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Biodegradation of carcinogenic textile azo dyes using bacterial isolates of mangrove sediment

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the degradation/decolouration pattern by novel bacteria, *Paenibacillus* sp. The decolouration effects, growth kinetics were assessed. In addition, potent dye degrading property of the bacteria can be used by the scientific community for the welfare of our society.
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ABSTRACT

Objective: To evaluate the biodegrading property against carcinogenic azo dyes using bacterial isolates of mangrove sediment.
Methods: The bacterial isolates were subjected to submerged fermentation and their growth kinetics were studied. The potential strain was characterized using 16S rDNA sequencing.
Results: In the present study, dye degrading bacterial colonies were isolated from the mangrove sediment samples of Parangipettai estuarine area, Tamil Nadu. Of the 30 morphologically different strains isolated, 5 showed antagonistic property. The growth kinetics of the two strains, P1 and G1, which showed potent activity were calculated. One particular isolate (P1) showing promising dye degrading potential in the submerged fermentation was further characterized. The strain was identified as *Paenibacillus* sp. by 16S rDNA sequencing.
Conclusions: This study reveals the less explored microflora of mangrove sediments. The novel strain may further be analyzed and used in the treatment of effluent from dye industry so as to reduce the impact of carcinogenic contaminants.

KEYWORDS
Biodegradation, Azo dye, Mangrove, 16S rDNA, *Paenibacillus* sp.

1. Introduction

Until the end of the 19th century, all colors used for dyeing were obtained from natural sources, but today the number of synthetic colorants exceeds 7000. Synthetic dyes are cost effective, offer a wide range of new colors, and they impart better properties upon the dyed materials. During the dyeing process around 30% of the dye quantity remains in the aqueous phase, mainly in the hydrolyzed form, leading to the colorization of the resulting effluent system[1].

Even though azo dyes are in general less harmful than older types, many are found to be toxic to fish, mammals, as well as to different kind of microorganisms[2-4]. Azo dyes and their metabolites are reported to be mutagenic or carcinogenic[5,6]. Azo dye compounds have been reported to cause bladder cancer in humans and hepatocarcinoma,

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nuclear anomalies in intestinal epithelial cells in mouse models[7-10]. Aromatic amines, which are known human carcinogens, have been found in the urine of dyestuff workers and also in test animals following the administration of azo dyes[8,11,12].

Synthetic dyes are common contaminants of water, it is estimated that the production of these compounds is around of 10000 tonnes per year and it is assumed that the quantity of dye discharged in the environment is about 1%-10%[13]. The discharge of this wastewater to the environment causes aesthetic problems due to the remaining color and also damages the quality of receiving water. The color impedes sunlight penetration disturbing the ecology, and the dyes and/or their degradation derivatives can prove toxic to aquatic life [2,4,14-19].

In the last few years, environmental legislation, about the appearance of colors in discharges, combined with the increasing cost of water for the industrial sector, has made the treatment and reuse of dyeing effluents increasingly attractive to the industry. The conventional treatment produces a lot of sludge, but does not remove all dyes, thus preventing recycling of the treated wastewater. Scores of physico-chemical methods have been used for the treatment of dye with effluent[20,21]. Physico-chemical methods such as coagulation/flocculation, activated carbon adsorption and reverse osmosis technique have been developed in order to remove the color[20,22]. However the latter methods are not economically feasible can only transfer the contaminants (dyes) from one phase to other leaving the problem essentially unsolved. Microorganisms have now been preferred to other sources because of their unique ability to degrade the toxic chemicals using their cytoplasmic enzymes. Bacterial enzymes like peroxidases, nitro reductases and azo reductases were found to decolorize dyes. These enzymes possess significant potential for the treatment of waste waters, which are colored by azo dyes. Therefore microbial dye degradation has become a very promising approach for effluent treatment.

Various bacterial strains reduce azo dyes under anaerobic conditions. The most generally accepted hypothesis for this phenomenon is that many bacterial strains possess rather unspecific cytoplasmic enzymes, which act as azo reductases and under anaerobic conditions transfer electrons via soluble flavins to the azo dyes[23]. Microbial metabolites are preferred over plant and animal source due to their economic production, consistency, ease of process modification and optimization. They are relatively more stable than corresponding metabolites from plant or animals.

Diverse categories of microorganisms, such as the actinomycetes, fungi and anaerobic and aerobic bacteria, bring about the degradation of dyestuff[24,25]. Various lignolytic fungi were shown to decolorize azo dyes using ligninases, manganese peroxidases, and laccases. Wood rotting fungi have been found to effectively degrade a variety of azo dyes under aerobic conditions[26]. Dye degrading fungi have been frequently used in bioreactors for the decolorization and degradation of azo dyes[27]. Immobilized enzymes could have potential effect for dye decolorization and recycling of effluents[28] without the need of growth substrates. Aerobic bacteria have been described to oxidatively decolorize several classes of dyes, among which azo dyes always turned out to be the most recalcitrant compounds. In contrast, under anaerobic conditions, the decolorization of many azo dyes via reduction of the azo bond has been shown by anaerobic (*Bacteroides* sp., *Eubacterium* sp., and *Clostridium* sp.) and facultative anaerobic bacteria (*Proteus vulgaris* and *Streptococcus faecalis*)[29-33].

The marine biosphere is one of the richest of the earth's innumerable habitats, one of the least studied and characterized fauna[34]. Marine bacteria require sodium, potassium, magnesium ions and some require chloride and ferric ions[35]. Yet one another fact which is leading an increasing interest for exploring and exploiting the marine microfauna for industrial application is their high salt tolerance ability[34]. Most biological degradation of azo dye is carried out by anaerobic bacteria[32,36,37]. Mangrove ecosystems are rich in bacterial flora. The mangrove bacteria exist as symbionts with the plants and animals, saprophytes on dead organic matter, and as parasites on living organisms. Fertility of the mangrove waters results from the decomposition of organic matter and recycling of nutrients[38]. They perform varied activities like photosynthesis, nitrogen fixation, methanogenesis, production of antibiotics and enzymes, *etc*. Mangrove microbes have been reported to degrade a varied range of pollutants such as PAH, crude oil, *etc*[39,40]. Although reports on the dye degrading ability of mangrove are available[41], works on the mangrove associated microbes are very scanty. With this view, the present study was targeted to identify novel dye degrading aerobic bacteria from mangrove environment.

2. Materials and methods

2.1. Sample collection

Mangrove sediment samples were collected from Parangipettai estuarine area (Latitude 11° 29′ N; Longitude 79° 46′ E) in sterile containers. The pH of the sample was analysed and found to be 8.0. The azo dyes Nitomill Brill

crimson, nito green B and methyl red were collected from the retail vendors (Kumbakonam).

2.2. Screening for dye degrading bacteria

One gram of sediment sample was added to a flask containing 1% peptone in distilled water/sea water (1:1) with 25 mg of azo dye. The flask was incubated at room for 72 h in an orbital shaker. The decolorization of the sample was checked at different time points. All the studies were carried out with the distilled water /sea water in the ratio of 1:1.

2.3. Isolation of dye degrading bacteria

The sediment which showed decolorization was serially diluted using sterile distilled water up to 10^{-5} dilutions. An aliquot of 0.1 mL from 10^{-2} to 10^{-5} dilutions was taken and spread on sets of nutrient agar plates containing 25 mg of Nitomill Brill crimson, Nito green B and methyl red. The uninoculated plate was kept as control. The plates were incubated at room temperature for 24 h and observed for growth. Decolorization in plates was observed by fading of dye surrounding the bacterial colonies, compared with the original dye medium as control. Colonies which were morphologically different were counted and streaked on nutrient agar slants. All the strains were preserved at 4 °C until further investigation.

2.4. Evaluation of decolorization

Based on the above observation, the dye degrading bacterial strains were selected and further studied by adopting shake flask technique. The strains were inoculated in flasks containing the azo dyes and incubated in a orbital shaker at 200 r/min for 3 d at 28 °C. The biomass, decoloration and pH were observed every 6 h. The biomass and decoloration were calculated by measuring the OD at specific wavelengths (520 nm, 640 nm and 560 nm for Nitomill brill crimson, Nito green B and methyl red respectively). Decoloration OD was measured by filtering the biomass using 0.2 μm syringe filter[42].

2.5. Growth kinetics

The growth kinetics of the 2 strains which showed promising results in the screening were calculated.

2.5.1. Specific growth rate

The rate of biomass growth per unit biomass concentration is called specific growth rate. This can be formulated based on the following equation

$\mu=(1/X)*(dX/dt)$

On integration the above equation results

$\ln X/X_0=\mu t$

Where, μ=specific growth rate of biomass (h^{-1}), X=Biomass concentration (g/L), X_0=Initial biomass concentration, (dX/dt)=Biomass growth rate (g/L/h).

2.5.2. Doubling time

The time required to double the biomass concentration is the doubling time. This can be calculated using the following equation

$t_d=\ln 2/\mu$

Where, t_d=doubling time (h), μ=specific growth rate of biomass (h^{-1}).

2.6. Characterization of dye degrading bacterial strains

2.6.1. Phenotypic characterization

The colony morphology, gram's staining and the motility of two strains showing antagonistic dye degrading activity were demonstarted.

2.6.2. Biochemical characterization

Cultural and biochemical characters like indole, methyl red, voges prousaker, citrate utilization and oxidase, of the potential strains were studied by adapting standard procedure recommended by Bergey's Manual of Systematic Bacteriology.

2.6.3. Molecular characterization

The potential bacterial isolate (P1), was characterized by 16S rDNA analysis for phylogenetic classification. The exponential phase culture of the isolate was centrifuged at 9 000×g for 5 min. Supernatant was discarded and the pellet was dissolved in 567 μL TE buffer, 30 μL of 10% sodium dodecyl sulfate, and 3 μL proteinase K (60 μg). After 1 h incubation at 37 °C, 100 μL of 5 mol/L NaCl was added and mixed thoroughly[43]. The DNA was then purified as previously described[44]. The 16s rDNA sequences were amplified by polymerase chain reaction (PCR) using universal primers of 27f (AGA GTT TGA TCC TGG CTC AG) and 1490r (AAG GAG GTG ATC CAG CC)[45]. All the primer sequences are presented in 5′ to 3′ orientation. The reaction mixture consisted of 5 μL of 10x buffer (Mg^{2+} free), 5 μL of $MgCl_2$, 10 μL of dNTP mix, 1 μL of each primer 2 μL of template DNA and 0.5 μL of Taq DNA polymerase (5U/μL) (Genei, Bangalore), in a final volume of 50 μL. The amplification conditions were, initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min. Annealing at 50

°C for 1 min, 72 °C for 90 seconds and final extension at 72 °C for 7 min. PCR products were electrophoresed on 1% agarose gel and documented under UV- transillumination. The sequence was generated using an automated DNA sequencer (Applied Biosystems, USA). The work was carried out at Bioserve Biotechnologies, Hyderabad. The partial 16S rRNA gene sequence of the potent bacterial isolate was submitted in NCBI and an accession number was assigned.

3. Results

3.1. Preliminary screening for dye degrading bacteria

Dye decoloration was observed in the flask from the second day which indicated the presence of dye degrading aerobic bacteria in the mangrove sediment samples.

3.2. Isolation of dye degrading bacteria

Morphologically different bacterial colonies which were found to decolorize the specific dyes were observed on the plates. Colony forming units were 34×10^{-4} CFU/g, 45×10^{-4} CFU/g and 58×10^{-4} CFU/g for plates incorporated with Nitomill Brill crimson, methyl red and nito green B respectively. Thirty colonies were found to be effective in decolorising the dye as evidenced by the zone clearance. Among them, five strains (P1, P3, G1, M1 and M2) which were observed in all the three plates were selected for further studies.

3.3. Evaluation of decolorization

Decolorization of dye in the flasks was observed (Figure 1-4). The pH variation during the study was noted given in Table 1. The rate of decoloration of the three dyes by all the five strains with respect to their biomass is graphically represented in Figure 5 to 11. Two strains P1 and G1 exhibited maximum degrading potential.

Figure 1. Nito Green B decolorization by P1.

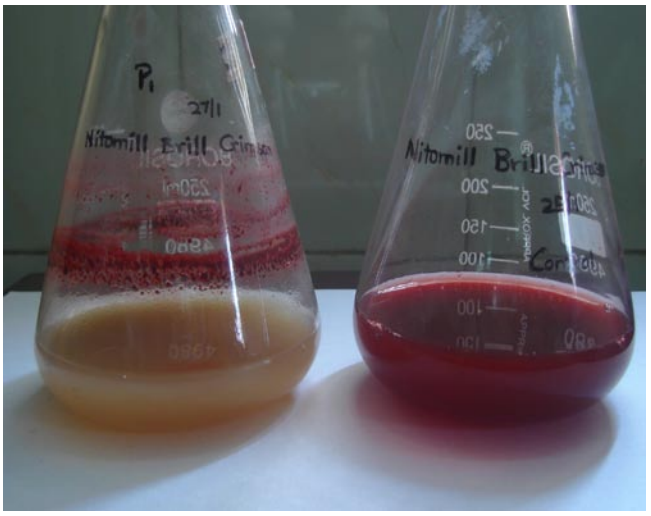

Figure 2. Nitomill Brill crimson decolorization by P1.

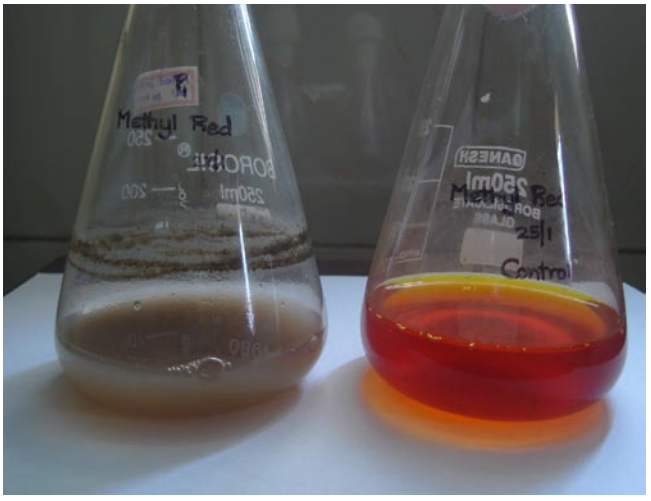

Figure 3. Methyl red decolorization by P1.

Figure 4. Dye decolorization by P1 after shake flask fermentation.

Table 1
pH variation during decoloration.

Stains	1st Day pH (initial)	2nd Day pH	3rd Day pH
P1	8.0	9.3	7.6
P3	8.0	9.1	7.8
G1	8.0	9.7	7.9
M1	8.0	9.5	7.3
M2	8.0	9.4	7.2

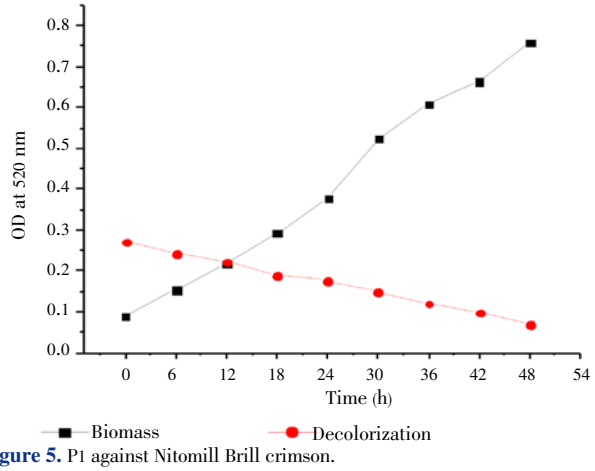

Figure 5. P1 against Nitomill Brill crimson.

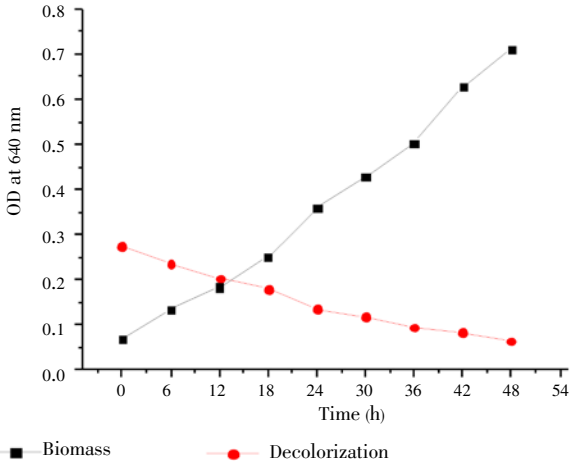

Figure 6. P1 against nito green B.

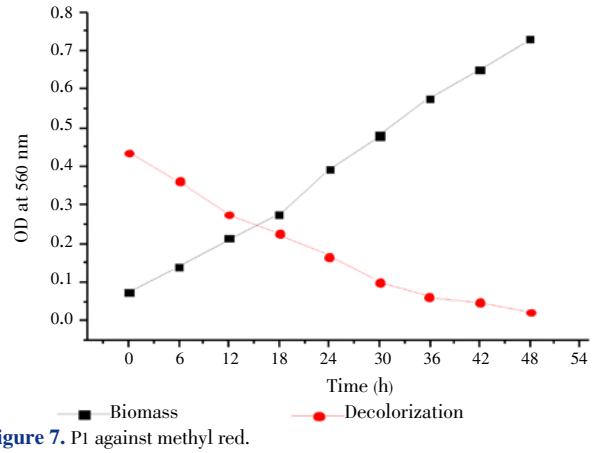

Figure 7. P1 against methyl red.

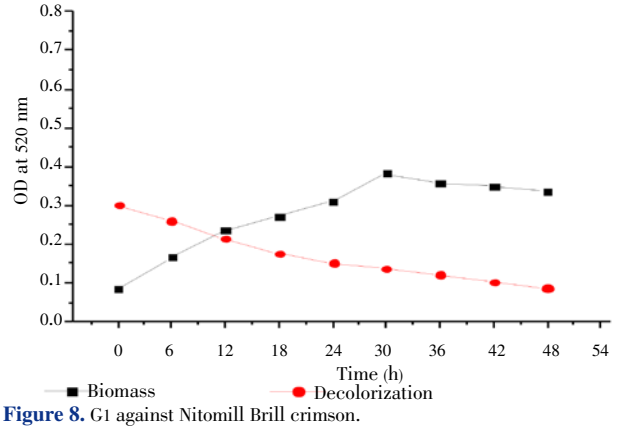

Figure 8. G1 against Nitomill Brill crimson.

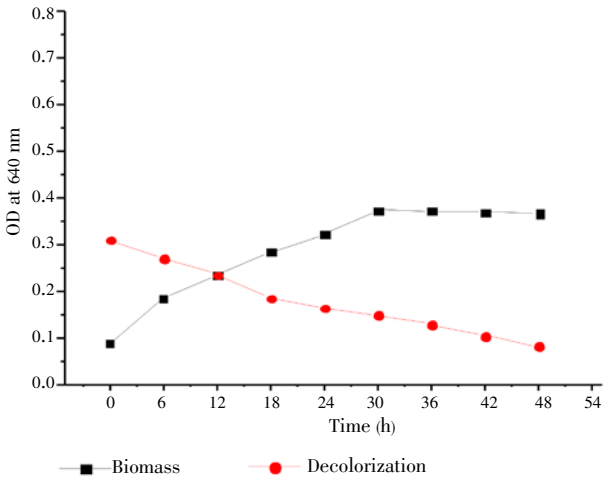

Figure 9. G1 against nito green B.

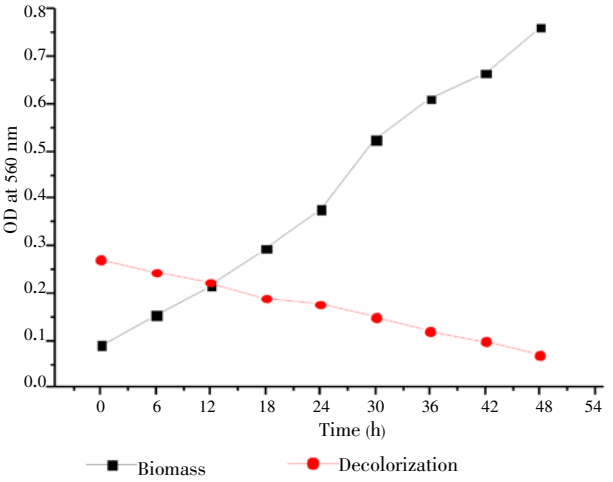

Figure 10. G1 against methyl red.

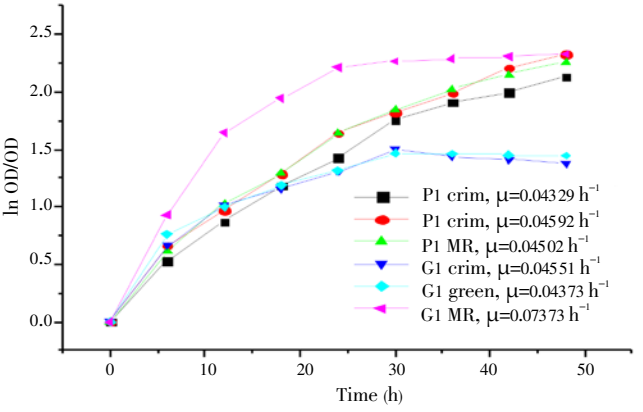

Figure 11. Specific growth rate of the isolates.

3.4. Growth kinetics

The specific growth rate and the doubling time of the two strains P1 and G1 was estimated and plotted graphically (Figure 7). Plotting ln X/X_0 against 't' results in a straight line at the exponential growth phase. μ value was found to be 0.04329, 0.04592, and 0.04502 (h^{-1}) for P1 and 0.04551, 0.04373 and 0.07373 (h^{-1}) for G1 against Nitomill Brill crimson, methyl red and Nito green B respectively.

Table 3
16S rDNA sequence homology.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF415644.1	*Paenibacillus* larvae isolate A 16S ribosomal RNA gene, partial sequence	792	792	99%	0.0	96%
AY530295.1	*Paenibacillus* larvae strain DSM 3615 16S ribosomal RNA gene, complete sequence	792	792	99%	0.0	96%
AY530294.1	*Paenibacillus* larvae subsp. larvae strain DSM 7030 16S ribosomal RNA gene, complete sequence	792	792	99%	0.0	96%
DQ079621.1	*Paenibacillus* larvae isolate 00-1163 16S ribosomal RNA gene, partial sequence	792	792	99%	0.0	96%
DQ079620.1	*Paenibacillus* larvae isolate 03-525 16S ribosomal RNA gene, partial sequence	792	792	99%	0.0	96%
AY030080.1	*Paenibacillus* larvae subsp. pulvifaciens 16S ribosomal RNA gene, partial sequence	792	792	99%	0.0	96%
AB073204.1	*Paenibacillus* larvae gene for 16S rRNA, partial sequence	792	792	99%	0.0	96%
EF187246.1	*Paenibacillus* larvae 16S ribosomal RNA gene, partial sequence	789	789	99%	0.0	96%
EF415646.1	*Paenibacillus* larvae isolate PLP 16S ribosomal RNA gene, partial sequence	789	789	99%	0.0	96%
EF415645.1	*Paenibacillus* larvae isolate B 16S ribosomal RNA gene, partial sequence	789	789	99%	0.0	96%

3.5. Characterization of potential strain

Table 2 shows the phenotypic characteristics such as microscopic appearance, Gram's staining and motility of P1. Cultural and biochemical characteristics of P1 were tabulated (Table 3) and found to be G+ve rod. The amplified 16S rDNA sequence was found to exhibit 96% homology with *Paenibacillus* sp. (Table 3). The new subspecies was named as *Paenibacillus* DD AU. The GenBank accession number for the 16S rDNA sequence of *Paenibacillus* DD AU is GQ327970. Phylogenetic tree was constructed for the same.

Table 2
Screening of isolated potential stains.

Phenotypic Characters	P1
Shape	Rod
Gram's staining	G+ve
Motility	Motile

4. Discussion

Treating waste water from textile industries has become a real challenge in recent years. The main problem occurring is that the color that remains due to the dyestuff used may cause disturbance to the ecological system of the receiving water[15-17]. The removal of dyes from aqueous effluent has received considerable attention within environmental research[19]; furthermore a recent review reveals that biodegradation is a promising method for dye removal[46]. Different combinations of treatment methods have been proposed in order to effectively manage the above waste water.

Anaerobic digestion of the azo dyes decomposes due to the cleavage of the azo bond consequently eliminating the color of waste water[47]. The reduction products (aromatic amines) should then be further treated using aerobic biological treatment methods[18,48-52]. However works on aerobic degradation are scanty. In that view, the present study was attempted to identify bacterial strains that effectively degrade azo dyes.

Mangrove sediments from Parangipettai estuarine area were used for isolation of dye degrading bacteria. Results showed that the microbes of mangrove sediment were potent in degrading both the industrial and laboratory dyes used. Sediment samples showed relatively less growth and the task of recovering the isolates was thriving. Results demonstrated that it is essential to provide the dye for the survival of these bacterial isolates and also the dye alone can supplement these organisms for their further endurance. Results revealed the existence of the dye degrading bacteria in aerobic conditions. Categorization of the colony was done by naked eye observation.

Nutrient source plays a vital role in the isolation of microbes as evident from the present study. Many reports on aerobic degradation of dye were noted. Components like peptone, beef extract, yeast extract, nutrient broth medium[53,54], supplemented with mineral sources like Na_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, $CaCO_3$ and H_3BO_3 have been used for microbial growth[53]. Other enrichment sources include P_2O_5, K_2O, MgO with sulfate salts (Fe, Cu, Mn, B, Zn, Mo)[47]. In the present study 1% peptone along with the dye exhibit bacterial colonies of different size, color and texture. This indicates that the trace amount of peptone with dye has accentuated the microbes to take up the dye. Once the peptone in the media gets depleted, the microbes start utilizing the azo dyes as the sole source of carbon and nitrogen[55]. The dyes used in this study are powdery in nature, grouped under the aryl azo groups in which the azo dyes have a double bonded (N=N) structure for its stability and binding. Possibly these bacterial strains could have cleaved the double bonding thereby using the carbon from the pyrrole rings of the chromophore (dye)[18,48,52].

In the study, the media were prepared using sea water to substitute the low nutrient source. Carbon, oxygen, hydrogen, nitrogen, sulfur and phosphorus are the major elements in the cell's most important macromolecules namely nucleic acids, proteins, carbohydrates and lipids. A number of cations (K, Ca, Mg and Fe) are also required in significant

amounts to maintain the structure and function of various cellular components such as enzymes and co-enzymes. In addition to these major nutrients, most bacteria require small quantities of trace elements, such as manganese, cobalt, zinc, molybdenum, copper and nickel. Some heterotrophs require low concentration of preformed growth factors or micronutrients such as aminoacids, pyrimidines, purines, and vitamins because they lack the biochemical pathways for the synthesis of these key intermediates. Results established that it is essential to provide the naturally occurring microelements as added nutritive source so as to fulfill the eco-physiological parameter that the particular population requires. A particular feature of the marine Bacteria and the Archaea is their requirement for sodium. True marine prokaryotes have an absolute requirement for Na+ ions (usually in the range of 0.5% to 5.0%) and fail to grow in the culture medium substituted with K+. This distinguishes the marine microbes from the closely related terrestrial and freshwater species. As expected, most of the marine microbes grow at a concentration of NaCl similar to that of sea water (3.0%-3.5% NaCl). The majority of the marine prokaryotes are moderate halophiles and grow in the media supplemented with up to 15% NaCl[56].

Another important criterion is pH. After 24 h, there was arise in pH and this may be due to the liberation of ammonia which increased the ionic concentration of hydrogen. This shows the idiophase of the culture. On the second day, there was a fall in pH due to the depletion of ammonia utilized by the culture. This indicated the production of metabolites of the bacterial isolates which require high nutrients. After 3rd day the increase in pH can be attributed to the excess ammonia liberated. This shows that when working with sediment from an extreme environment like mangrove it has to be seen that the initial pH should be taken with the native pH at which the population survives. Furthermore, the strains after the idiophase might have utilized the ammonia liberated in the first phase as the nitrogen source. The biomass and decoloration data showed that they were positively correlated in the present study. This is concordant with the previous reports[14,47,57,58]. However, those studies dealt with the effluents rather than dye as a whole.

As per 16S rDNA sequence analysis, the dye degrading strain reported in this study shared a 96% similarity with *Paenibacillus* sp. Phylogenetic tree construct reveals that the strain coincides only with the Genus *Paenibacillus* sp. and not any specified species. Reports on dye degrading *Paenibacillus* are[59] very few and that too from the mangrove ecosystem reports are diminutive.

Hence, this strain *Paenibacillus* sp. DD AU can be used as a candidate strain for dye degradation and can be taken for scale up processes. Therefore, the bacterial strain P1 with potent dye degrading property can be exploited in a manner so as to stamp out the chore of scientific community and for the welfare of human and marine biosphere.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Synthetic dyes are common contaminants of water especially, azo dyes and their metabolites are carcinogenic nature. So, there is need to detect the biological agents to degrade/convert the azo dye.

Research frontiers

The present research work was targeted to identify potential and novel dye degrading bacteria from mangrove environment.

Related reports

Carliell *et al*. (1995), Georgiou *et al.* (2004), Pandey *et al*. (2007) and Murty *et al.* (2012) have conducted a research on the degradation of various dyes using microbes and have also reported different ratio of dye decolouration/degrading capabilities.

Innovations and breakthroughs

Various reports are available on azo dye degrading bacteria from industrial and terrestrial environment with different range of results. But, the present study reported high decolouring/degrading effects expressed by bacteria. Further, the strain was isolated and identified from the marine region which is novel.

Applications

From this research, we could rectify the dye degrading

problems on the waste water treatments of textile industries. Further, the ecological systems of the receiving water bodies (River, canal, *etc*) might be protected from the discharge of dye pollution.

Peer review

This is a valuable research work in which authors have demonstrated the degradation/decolouration pattern by novel bacteria, *Paenibacillus* sp. The decolouration effects, growth kinetics were assessed. In addition, potent dye degrading property of the bacteria can be used by the scientific community for the welfare of our society.

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