated from textile soil for decolorizing a solution containing a Bromophenolblue dye at various optimized conditions like dye concentration, temperature, pH, and carbon source.

**MATERIALS AND METHODS**

**Sample collection**

Soil sample was collected from contaminated sites of Mangalagiri textile industry, Guntur (DT), for isolating Bromophenolblue (BPB) degrading organism. Using sterile spatula, removed a wedge in the shape of V which is approximately 6 inches depth. The collected soil sample was placed in a sterile polythene bags and stored at 4°C until use.

**Isolation and screening of bacterial strains**

The soil sample collected from textile industry was subjected to serial dilution (Madigan *et al.* 2000)\(^\text{13}\). From that 10\(^{-5}\) and 10\(^{-6}\) dilutions are plated on Nutrient agar (peptone 5g/l, NaCl 5g/l, yeast extract 1.5g/l, beef extract 1.5g/l, agar20g/l temperature-25°C, pH7.4±0.2) by spread plate method. The single isolated colonies were sub cultured on NB (peptone 5g/l, NaCl 5g/l, yeast extract 1.5g/l, beef extract 1.5g/l, agar20g/l temperature-25°C, pH7.4±0.2) agar plates. Pure culture was maintained on LB (Luria Bertani) agar plates.

The three purified colonies from 10\(^{-5}\) and 10\(^{-6}\) dilution were streaked on the screening media (Prepare 30ml LB agar medium and 5mg/l Bromophenolblue dye was added to medium) and were kept for incubation for 1day for preliminary identification of dye degrading bacteria. A control plate was also maintained for comparison.

**Genomic DNA isolation**

Genomic DNA was isolated using the Method described by Krsek and Wellington (1999)\(^\text{14}\), with some modifications. The 1.5 ml overnight grown culture was suspended in 2 ml of a lysozyme solution (150mM NaCl, 100 mM EDTA, 5 mg/ml lysozyme). The tube was inverted several times to mix the contents and placed in a 37°C water bath for 2hr. Subsequently, 500µl proteinase K (2.5mg/ml) was added, and the contents of the tube gently mixed and placed in a 55°C water bath for 15min and centrifuged at 10,000rpm for 2min. From the supernatant 500µl was transferred into a fresh tube to that an equivalent volume(0.5ml) of phenol-chloroform-isoamyl alcohol (25:24:1) was added and the mixture gently vortexed for 1 min. To precipitate DNA, 0.7 vol. cooled isopropanol and 1/10 vol. 3M sodium acetate were added to the supernatant. The mixture was gently mixed and kept at -20°C overnight. The sample was centrifuged at 4100rpm for 10min, and the pellet was washed three times with cold 70% ethanol and then resuspended in 100µlTE buffer 10/0.1 (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). For visualizing the DNA extract, 10µl of each extract was electrophoresed on 1% agarose gel in 1X TAE buffer and examined under UV light.

**Strain characterization**

The Genomic DNA was isolated from the overnight grown bacterial culture was amplified with universal bacterial primers. The 25µl of reaction mixture contains, 15µl of master mix(NEB,USA) (10X assay buffer, DNTP’s, Taq), 1µl of forward primer (GGCGAACGGGTAGTAA), 1µl of reverse primer (ACTGCTGCCTCCCGTAG), 2µl of template DNA and 6µl of distilled water using thermal cycler (Corbett Research, Australia). PCR was carried out by using the Thermal cycler under the following conditions- 30 cycles of 94°C for 4min, 94°C for 1min, 52°C for 1min, 72°C for 1.15min followed by final extension at 72°C for 1min and holding temperature at 10°C for 1min. The amplified DNA fragments were observed by agarose gel electrophoresis in 2% agarose gel and sequenced. The unknown bacterium was identified using GenBank database.

Partial 16S rRNA sequences obtained from isolates were assembled in a contig using the phred/Phrap/CONSED program\(^\text{15,16}\). Identification was achieved by comparing the contiguous 16S rRNA sequences obtained with the 16S rRNA sequence data and type strains available in the public databases GenBank using the BLASTn sequence match routines. The sequences were aligned using the CLUSTAL X program and analyzed with the MEGA software 2001\(^\text{17,18}\). Evolutionary distances were derived from sequence-pair dissimilarities, calculated as implemented in MEGA, using Kimura’s DNA substitution model\(^\text{19}\).

The phylogenetic reconstruction was done using the neighbourjoining (NJ) algorithm, with bootstrap values calculated from 1000 replicate runs, using the routines included in the MEGA software\(^\text{20}\).

**DECOLORIZATION EXPERIMENTS**
**Effect of initial dye concentration on decolorization**

The decolorization of Bromophenolblue blue was studied at different concentrations of the dye (5, 10, 20, 30, 40, 50 and 60 mg/l) as described by El-Naggar MA, (2004)\(^{21}\). The Bromophenolblue blue dye was added separately to overnight incubated bacterial (*Bcillus subtilis*) culture, isolated from Industrial Textile soil. The 3ml of the culture media was withdrawn at different time intervals. Aliquot was centrifuged at 10000rpm for 5minutes to separate the bacterial cell mass, clear supernatant was used to measure the decolorization at the absorbance maxima of the respective dyes\(^{22}\). Controls were carried out in the same conditions but without inoculum. Concentration of dyes were measured by observing OD at 620nm.

The percentage decolorization was calculated as follows:

\[
\text{% Degradation} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100
\]

**Effect of pH on decolorization**

Decolorization of dyes was determined as relative in decrease in absorbance for each dye at their absorbance maximum at particular time interval. The effect of pH on Bromophenolblue dye decolorization was studied by performing the experiment at different pH values from pH 6-pH 10 using 1M NAOH and 1M HCL and at 620nm absorbance was measured by spectrophotometer\(^{23}\).

**Effect of Temperature on decolorization**

For studying the effects of temperature on decolorization of Bromophenolblue dye, the bacterial culture was incubated at different temperatures from 10ºC-40ºC. After 24hrs incubation the Bromophenolblue dye was added separately to cultures. Dye absorbance was measured by spectrophotometer at 620nm at time intervals.

**Effect of carbon source on decolorization**

For enhancing the decolorization of Bromophenolblue was observed by addition of different carbon sources to culture media. The rate of decolorization was decreased with increasing concentration of dye. Absorbance was measured by spectrophotometer at 620nm\(^{24}\).

**RESULTS AND DISCUSSION**

**Isolation and screening of bacterial strains**

Soil sample collected from the Mangalagiri textile industry, Guntur dist, AP, India contaminated sites, led to the isolation of 3 morphologically different bacterial isolates. The pure cultures were grown and preserved on LB (Luria Bertani) agar medium at 4ºC. All 3 isolates were tested individually for their ability to decolorize Bromophenol blue on LB Agar plates.

In LB agar medium, after overnight incubation the plates were observed for decolorization of the dye by growing the culture on comparison with control. The disappearance of color and change in original color in the around the bacteria growth on agar plates were observed. The evaluation of decolorization was assessed as the disappearance of color during the growth of the bacterium. Bromophenol blue was decolorized by Gram-positive isolate, and further characterized by molecular level.

**Genomic DNA isolation**

The genomic DNA extracted with enzymatic lysis method (Fig.1). The highest yield of DNA to be obtained and preserved with TE buffer. The extracted DNA was electrophorised on 1% Agarose gel with etidium bromide. The bands were observed under UV-transilluminator.

**Fig 1:** Agarose gel electrophoresis of genomic DNA. Lanes 1 and 2 were isolated Genomic DNA of soil sample collected from dye industry.

**PCR amplification of bacterial DNA**

The DNA extraction provided a good yield of DNA, which could be used in 16S gene amplification with bacterial universal primers (Figure 2). The amplified sample and ladder was electrophorised on 2% Agarose gel and compared. The bands were observed under UV-transilluminator.
Phylogenetic tree: The dye degrading bacterial culture was molecular characterized based on the 16sr RNA gene was sequenced at MWG Eurofins Bangalore by using ABI 3600 system.

16s r RNA sequence report

>seq1
GCTATACATGCAGTCCGAGCGGAACAGATGGGA
GCTTGCTCCCTGATGTTAGCGGCGGACGGGT
GAGTAACA
CGTGGGTAACCTGCCTGTAAGACTGGGATAA
CTCCGGGGAAAAACCGGGGCTAATACCGGATGGT
TGTTTGAA
CCGCATGGTTCAAACATAAAAAGGTGGCTTGCG
GCTACACTTACAGATGGACCCGCGGCGAT
TAGCTAGT
TGGTGAGGTAATGGCTCACCAAGGCACGTG
AGCTGGACT
GGGATTAGGAGGAGGAGCTGGGG
GGCGAGGAC
AGGAGGAGATGGGAACACCAGTGCGAAGG
AGTGCTCGTAAGCTCGTAGCTCAGCAAGCGCT
GGCTACAAAAAGGTGGCTTCAG
GCTACACTTACAGATGGACCCGCGGCGCAT
TAGCTAGT
TGGTGAGGTAATGGCTCACCAAGGCACGTG
GCTACACTTACAGATGGACCCGCGGCGCAT
TAGCTAGT
TGGTGAGGTAATGGCTCACCAAGGCACGTG
GGCTACAAAAGGTGGCTTCAG
GCTACACTTACAGATGGACCCGCGGCGCAT
TAGCTAGT
TGGTGAGGTAATGGCTCACCAAGGCACGTG
GGCTACAAAAGGTGGCTTCAG
GCTACACTTACAGATGGACCCGCGGCGCAT
TAGCTAGT
TGGTGAGGTAATGGCTCACCAAGGCACGTG
GGCTACAAAAGGTGGCTTCAG
GCTACACTTACAGATGGACCCGCGGCGCAT
TAGCTAGT
Effect of initial dye concentration on decolorization
The decolorization of BPB was studied at various increasing concentration of dye i.e. from 5, 10, 20, 30, 40, 50, 60 mg/L. The rate of decolorization was decreased with increasing concentration of dye. 65% decolorization of BPB was observed at 5mg/L, concentration within 24 h respectively. Only 51%, 33%, 29%, 24%, 23% and 16% decolorization was observed at 10, 20, 30, 40, 50, 60 mg/L dye concentration respectively. These results indicate toxicity of BPB at higher dye concentration.

Effect of PH on decolorization
The effect of pH on dye decolorization was studied by performing the experiment at pH6-pH11. 70% decolorization of BPB was observed at Ph8, within 12h respectively. Only 39, 67, 64%, 61% and 4% decolorization was observed at ph 6, 7, 9, 10 and 11 dye concentrations respectively. The maximum degradation was attained at pH 8.0 with in 12 hrs and the decolorization percentage was reported as 70% which was showed in the Graph (2).

Effect of Temperature on decolorization
For studying the effects of temperature on decolorization of Bromophenolblue the culture was incubated at 10-45ºC. 88% decolorization of BPB was observed at 40ºC within 12h. Only 83%, 52%, 27%, 10% degradation was observed at 20ºC, 30ºC, 37ºC, 45ºC respectively and no decolorization was observed at 10ºC temperature.

Effect of carbon source on decolorization
In LB medium, only 70% decolorization of BPB was observed in 24 h. In an attempt to enhance decolorization performance with extra supplements of carbon source in LB medium, found 92% decolorization was observed with glucose in 12h. Only 68% and 46% decolorization observed at fructose and starch respectively.
DISCUSSION

The textile industries are multi-chemical utilizing concerns of which dyes of various types are of importance. During the dyeing process a substantial amount of dyes and other chemicals are lost in the waste water. Estimates put the dye losses at between 10–15% (Vaidya and Datye, 1982). Though not generally toxic to the environment, dyes color water bodies and may hinder light penetration thereby affecting aquatic life and limiting the utilization (Ajayi and Osibanjo, 1980; Goncalves et al.,2000). In the present study the isolated bacterium decolorized the dye substrates. The decolorizing efficiency was dependent on the growth of the isolate. There was neither growth nor decolorization in the control flasks; this showed that the decolorization was due to the metabolic activity of the organisms and not due to any Abiotic factors.

The isolated strain from the soil was Bacillus subtilis is a gram positive, rod shaped. Decolorization of synthetic dyes is due to the cleavage of the chromophoric group which generates colourless metabolic intermediates. These intermediate metabolites of the dye substrates are aromatic amines (Ganesh, 1992; Brown and DeVito, 1993). The results obtained in Table 3 show that the chromophoric groups of the dye were cleaved. The cleavage of the chromophoric group of dyes is a reduction process which requires redox equivalents (electron donars) that transfer electrons to the chromophoric group (electron acceptor) of dyes (Russ et al., 2000).

It was found that change in pH significantly affect the decolorization rate. The culture shown growth at pH 7, 8, 9 and 10 while at 3 and 5 was found to be inhibitory for the growth. Bacterial cultures generally exhibit maximum decolorization at pH values near 7.0 - 8.0. Decolorization was the fastest at pH 10 and the pseudo-first-rate constant gradually decreased with the lowering of pH. Similar results were also reported by Hustert and Zepp (1992) and Tang and An (1995) for acidic azo dyes bearing sulfonate groups, whose degradation was the fastest at more alkaline pHs.

The dye decolorizing ability of culture found to increases in incubation temperature with maximum activity attained at 40ºC, further increase in incubation temperature to 50ºC results in many fold reduction in decolorization activity of the culture. The dye decolorization activity of mix consortia was found to increase with increase in incubation temperature with maximum activity attained at 40°C. Although a lag phase was observed and the decolorization rate was comparatively low at 22°C, Decolorizing activity was significantly suppressed at 42°C, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 42°C (Cutin and Donmez, 2006; Panswad and Luangdilok, 2000).

In preliminary studies, it was observed that for the initial biodegradation of the synthesized azo dyes, an additional carbon source must be present in the culture medium confirming the results obtained by other authors (Blondeau, 1989; Morgan et al.,1993). Different carbon sources were supplied with yeast extract like glucose, fructose, and starch at 5% concentration. The culture exhibited maximum decolorization activity in presence of glucose and as compared to fructose and starch, the activity possibly due to glucose, serves as a source of reducing equivalent and at lower glucose concentration the reducing equivalent generated decolorization, alternately glucose may enhanced decolorization by allowing faster growth of actively respiring bacteria resulting in rapid decolorization.

This rate of decolorization may be due to the high metabolic diversity being seen in the halophiles due to their extremophilic nature.

CONCLUSION

The present research indicates the potential aspects of Bacillus subtilis to 92% decolorize and degrade bromophenol blue. The culture has ability to decolorize Bromophenolblue in optimizing the various parameters like pH, temperature and carbon source within less time, which is significant for its
commercial and industrial application. The results showed that the decolorization depend on dye concentration, initial inoculum size, pH and temperature. Over the range of 10–45°C the decolorization rate increased with the temperature rise. The optimal decolorization pH was attributed at pH 8. These results indicate that the color removal by Bacillus subtilis may be largely attributed to biodegradation. This strain has also the ability to decolorize other dyes including textile dyes and the use of cheap sources for decolorization. Overall results suggested the ability of Bacillus subtilis for the decolorization of azo dye and ensured the ecofriendly degradation of Bromophenol blue.

REFERENCES


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