International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 8, Issue 5, 2016, pp.-754-758.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

Research Article

DECOLOURIZATION OF REACTIVE VIOLET 13 AND REACTIVE BLUE 171 BY *PSEUDOMONAS STUTZERI* RJVL 1514 ISOLATED FROM DYE CONTAMINATED SOIL

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Received: March 17, 2016; Revised: April 14, 2016; Accepted: April 21, 2016; Published: May 07, 2016

Abstract- Azo dyes are the synthetic dyes, which resist degradation and cause many environmental problems. Among different decolourizing microorganisms, bacteria are effective in degradation and complete mineralization of many dyes under optimal environmental conditions. In this research work, it was planned to examine the potential of a total of 22 newly isolated bacterial species for decolourization of azo dyes such as Reactive Violet 13 and Reactive Blue 171. Of these isolates, based on two levels of screening for their ability to degrade the selected dyes efficiently and rapidly, one isolate has been selected for further studies. The isolate showed rapid growth and decolourization of both dyes in LB than MS medium under specific growth conditions. Based on morphological, biochemical and molecular characterization (16s rRNA sequencing), the selected strain was identified as *Pseudomonas stutzeri*. It exhibited 70-80% decolourization of dyes at 100ppm concentration in Luria Bertani (LB) broth and 50-60% in Mineral Salt medium (MSM) within 24 h. An improved decolourization activity was observed with the increase in biomass concentration.

Keywords- Azo dyes, Recalcitrant Biodegradation, Decolourization, Molecular characterization

Citation: Gangavarapu Vijaya Lakshmi and Ravuri Jaya Madhuri, (2016) Decolourization of Reactive Violet 13 and Reactive Blue 171 by *Pseudomonas stutzeri* RJVL 1514 Isolated from Dye-contaminated Soil. Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 8, Issue 5, pp.-754-758.

Academic Editor / Reviewer: K. V. Mohanan, Zeinab Shawky

Introduction

Azo dyes are the complex synthetic colouring agents containing one or more azo group (-N= N-). The dyeing industries generate and release large quantities of effluents into the environment. All most 100 litres of water are generally consumed by the textile dyeing units for dying about 1 Kg of textile material. During industrial processing up to 40% of the used dyestuff is released into the process water [1]. The effluents from dyeing industries are complex, contain a wide variety of residual dyes and other products such as dispersants, acids, bases, salts, detergents, humectants, oxidants, etc.[2]. The discharge of these coloured effluents into rivers and lakes results into reduced dissolved oxygen concentration, thus creating anoxic conditions that are lethal to resident organisms. Thus, dye decolourization has become a primary goal of dye wastewater treatment processes [3]. The recalcitrance of these dyes is attributed to azo groups (-N= N-). Various physical and chemical methods such as flocculation, coagulation, adsorption, membrane filtration, precipitation, irradiation, ozonization and Fenton's oxidation are available to treat dye effluents [4, 5] but are not widely employed since these methods are expensive, and produce huge quantity of sludge, which in turn causes the second level of soil pollution. Therefore, economic and safe removal of the polluting dyes is still an important issue. Bioremediation through microorganisms has been identified as a cost effective and environment friendly alternative for disposal of textile effluent [6] [7]. The biological mode of treatment of dye bath effluents offers distinct advantages over the conventional modes of treatment. This method is more economical and leads to less accumulation of relatively harmless sludge. Most importantly, it causes mineralization of dyes to simpler inorganic compounds, which are not lethal to life forms.

Different groups of microorganisms such as bacteria, fungi, actinomycetes yeast and algae have been found effective in decolourizing azo dyes. Among them, the fungal members, especially white rot fungi, *Trametes species and* non white rot fungi such as *Aspergillus species* have been proved as potential agents of biodegradation [8,9]. Many bacteria are also capable to degrade azo reactive dyes aerobically and anaerobically [10].

The microbial enzymes directly cleave the azo bonds present in the chromophore group of azo dyes, which ultimately results in colour removal Azo dyes are known to undergo reductive cleavage whereas the resultant aromatic amines are metabolized under aerobic conditions [11]. At present, a number of studies have focused on microorganisms, which are able to decolourize and biodegrade these dyes. Alternatively, dye decolourization using microbial enzymes has received great attention in recent years due to its efficient application [12]. Colour removal processes with active microorganisms have two different simultaneous steps: an adsorption of dyes on the surface of the organisms and a degradation of dyes by the enzymes produced by these organisms [13]. The present study was aimed to isolate and identify a bacterial strain capable of decolourizing Reactive violet and Reactive Blue azo dyes commonly used in textile industries of Andhra Pradesh, India. In addition, decolourization efficiency in different media was studied.

Materials and Methods

Sample collection

The dye amended soil samples were collected from the sites nearer to the textile dyeing units located in Desaipeta, Mangalagiri, Vetapalem, Andhra Pradesh (India) as described earlier in reference[14].

Dyes and chemicals

The azo dyes Reactive Violet 13 and Reactive Blue 171 used in this study are of industrial grade and all other chemicals used were of analytical grade. Details of the dyes were given in [Table-1] and structural information was represented in [Fig-1 & 2]. The concentrations of dye used for the present study are 50 ppm and

100 ppm.

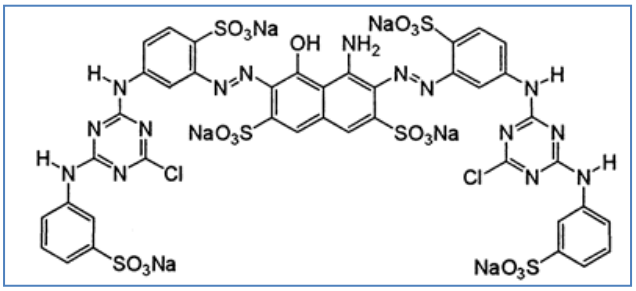


Fig-1 Structure of Reactive Blue 171

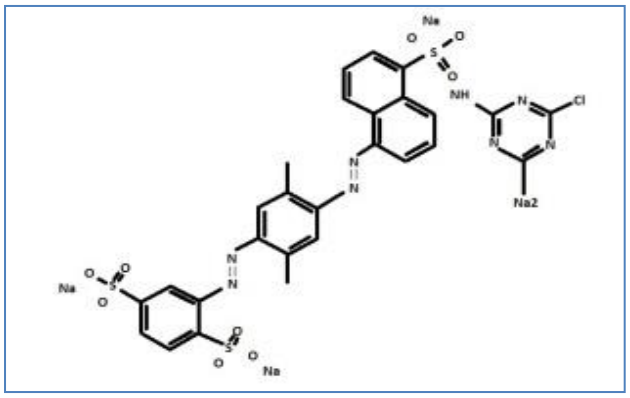


Fig-2 Structure of Reactive Violet 13

Isolation of Bacteria from soil

For the isolation of bacterial strains, the soil sample was analyzed by serial dilution agar plate method [15]. 0.1 ml aliquot of 10^{-2} to 10^{-7} dilution was added into sterile petri plates containing 15 ml of Nutrient agar medium by spread plate technique. Triplicates were maintained for each dilution. The plates were incubated for 24-48 h in inverted position at 37˚C. After incubation, morphologically distinct colonies were selected, isolated and maintained as pure cultures at 4˚C for further use.

Screening for Dye decolourizing bacteria

The bacterial isolates were subjected to two level screening processes to identify the potential dye degraders among them. Two types of screening methods employing both liquid and solid media were adapted.

Screening in liquid medium: A loop full of isolated bacterial cultures was inoculated into separate culture tubes containing Nutrient broth. The inoculated tubes were incubated at 37°C under shaking conditions for 24hrs.

Table-1 *General properties of the dyes*

S. No.	Properties	Reactive Blue	Reactive Violet
1.	Commercial Name	Navy Blue HER	Magenta MB
2.	Generic Name	C.I. Reactive Blue 171	C.I. Reactive Violet 13
3.	Chemical class	Double Azo class	Double Azo class
4.	Molecular Formula	$C_{40}H_{23}Cl_2N_{15}Na_6O_{19}S_6$	--
5.	Molecular weight	1418.93	--
6.	CAS Registry Number	77907-32-5	12270-87-0
7.	λmax	620nm	520nm

2 ml of cultures were taken in separate test tubes and 50ppm of the dye, Reactive Violet 13 was added to each tube. Similarly, the same procedure was followed for the other dye Reactive Blue 171. The test tubes were incubated under shaking conditions at 37°C. The Nutrient broth containing the dye of same concentration was taken as the control. After 24 hours, the test culture tubes with apparent decolourization were selected by comparing with the control [16].

Screening on solid medium: The isolated bacterial strains were screened on solid medium in two different methods

Well diffusion method: 5 mm wells were made in Mineral salt- dye agar medium and were loaded with 0.1 ml broth culture of respective strains and subjected to incubation at 37°C for 1-2 days. After incubation based on the extent of zone formed, the test organisms were selected for further study [17].

Spread plate method: 0.1 ml of isolated bacterial broth culture was inoculated on Mineral salt agar medium incorporated with dye by spread plate technique. Respective dye incorporated agars without any inoculums were used as controls and the decolourization was estimated visually by comparing the inoculated plates with those of the control plates after 24 to 72 h [18].

Morphological and Biochemical Characterization

Selected isolates were grown on Nutrient agar plates (Himedia, India). Based upon the growth characteristics, staining reactions and biochemical tests the isolates were identified according to Bergey's Manual of Determinative bacteriology [19, 20].

Identification of bacteria using 16S r RNA sequencing

The 16S rRNA analysis method used to identify the selected bacterial strains was accomplished by Yaazh xenomics Pvt Ltd. Chennai. First the Bacterial Genomic DNA was isolated using the Insta Gene TM Matrix Genomic DNA isolation kit. Using 16S rRNA Universal primers, 27F (AGAGTTTGATCMTGGCTCAG) & 1492R (AGAGTTTGATCMTGGCTCAG). Nearly full length 16SRNA gene was amplified using MJ Research Peltier Thermal Cycler. The purified PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using an ABI PRISM® Big Dye TM Terminator Cycle Sequencing Kits. Single-pass sequencing was performed on each template using 785F (GGATTAGATACCCTGGTA) and 907R (CCGTCAATTCMTTTRAGTTT) 16s rRNA universal primers. The 16s rRNA sequence data was aligned and subjected to blast analysis by using NCBI blast similarity search tool. The program MUSCLE 3.7 was used for multiple alignments of sequences [21]. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks removes alignment noise [22]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. The program Tree Dyn 198.3 was used for tree rendering [23]. The bacterial strain was deposited in Genbank, NCBI and accession number was obtained.

Biodegradability assaZ

Biodegradability assay is generally used to measure the dye degrading ability of selected strains. It was carried out in 250 ml conical flasks containing 100 ml of MS broth amended with 50ppm and 100ppm of the dye inoculated with 10% (v/v) of 24 h culture and incubated at 37˚C under static conditions. The same experiment was repeated with another set of flasks containing LB broth. The experiment was carried out in triplicate with an abiotic control and a blank. Abiotic control without bacterial culture was kept to analyze abiotic loss of the dye. The aliquots (4 ml) were withdrawn at regular intervals and centrifuged (10,000 rpm/10 min) to separate bacterial biomass and absorbance of the supernatant was measured at 520nm for RV 13 and 620nm for NB 171 by using UV-visible spectrophotometer to calculate the percent of decolourization [24,25].
The percent decolourization was calculated using the formula;

$$\% \text{ Decolourization} = (A-B)/A \times 100;$$

Where A is initial absorbance of control dye and B is observed absorbance of degraded dye.

Results and Discussion

Isolation and screening of Dye degrading Bacteria

A total of 30 morphologically distinct bacterial strains were isolated [Fig-3] from dye amended soil samples collected from the sites around dyeing industries.

When screened for decolourizing capability, using liquid medium containing 50 ppm of selected dyes, nine strains recorded apparent preliminary decolourization of both Reactive Violet 13 and Reactive Blue 171 [Fig-4], whereas ten isolates decolourized only Reactive Violet 13 and seven species showed discolouration in only Reactive Blue 171 containing tubes. Four bacterial isolates remained incapable and exhibited no degradation of dyes tested. The nine strains having the capability of degrading both the dyes were subjected to second level of screening employing solid medium by well diffusion technique and spread plate method [Fig-4 & 5].

Finally, based on the maximum rate of decolourization shown on inoculated spread plates and the greater zone of decolourisation formed around the wells in well diffusion method, two best isolates (RJV1 and RJV2) effective in degrading textile dyes were selected. Out of these two, RJV1 formed the greater zone of decolourization so; it was selected for further study.

Fig-3 Sample collection site & Isolation of Soil Bacteria

Fig-4 Well diffusion method & Screening in liquid medium

Fig-5 Screening by Dye agar plate method

Identification of selected bacterial species.

The selected strain RJV1 was identified by using standard Procedures as mentioned in Bergey's manual of systematic Bacteriology which mainly include Morphological, Biochemical and Molecular characterization.

Morphological characterization

The colonies on agar media are strongly coherent and can easily be lifted off as a unit. The consistency is dry in case of fresh isolates later becomes mucoid. The shape of the colonies is neither uniform nor necessarily constant. Mucoid protuberances observed at the periphery. On repeated transfers in laboratory media, colonies become smooth, and pale in colour. A surface film is formed generally in liquid cultures, when destroyed; the film breaks up into flocks that settle rapidly. The cells are rod shaped, about 1_3µ by 0.5 µ Gram negative, [Fig-6] non spores forming and have a single polar flagellum.

Biochemical characterization

Several biochemical tests have been performed to identify the genus of the organism under study. The isolated culture recorded positive results for Oxidase test, Catalase test, Nitrate reduction test, Citrate utilization test, Methyl red test and Glucose fermentation test [Fig-7]. Based on morphological and bio chemical characterization, the pure culture was identified as *Pseudomonas* sp.

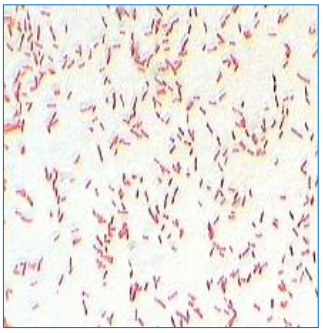


Fig-6 Gram negative rods of *Pseudomonas*

Fig: 7 Biochemical test results

a) Nitrate Reduction test **c) Citrate Utilisation test**

b) Methyl Red test **d) Glucose Fermentation test**

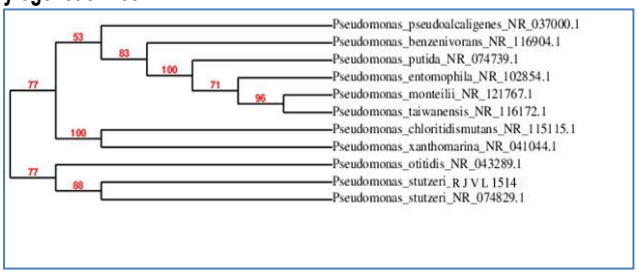
Molecular characterization

The 16S rRNA gene sequence of the strain RJVL was determined and compared with 16S rRNA gene sequences in the Genbank nucleotide databases. The strain was phylogenetically placed in the genus Pseudomonas. The gene sequence determined in this study was deposited in the Genbank database under the accession number KT873299 *Pseudomonas stutzeri* RJVL 1514.

CTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGA
GTGGAGCTTGCTCCATGATTCAGCGGCGGACGGGTGAGTAATGCGTAGGAA
TCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATAC
GTCATACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGC
CTAGGTCGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATC
CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCA
GACTCTTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAATCCT
GATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTT
AAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAA
CAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTT
AAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTG
GCGAGCTAGAGTATGGCAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAA

TGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGGCTGA
TACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGTCTACTAGCCGTTGGAATCCTTGAGATCT
TAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTGGGGAGTACGGCCGCAA
GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGA
ACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCAT
GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGC
GCAACCCTTGTCCTTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGGAGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC
CTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCA
AGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAG
TCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAAT
GTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAACCATGG
GAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCTTCGGGGGGACGGTACCA
CGGAGTGATCATGATGGG

Phylogenetic Tree



Biodegradability assay

The ability of *Pseudomonas stutzeri* RJVL1514 to decolourize two Azo dyes Reactive violet 13 and Reactive Blue 171 was evaluated in two different growth media such as Mineral salt broth and Luria Bertani broth. *Pseudomonas stutzeri* showed remarkable decolourization of both the dyes in both media used (MSM and LB) [Table-2] [Fig-8]. However, LB medium proved to be more supportive for obtaining maximum decolourization. The percentage of decolourization recorded by *Pseudomonas stutzeri* with Reactive violet was 64% and with Reactive Blue was 66%, whereas, in MS broth, 82% and 88 % decolurization was observed with Reactive violet and Reactive Blue respectively in LB medium.

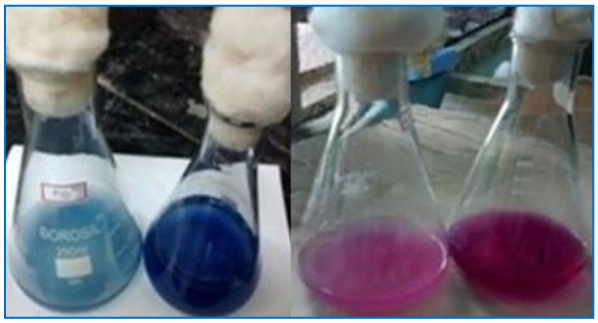


Fig-8 Decolourization of reactive Blue and Reactive violet by *Pseudomonas stutzeri*

The chemical complexity of the dye influences the decolourization rates [26]. The low molecular weight dyes with less structural complexity (mono azo group) were easily prone to degradation by microorganisms whereas, high molecular weight dyes with heavy structural complexity (containing two or more azo bonds) are less susceptible to biodegradation.[27]. In the present study, decolourization was greatly influenced by concentration of dyes employed. *Pseudomonas stutzeri* reported decreased levels of decolourization with the increasing dye concentrations. The gradual reduction in rates of decolourization with the increasing concentration of dye was due to the toxic effect of dyes on degrading microorganisms or the blockage of active sites of azo reductase enzymes by dye molecule with different structures [28]. Reference [29] reported that the decolourization rate of Reactive Black 5 by *Enterobacter* sp. EC3 was decreased with the increase in initial concentration.

The dye decolourization efficiency of the bacteria was highly related with the supplement of the carbon and nitrogen co substrates. When grown in basal medium like MSM, the organism utilizes the azo compounds as sole source of carbon and nitrogen for its growth and metabolism. Number of studies revealed that decolourization rate was enhanced with the addition of Yeast extract [30,31]. Similar to our findings, Telki et al [32] reported that yeast extract was effective co substrates for decolourization of Reactive Red 141 by *Rhizobium radiobacter*, which is in agreement to our finding. In the present study LB broth was rich in nitrogen source when compared to MSM broth.

Many studies reported that the first step in the bacterial biodegradation of Azo dyes was the reduction of azo compounds into aromatic amines catalyzed by azo reductase and the resultant amines were further degraded either aerobically or an-aerobically [33,34]. The organic nitrogen sources such as yeast extract , beef extract pyruvate etc are considered as media supplements for regenerating NADH, the essential electron donors required for the reduction of azo dyes by micro organisms [35]. In our present study, maximum decolourization of dyes by *Pseudomonas stutzeri* was observed in LB broth than MSM broth because of the abundant availability of carbon and nitrogen source in it.

Table-2 *The Dye decolourization by Pseudomonas stutzeri in different media*

Medium used	Reactive violet		Reactive blue	
	50 ppm	100ppm	50ppm	100ppm
MSM	64%	53%	66%	58%
LB	82%	78%	88%	79%

Conclusion:

The strain RJV1 isolated from the dye contaminated sites around the dyeing units located in Desai pet, Mangalagiri, AP, India was identified as *Pseudomonas stutzeri* RJVL 1514. The two dyes tested were decolorized rapidly with some difference in rates of decolourization depending on the concentrations of dyes involved. Decolourization was strongly dependent on the presence of Yeast extract in the medium, indicating the need for additional vitamin and nitrogen sources. Thus, the isolated strain proved to be a good degrader of azo dye and further the culture conditions should be made optimized to get effective decolourization.

Conflict of Interest: There is no conflict of interest for both the authors.

Author Contribution: Equal contribution was extended by both the authors in preparing, editing and submitting the manuscript.

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