Original Research Article

Studies on Pseudomonas otitidis SA1 as a novel azo dye (Congo red) degrading strain isolated from grit tank of tannery effluent treatment plant

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Abstract
Azo dyes represent a major group of dyes causing environmental concern because of their colour, biorecalcitrance nature and potential toxicity to living beings. Various physico-chemical methods have been used to eliminate the coloured effluent from wastewaters. These methods have disadvantages of being highly expensive, coupled with the formation of huge amount of sludge and emission of toxic waste. Today is the era of bioremediation as biological methods are eco-friendly. In the present investigation, the bacterial isolate Pseudomonas otitidis SA1 (isolated from Grit Tank of Tannery Effluent Treatment Plant) has been reported to decolourize the dye (Congo red) at a concentration of 100 mg L\(^{-1}\) upto 94% within 24 h in static conditions. The temperature and pH for optimum growth and activity of the isolate were found as 37 °C and 7.0, respectively. The isolate (SA1) may be a potential strain for biological treatment of effluents of tannery industries in future.

Keywords: Biodegradation, Decolourization, Congo red, Tannery effluent, Pseudomonas otitidis

1. Introduction
The disposal of industrial waste is becoming trouble all over the world. In recent years, industrial effluents have been regarded as common source of pollution due to lack of proficient treatment methods and inappropriate disposal modes generated by industries. Dyes and dyestuffs are extensively used within the food, pharmaceutical, cosmetic, textile and leather processing industries. Approximately 75% of the dyes discharged by industries belong to the classes of reactive (~36%), acid (~25%) and direct (~15%) dyes. It has been reported that 10-15% dyes used are released into wastewaters. Their release in the aqueous bionetworks causes serious environmental concerns along with health (Fang et al., 2004; Asad et al., 2007; Elisangela et al., 2009). Among these, azo dyes are the most widely used; accounting for more than 60% of the total number of dye structures known to be produced (Zollinger et al., 1991). These do not bind to the fibers and thus get released into sewage treatment system or the environment (Clarke et al., 1980). Tannery effluent is one of the major industrial pollutants having BOD, COD, pH, dyes and metal ions. Their discharge into surface water obstructs light penetration and oxygen transfer into water bodies (Pinheiro et al., 2004). Under these conditions aquatic life suffers, resulting in loss of productivity and deterioration of water quality to such an extent that it becomes unusable. Also, dyes impart colour to wastewater which is highly visible and affects its esthetics, transparency and gas solubility. Therefore dye wastewaters have to be treated (Banat et al., 1996). In current practices, new processes for dye degradation and wastewater reutilization have been developed.

Various physical and chemical methods have been recommended for the treatment of dye contaminated wastewater but due to high input cost along with secondary pollution generation; such methods are less commonly preferred (Jadhav et al., 2007). Microbial decolourization and degradation has appeared as an environmentally pleasant and cost-competitive alternative to chemical decomposition processes (Pearce et al., 2003; Ozdemir et al., 2008).

Several bacterial strains have been reported (Kheha et al., 2005; Olukanni et al., 2006; Lin and Leu, 2008) for decolourizing azo dyes under aerobic (Xenophylyusazovorans KF46F, Kerstersia sp. strain VKY1, Bacillus sp., and Staphylococcus sp.) and anaerobic (Eubacterium sp., Clostridium sp., Sphingomonas xenophaga BN6, Butyrivibrio sp. or Bacteroides sp.) conditions. Apparently there is a need to expand novel biological decolourization processes leading to the more efficient clean-up of azo dyes using a single microorganism. Thus, the
main objective of present investigation was to observe the azo dye degradation (congo red) using novel bacterial isolate from grit tank of tannery effluent treatment plant under optimized cultural conditions.

2. Materials and Methods

2.1. Dyestuff and chemicals

Congo red commonly used azo dye, was chosen for the screening of dye degradative bacteria. All the chemicals used were of highest purity and of analytical grade.

2.2. Sample Collection

Effluent and soil samples were collected from the effluent treatment plant present at Jajmau, Kanpur (88° 22'E longitude and 26° 26'N latitude) in Uttar Pradesh (India). All the samples were collected in sterile containers and bags.

2.3. Isolation

Bacteria were isolated from tannery effluent and soil irrigated with these effluents using serial dilution technique. Approximately 0.1 ml aliquots of appropriate dilutions were poured on nutrient agar plates and incubated at 37 °C for 24 to 48 h. Individual bacterial colonies which varied in shape and colour were picked and purified by repeated sub culturing on the respective medium.

2.4. Screening

2.4.1. Primary Screening

Inoculum was prepared aerobically by growing the cells at 37 °C for 24 h in Luria Bertani (LB) media at pH 7.0. For screening of isolates, LB media containing dye (100 mg L⁻¹) was inoculated with 24 h old precultured cells 1.0% (v/v). The decolourization of dye was monitored after every 24 h interval. Primary screening was done only on the visibility basis i.e. change in colour of media containing respective dye.

2.4.2. Secondary Screening

In secondary screening, decolourization (%) was measured as decrease in optical density using spectrophotometer (Systronics PC based double beam spectrophotometer 2202). Decolourization (%) was calculated by following formula: (Parshetti et al., 2010).

\[
\text{Decolorization (\%)} = \left[ \frac{(A₀-Aᵣ)}{A₀} \right] \times 100 \\
\]

where, \(A₀\): Initial absorbance; \(Aᵣ\): Final absorbance

2.5. Optimization of cultural conditions

2.5.1. Effect of temperature

The effect of temperature on the decolourization was studied by incubating the LB media containing dye (100 mg L⁻¹) under a range of temperature (5 °C to 55 °C) at pH 7.0.

2.5.2. Effect of pH

The effect of pH on decolourization was studied by inoculating LB media containing dye (100 mg L⁻¹) at different pH values (5.0 to 10.0) keeping temperature constant 37 °C.

2.6. Molecular Characterization

Total genomic DNA of bacteria was isolated using Charles and Nester method (1993) with slight modifications. Pure bacterium culture was grown in 10 ml nutrient broth for 18 to 24 h. The bacterial pellet was washed in 1.5 ml of 0.85% NaCl, centrifuged for 2 min at 12,000 rpm and was resuspended in 0.4 ml Tris-EDTA buffer. Cell lysis was done by adding 20 µl of 25% SDS, 50 µl of 1% lysozyme and 50 µl of 5M NaCl followed by incubation at 68 °C for 30 min in a circulatory water bath. For protein precipitation, 260 µl of 7.5M ammonium acetate solution was added to the microcentrifuge tubes and kept in ice for 20 min followed by centrifugation at 12,000 rpm for 15 min at 20 °C. Supernatant was carefully pipetted out in another fresh, sterile microcentrifuge tube in which 1 µl RNase (4 mg ml⁻¹) was added followed by incubation at 37 °C for 20 min. Equal volume of chloroform was added in the tubes and RNA was precipitated by centrifuging at 12,000 rpm for 1 min. The top layer containing total cell DNA was pipetted out in fresh microfuge tube and used for next step. DNA was precipitated by adding 0.8 volume of isopropanol followed by incubation on ice for 30 min and pellet down by centrifuging at 10,000 rpm for 15 min. DNA was further washed with 0.5 ml of 70% ethanol and spun down at 10,000 rpm for 1 min. Pure DNA sample was then suspended in 20 µl Tris-EDTA buffer or deionized water and stored at 4 °C for further use.

2.6.1. Agarose gel electrophoresis
The genomic DNA sample of bacteria was quantified though agarose gel electrophoresis by analyzing the migration on 0.8% agarose gel prepared in 0.5 M Tris-borate-EDTA (TBE) buffer and run in an electrophoresis tank filled with the same concentration of TBE buffer. The genomic DNA was diluted with Tris-EDTA buffer so as to achieve a concentration of 50 ng in 10 µL to be used as a template DNA in PCR amplification reaction.

2.6.2. 16S rRNA PCR-Amplification

The universal forward and reverse primers were custom synthesized from “Ocimum link biotech” Hyderabad, India. The sequences of the oligonucleotide primers used for amplification of 16S rRNA genes were:

Forward primer
16SF (5'-AGAGTTTGATCCTGGCTCAG-3’)

Reverse primer
16SR (5'-ACGGCTACCTTGTTACGACTT-3’)

The stock solution (100 ng ml⁻¹) of primers was prepared by reconstituting lyophilized primers in sterilized deionized (milliQ) water and stored at 20 °C.

2.6.3. 16S rRNA sequencing and Phylogenetic relationship

Phylogenetic identity of bacteria was determined by BLASTn result and sequences were aligned using alignment software i.e. ClustalW. Phylogeny calculations and dendrogram was constructed by Mega 5.05 software package using UPGMA method. Bootstrap analysis (Felsenstein, 1985) was conducted using 1000 replicates samplings of data.

3. Results and Discussion

3.1. Isolation

From the samples collected, total 136 different bacterial isolates were obtained. About 46 isolates were obtained from tannery effluent and remaining 90 from soils and fields irrigated with this effluent.

3.2. Screening

3.2.1. Primary Screening

Out of 136 isolated bacteria, only 4 bacteria (Fig 1.) possessed the capability of showing visible decolourization of congo red (100 mg L⁻¹) within 5 days. Then these 04 selected bacteria were used in secondary screening.

3.2.2. Secondary Screening

Four isolates selected from primary screening were further used in secondary screening. The rate of decolourization was calculated by decolourization assay. Among 4 selected isolates, the isolate SA1 (Fig 2.) showed significant decolourization (88.99%, 5 days). This potent isolate was further selected for optimization of cultural conditions.

3.3. Optimization of cultural conditions

3.3.1. Effect of Temperature

The isolate SA1 showed maximum decolourization at 37 °C (93.31% ± 0.32). It was observed that rate of decolourization increased from 20 °C to 40 °C and afterwards it has been significantly affected with temperature change (Fig 1.). Wong et al. (1998) reported methyl red (MR) degradation by Klebsiella pneumoniae RS-13 and Acetobacter liquefaciens S-1. Their study indicated that both K. pneumoniae RS-13 and A. liquefaciens S-1 decolourized MR by cleaving it into 2-aminobenzoic acid (ABA) and N, N-dimethyl-p-phenylenediamine (DMPD). Maximum degradation of DMPD was found in temperature range of 30 to 37 °C whereas no decolourization was observed at 45 °C. Also, Bacillus subtilis (RA-29) showed significant (95.67%) congo red decolourization at 37 °C and increase in temperature beyond 37 °C led to decline in decolourization activity of the strain (Kumar and Sawhney, 2011).

3.3.2. Effect of pH

The effect of pH was studied by inoculating the 1% culture (v/v) into LB media containing dye (100 mg L⁻¹) at different pH (5.0 to 10.0) and decolourization was measured at 560 nm. The optimum pH for maximum decolourization (94% ± 0.69) was found to be 7.0 (Fig 4.). Decolourization of congo red increased with increase of pH and has been drastically affected after pH 8.0. Similar observations were reported by Moosvi et al. (2005). The gradual increase in congo red decolourization was observed from pH 5.0 to 8.0 by Bacillus subtilis also and maximum decolourization was found at pH 8.0 (Kumar and Sawhney, 2011). Mali et al. (2000) found that pH between 6.0 and 8.0 was optimum for decolourization of triphenylmethane and azo dyes by Pseudomonas sp. Also, Pseudomonas putida has been reported as the best decolourizer (Acid Orange 10; 90%) (Tripathi and Srivastava, 2011).
3.4. Molecular Characterization

3.4.1. 16S rRNA sequence analysis

PCR amplification of 16S rRNA gene for isolate SA1 produced an amplification product of approximately 1489 bp. The partial 16S rRNA gene has been sequenced and submitted to NCBI Genbank (Accession number KC540914). The alignment of the retrieved sequences from the NCBI database with 16S rRNA gene of SA1 showed sequence homology to Pseudomonas otitidis (Fig 5.).

4. Conclusion

The congo red decolourizing isolate SA1 (isolated from grit channel of Tannery Effluent Treatment Plant) has been identified as Pseudomonas otitidis. The azo dye was maximally (94%) decolourized at optimal conditions (pH 7.0; Temp. 37 °C) under static conditions in 24 h. Thus SA1 could be exploited for its bioremediation ability to treat azo dye contaminated aqueous ecosystem. Moreover, further studies on this isolate could explore new tools and techniques to evolve commercially viable and ecofriendly microbial solutions for treatment of dye industry effluents.

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References

Fig. 1: Decolourization (%) of congo red

Fig. 2: Decolourization of congo red

A: Control, B: Remediated dye

Fig. 3: Effect of temperature on decolourization

Fig. 4: Effect of pH on decolourization

Fig. 5: Phylogenetic tree showing interrelationship of SA1 with the other closely related species


