*Int.J.Curr.Microbiol.App.Sci* (2019) *8*(4): 1742-1754

*International Journal of Current Microbiology and Applied Sciences*
***ISSN: 2319-7706* Volume 8 Number 04 (2019)**
Journal homepage: http://www.ijcmas.com

**Original Research Article** https://doi.org/10.20546/ijcmas.2019.804.203

# Decolourization of Textile Azo Dye Direct Red 81 by Bacteria from Textile Industry Effluent

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**Keywords**

Textile effluents, Azo dye, Decolorization, Bacteria

**Article Info**

***Accepted:***
15 March 2019
***Available Online:***
10 April 2019

**A B S T R A C T**

Isolation and identification of the bacteria from textile effluent and evaluation of their ability to decolorize toxic sulfonated azo dye, Direct Red 81 were studied. A total of four bacterial strains were isolated from textile wastewater and their decolorizing activity was measured spectrophotometrically after incubation of the isolates for 24 h. in mineral salt medium modified with 100 ppm Direct Red 81 and supplemented with yeast extract. The bacterial strains were identified belonging to *Raoultella planticola* strain ALK314 (DR1), *Klebsiella* sp. SPC06 (DR2), *Pseudomonas putida* strain HOT19 (98.68%) (DR3) and *Pseudomonas* sp. strain 2016NX1 (DR4) respectively. Among the isolates *Pseudomonas aeruginosa* sp. strain ZJHG29 (DR4) was the most efficient bacteria to decolorize direct red 81 (100ppm) and showed 95% color removal efficiency at 36°C temperature in 24 hours. This study thus reveals that some bacteria inhabit in textile effluent whereby utilize the dyes as their source of energy and nutrition and imply their importance in the treatment of industrial effluents.

## Introduction

Textile industry generated waste water is a complex mixture of many pollutants such as heavy metals, chlorinated compounds, pigments and dyes (Saraswathi and Balakumar, 2009).

It is estimated that approximately 15% of the dyestuffs are lost in the industrial effluents during manufacturing and processing operations (Khaled *et al.,* 2009). Dyes are an important class of synthetic organic compounds, widely used in textile, leather, plastic, cosmetic and food industries and are therefore common industrial pollutants. Synthetic dyes are chemically diverse and divided into azo, triphenylmethane or heterocyclic/polymeric structures (Cheunbarn *et al.,* 2008).

These dyes are designed to be stable and long lasting colorants and are usually recalcitrant in natural environment. After release into

water bodies, these dyes have negative impact on photosynthesis of aquatic plants and the azo group (N = N) in dyes are converted to aromatic amines which are possible human carcinogens (Banat *et al.,* 1996). Some dyes and their breakdown products also have strong toxic and mutagenic effect on living organisms (Pinheiro *et al.,* 2004). Discharge of textile dyes without proper treatment may lead to bioaccumulation that may incorporate into food chain and effect on human health.

In recent years, numerous studies were carried out for the decolourization of textile effluent, including various physicochemical methods such as filtration, coagulation, chemical flocculation, use of activated carbon, advanced oxidation processes, ion exchange, electrochemical and membrane process. Few of them are effective but with high cost, low efficiency and lack of selectivity of the process (Maier *et al.,* 2004; Kurniawan *et al.,* 2006).

Biological treatment offers a cheaper and environment friendly alternative to dye decolourization and wastewater reutilization in industrial process (Santos *etal.,* 2007; Mondal *et al.,* 2009). The general approach for bioremediation of textile effluent is to improve the natural degradation capacity of the indigenous microorganism that allows degradation and mineralization of dyes with a low environmental impact and without using potentially toxic chemical substances, under mild pH and temperature conditions (Dhanve *et al.,* 2008; Khalid *et al.,* 2008).

Interest has developed in recent years in the ability of microorganisms to degrade and detoxify pollutants, which are introduced in the environment through industrial activities of man. Microorganisms are among the most metabolically diverse group on earth, which play the vital role in course of neutralizing the toxic effects of a large number of chemicals.

## Materials and Methods

### Source of the sample and dyes

Samples of effluent were collected in sterile plastic bottles from drainage canal of Textile Dyeing Industries located in Narshingdhi, Bangladesh. Samples were in the form of liquid untreated effluent and untreated sludge. Azo dye named Direct Red 81 was procured from ACCE department of Rajshahi University and which was purchased from Sigma-Aldrich, USA was used in the present experiment.

### Enrichment and isolation of dye decolourizing bacteria

All samples (untreated textile effluents) were used for isolation of dye decolourizing bacterial cultures by enrichment culture techniques using enrichment medium amended with 20 ppm of the test dyes (Direct Red 81) for the adaptation of the microorganisms. For this, 1ml of sample of textile effluent was first diluted with 9ml sterilized water in test tubes separately. Then, 1ml of diluted sample was transferred into each single test tube containing 9 ml autoclaved enrichment medium. Required amount of respective dye was added to adjust the concentration 20 ppm and incubated to observe dye decolourization. After 24 –72 hours incubation, the bacteria from the decolourized test tube were streak plated on enrichment agar medium and mineral salt (MS) agar medium having 20 ppm of respective dye. Bacterial colonies that showed a clear decolourization zone around them on enrichment agar medium were picked and cultured for 24 hours at 36°C in MS medium amended with 1ml/l TE solution. Then, 1 ml of the culture of individual colony was reintroduced into 9 ml enrichment medium. To observe decolourization activity by individual bacteria, 1 ml of the culture of

individual colony was added into 9 ml MS medium separately containing 100 ppm of respective dye, and then incubated for 24 hours at 36°C. Then, 2 ml of incubated media was taken out aseptically and centrifuged at 10,000 rpm for 10 minutes. The cell free supernatant was used to determine the percentage decolourization of the added dye. Isolate showing the most decolourization of the added dye was selected and preserved for further studies.

## Determination of optimum growth conditions

Bacterial optimum growth influenced by the various culture conditions such as pH and temperature. For the effects of *p*H, culture medium was adjusted to pH 6.0, 7.0 and 8.0. Incubation temperatures were varied at $28^0$C, $36^0$C and $45^0$C. Bacterial cell density of nutrient liquid culture was determined by measuring optical density at 660 nm with photoelectric colorimeter.

## Decolourization activity test

Decolourization activity was expressed in terms of percentage decolourization and was determined by monitoring the decrease in absorbance at absorption maxima (λ max) using UV-Visible spectrophotometer. Aliquot (2 ml) of culture media was withdrawn at different time intervals and centrifuged at 10000 rpm for 10 minute. The concentration of dye in the supernatant was determined by monitoring the absorbance at the maximum absorption wavelength (λ max) at 511 nm for Direct Red 81. All decolourization experiments were performed in triplicates. Abiotic control (without microorganism) was always included in each study. The % decolourization rate was measured (Saratale, 2009) as follows:

$$Dye Decolourization(\%) = \frac{Initial absorbance - Final absorbance}{Initial absorbance} \times 100$$

## Identification of dye-degrading bacteria by 16S rDNA gene sequence

Identification of the isolated strain was performed by 16S rDNA sequence analysis. Genomic DNA was extracted from the bacterial cells using Maxwell Blood DNA kit (Model: AS1010, Origin: Promega, USA).The 16S rDNA gene was amplified by PCR using the specific primers, 27F and 1492R which are capable of amplifying 16S from a wide variety of bacterial taxa. The sequence of the forward primer was 16SF 5'-AGA GTT TGA TCM TGG CTC AG-3'(Turner *et al.,* 1999) and the sequence of the reverse primer was 16SR 5'-CGG TTA CCT TGT TAC GAC TT-3'(Turner *et al.,* 1999). The PCR amplicons are separated electrophoretically in a 1% agarose gel and visualized after Diamond™ Nucleic Acid Dye (Cat: H1181, Origin: Promega, USA) staining. The PCR products were purified using SV Gel and PCR Clean Up System (Cat: A9281, Origin: Promega, USA) according to the manufacture′s protocol. The total DNA yield and quality were determined spectrophotometrically by NanoDrop 2000(Thermo Scientific, USA). The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The 16S rRNA genes in the Gene Bank by using the NCBI Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees were formed using Weighbor (Weighted Neighbor Joining: A likelihood-Based Approach to Distance - Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000.The 16S rRNA gene sequences were deposited to Genbank (Accession no. DR1-MK572807; DR2-MK572731; DR3-MK583692; DR4-MK574814).

## Statistical analysis

Unless indicated otherwise, all experiments were independently conducted three times and data were pooled for presentation as mean±SEM. All data were analyzed with Prism software (GraphPad, La Jolla, CA, USA) using two-tailed unpaired Student's t-tests. P-values <0.05 were considered significant.

## Results and Discussion

## Isolation of dye decolourizing bacteria

Dye decolourizingbacteria were isolated by plating onto an agar solidified MS medium supplemented with dye from effluents of the textile industries. The plates were incubated at 36°C for 24 hours and bacterial colonies were found to grow on the medium. Furthermore colonies with decolourized zone were isolated and then tested for dye removal capability using 100 ppm Direct Red 81 dye as the sole carbon source in the MS medium. Four morphologically distinct bacterial isolates (DR1, DR2, DR3 and DR4) were indentified for decolourization of Direct Red 81 dye.

The minimum inhibitory concentration (MIC) of Direct Red 81 dye for the isolates DR1, DR2, DR3 and DR4 were also studied and the results showed 200ppm for DR1, 200ppm for DR2, 200 for DR3 and 400ppm for DR4 respectively.

## Effect of $p^H$ and temperature on bacterial growth

To determine the effect of pH and temperature of growth medium on the growth rate of the bacteria was tested a series of investigation. The results of the investigations are presented in Figures 1 and 2, respectively. The optimum pH for the growth of the isolates was 8.0 and bacteria also grow in other pH value range to 6.0-8.0. The optimum temperature was 36ºC for the growth of bacterial isolates while the minimum growth rate was observed at 45 °C.

## Measurement of decolourization of Direct Red 81 dye

Azo dye decolourization efficacy by four bacterial isolates (DR1, DR2, DR3 and DR4) grown in nutrient media supplemented with 100 ppm Direct Red 81 dye was analyzed. The decolourization activity was measured after 24 hours incubation at 36°C and was monitored by UV spectrophotometer at 511 nm (Fig. 3) and also in order to enhance the decolourization of Direct Red 81 dye 0.5% of yeast extract supplemented into minimal salt medium and the decolourization rate monitored upto four days (Fig. 4). The data is a mean±SEM from three independent experiments.

## Phylogenetic analysis and identification of the strains

Phylogenetic tree were constructed from pairwise alignment between the BLAST related sequences for each DR strains. A total of 25 related blast sequences randomly select for constructing phylogenetic tree. Neighbour joining algorithm used to produce a tree from given distances (or dissimilarities) between sequences (Saitou and Nei, 1987). Distances between sequences were analyzed from the NCBI website (http://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi?) and the unrooted tree date downloaded as Newick format. The unrooted tree opened in MEGA VI phylogenetic tree software then edited and resizing (Tamura *et al.,* 2013). The phylogenetic positions of all isolates within different subgroups were investigated by comparing their 16S rDNA sequences to those representatives of various genera. It is evident from the phylogenetic tree that DR1 is

closely related to *Raoultella planticola* strain ALK314, DR2 to *Klebsiella* sp. SPC06, DR3 to *Pseudomonas putida* strain HOT19 (98.68%) and DR4 to *Pseudomonas aeruginosa* strain ZJHG29 (Fig. 5).

In this study, the sample of textile effluents were collected and used for isolation of dye decolorizing bacteria employing Direct Red 81 (DR81) dye as a sole source of carbon & energy. Pure culture of dye decolorizing bacteria were isolated by planting out on agar solidified MS medium contains 100 ppm DR81 dye. Despite repeated attempts we were not successful in isolating bacteria capable of decolorizing and utilizing DR81 dye as a sole source of carbon and energy. The obligate requirement of unstable carbon source for functioning of dye decolorizing bacteria has been reported, therefore, isolation was also attempted by employing glucose and yeast extract as co-substrates (Banat *et al.,* 1996; Coughlin *et al.,* 1997). Then, Four dye decolorizing bacteria were identified by both morphological & biochemical tests & this is further confirmed by 16s rRNA gene sequence analysis. Analysis of 16s rRNA gene sequence revealed that the isolated bacteria, DR1 is closely related to *Rautella planticola* strain ALK314 (97.06%), DR2 to *Klebsiella* sp. spc06 (97.72%), DR3 to *Pseudomonas putida* strain HOT19 (98.68%) and DR4 to *Pseudomonas aeruginosa* sp. strain ZJHG29 (97.83%).

There are previous reports on different strains of *Klebsiella* and *Pseudomonas*, which are able to decolorize different types of azo dye. *Pseudomonas* sp. decolorize Orange 3R and showed maximum decolourization of 89% at the end of 144 hours under optimum condition (Ponraj *et al.,* 2011). Prasad (2014) observed that *Pseudomonas aeruginosa* showed maximum textile dye degradation on the 8$^{th}$ day of incubation at 40 mg/l ofdye concentration under optimum condition ($40^0$C, pH 6 to 8). *Klebsiella* spp. DA26 had showed 86.9% Methyl Orange dye decolorizing activity under optimized condition within 48 hours (Radhakrishin and Saraswati, 2015). Godlewska *et al.,* (2015) discovered two *Klebsiella* strains (Bz4 and Rz7) which are decolorize Evans Blue and Brilliant Green at the rate of 95.4% and 100%, respectively.

During the present investigation it was recovered that all isolates could grow and decolorize the DR81 dye up to 200 ppm within 24 hour except DR4 (up to 400 ppm within 24 hour). Sahasrabudhe *et al.,* (2014) have identified a strain of *Enterococcus faecalis* YZ66 shows complete decolourization and degradation of toxic, sulfonated recalcitrant diazo dye DR81 (50 mg/L) within 1.5 hour of incubation under static condition.

Throughout the study it was found that, in nutrient broth medium above 90% decolourization rate achieved by DR1 (93%) and DR3 (95%) bacterial isolates at 60 hours incubation period on static condition, while these two takes 72 hour incubation period to reach 95% and 96% decolorizing ability respectively in MS medium supplemented with 0.5% yeast extract. In case of, DR2 and DR4 bacterial isolates 93% and 94% decolourization activity were shown at 48 hours, whereas, 95% decolourization rate achieved by the both isolates but it takes 72 hours for DR2 and only 24 hours incubation period required for DR4 in MS medium supplemented with 0.5% yeast extract. DR4 was found to be the most effective decolorizer among them.

Most pure cultures of bacteria like *Pseudomonas luteola* (Hu, 1998; Chang *et al.,* 2001), *Klebsiella pnuemoniae* (Wong and Yuen, 1996) *Aeromonas hydrophila* (Chen *et al.,* 2003) and different mixed cultures like

*Paenibacillus* sp. and *Micrococcus* sp. (Moosvi *et al.,* 2007), *Bacillus* sp. and *Clostridium* sp. (Knapp and Newby, 1995) have exhibited effective dye decolourization in presence of yeast extract.

The growth and decolorizing ability of the isolated bacteria were dependent on pHand temperature.The optimum pH for the growth of the isolates was 8.0 and also the isolates grow well on pH 7.0. The rate of decolourization for Direct Red 81 was optimum in the narrow pH range from 7.0 to 8.0. *Klebsiella pneumonia* RS-13 completely degraded methyl red in pH range from 6.0 to 8.0 (Wong and Yuen, 1996). Mali *et al.,* (2000) found that a pH value between 6 to 9 was optimum for decolourization of triphenylmethanes and azo dyes by *Pseudomonas* sp. The dye decolourization varies with pH. At the optimum pH, the surface of biomass gets negatively charged, which enhances the binding of positively charged dye. Binding occurs through electrostatic force of attraction and it results in a considerable increase in color removal (Daneshvar *et al.,* 2007). Below the optimum *p*H, $H^+$ ions compete effectively with dye cations, causing a decrease in color removal efficiency. At alkaline pH, the azo bonds will be deprotonated to negatively charged compounds and it results in obstruction of azo dye decolourization. In acidic pH, the azo bond will be protonated (-N=N- → [-NH-N=]$^+$ which leads to decreased dye decolourization due to change in chemical structure (Hsueh and Chen, 2007). Similarly azo dye decolourization was exhibited at *p*H 7 in case of *E.coli* and *P.luteola*(Chang and Lin, 2001). Most of the azo dye reducing species of *Pseudomonas luteola, Bacillus* and *Enterobacter*sp. EC3 (Chang *et al.,* 2001; Kalme *et al.,* 2007; Wang *et al.,* 2009) were able to reduce the dye at neutral pH. Due to the difference in genetic determinants for dye decolourization and bacterial physiology, the optimal pH varies with species and dyes (Chang and Lin, 2001).

It was recovered that the optimum temperature for the best growth of isolated bacteria was $36^0$C. So $36^0$C temperature is the most suitable temperature for the decolorizing of Direct Red 81 dye. The dye decolourization activity of our four isolated bacterial culture were found to increase with increase in incubation temperature from $28^0$ to $36^0$ with maximum activity attained at $36^0$C. Further increase in temperature resulted in marginal reduction in decolourization activity of four isolated bacteria. Enhanced dye decolourization of Direct Red 81 was observed at $36^0$C but it drastically decreased with increase in temperature (40°C). Reduced color removal beyond 35°C may be due to the loss of cell viability or thermal deactivation of decolorizing enzymes (Panswad and Luangdilok, 2000; Cetin and Donmez, 2006).

Decreased decolourization was exhibited at $45^0$C under static condition since the bacterium poorly grows at this temperature. It implies that the bacterium is mesophilic and the possible reason is that the enzyme responsible for decolourization has its activity between 30 - $40^0$C. Results obtained are also correlated with earlier studies by Khalid *et al.,* (2008) where the decolourization of Methyl Red and RBR X-3B by *Vibrio* sp. and *Rhodopseudomonas palustris* was maximum around 30-$35^0$C (Adedayo *et al.,* 2004; Liu *et al.,* 2006). Reports also show that *Klebsiella pneumoniae* RS - 13 and *Acetobacter liquefaciens*S-1 had no decolourization of methyl red at $45^0$C (Wong and Yuen ,1998). Previous reports indicate that rapid decolourization of Remazol Black B, Direct Red 81, Acid Orange 10, Disperse Blue 79, Navy Blue HER and Acid Blue 113 were observed at $37^0$C (Meehan *et al.,* 2000; Junnarkar *et al.,* 2006; Kolekar *et al.,* 2008; Gurulakshmi *et al.,* 2008).

**Fig.1** Optimum pH for growth of the bacterial strains DR1, DR2,DR3 and DR4at 36°C. The optimum pH of bacterial growth was determined at every 4-hours interval up to 48hours incubation at pH 6.0, 7.0 and 8.0 by measuring optical density at 660 nm

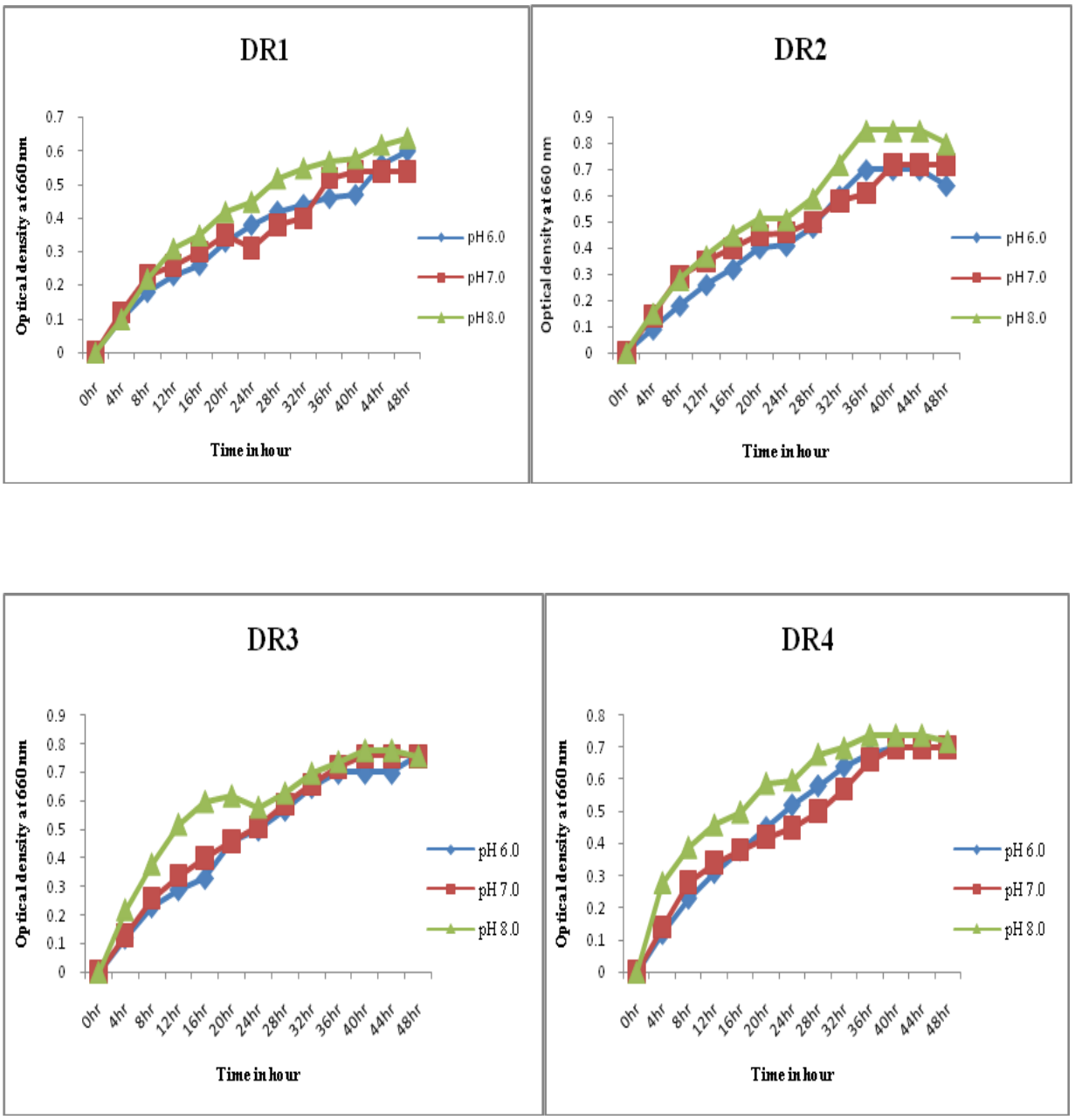


**Fig.2** Optimum pH for growth of the bacterial strains DR1, DR2, DR3 and DR4at pH 8.0.The optimum temperature of bacterial growth was determined at every 4-hours interval up to 48 hours incubation at 28 °C, 36 °C and 45 °C by measuring optical density at 660 nm

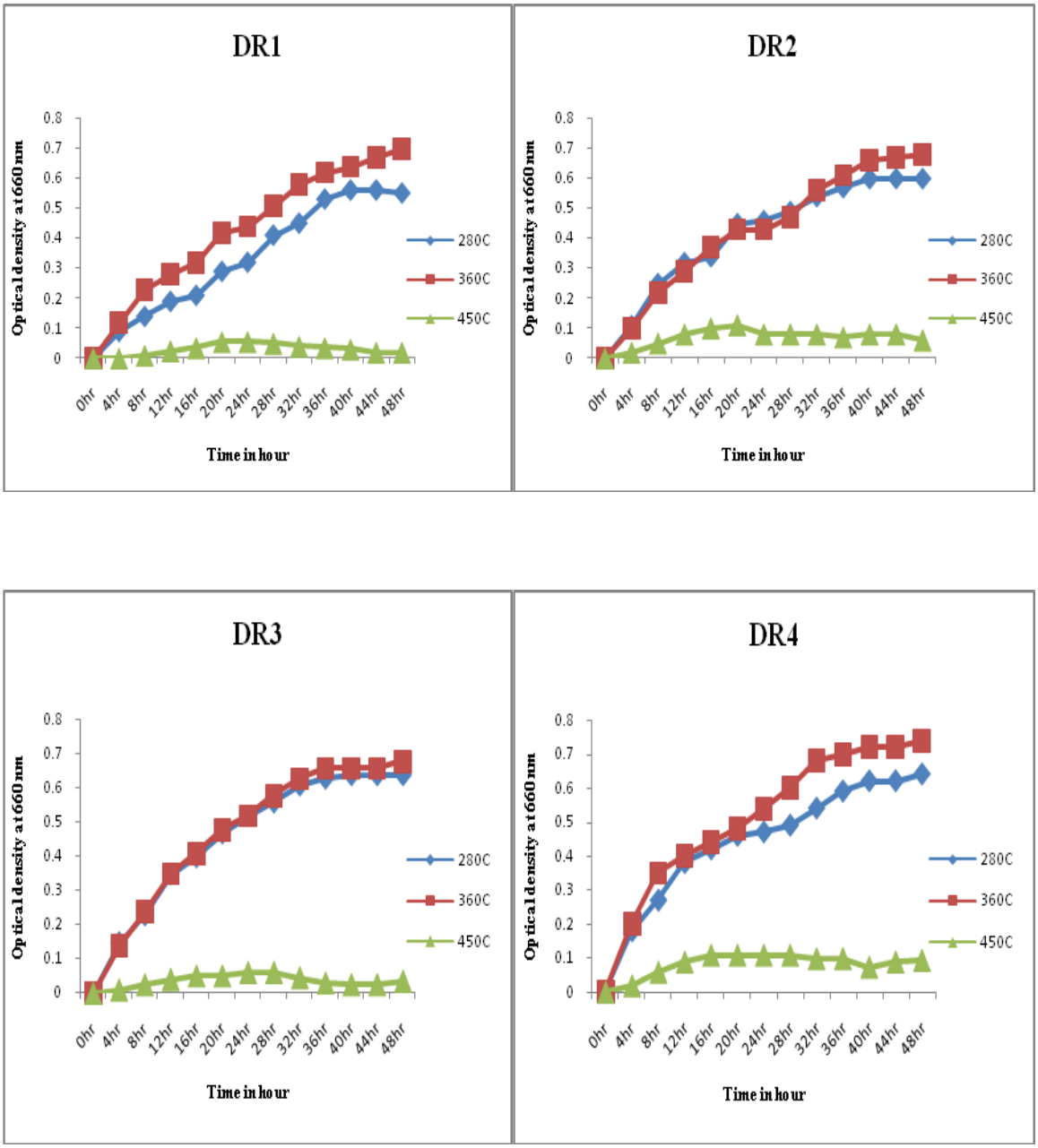


**Fig.3** Percentage of dye decolourization on DR81 in nutrient medium

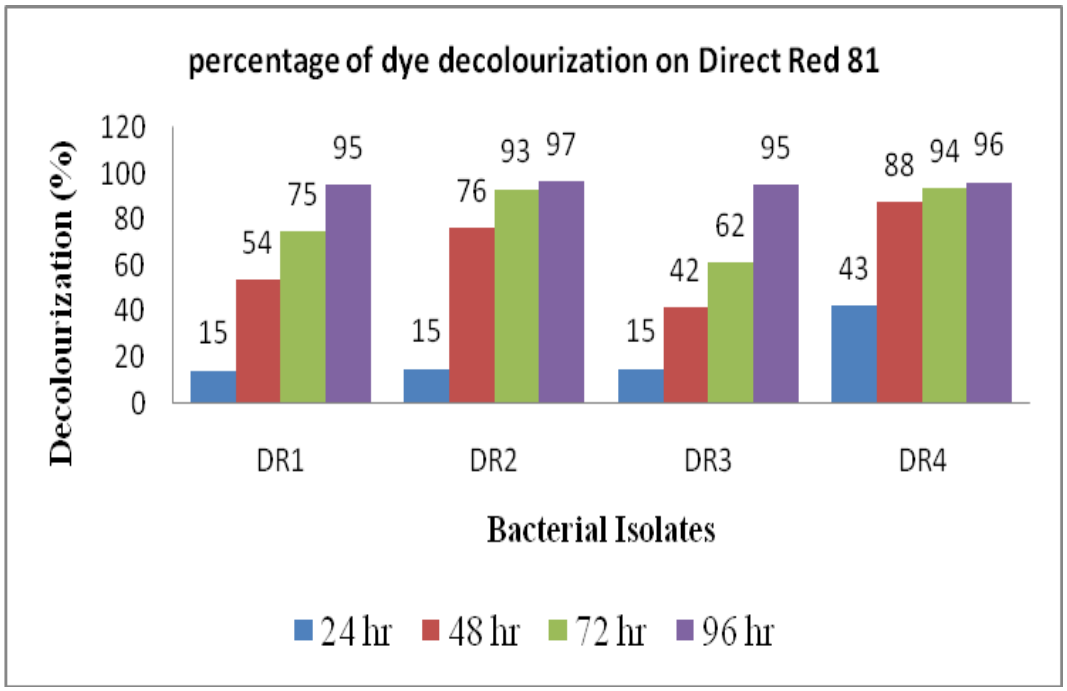



**Fig.4** Percentage of dye decolourization on DR81 in MS medium supplemented with 0.5% yeast extract

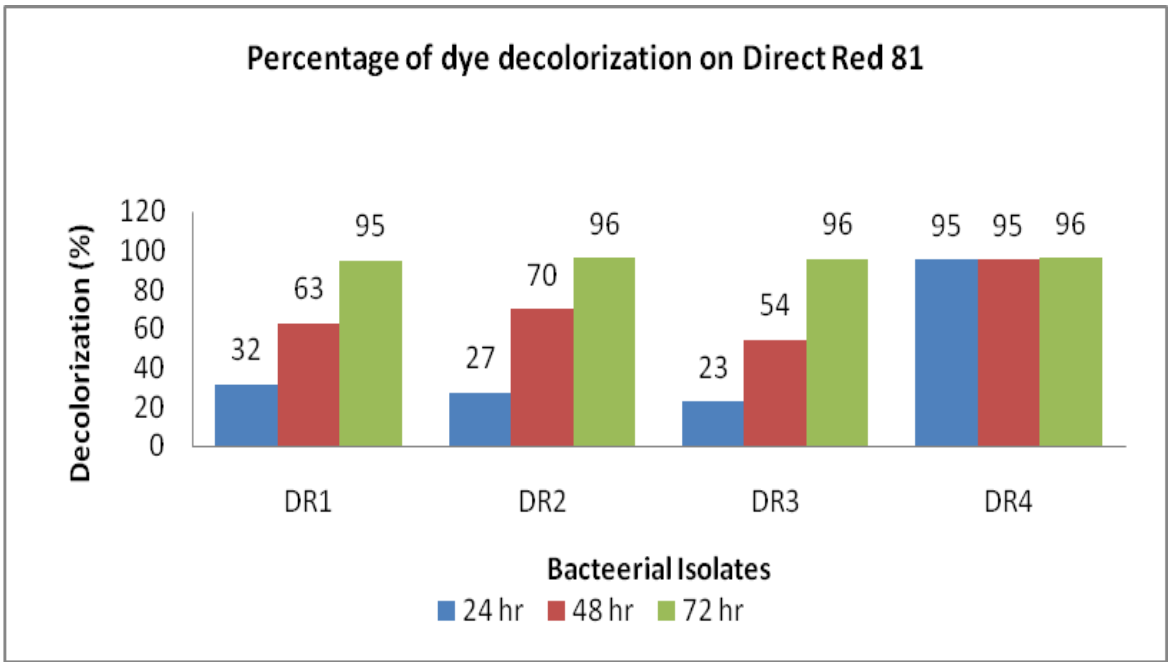
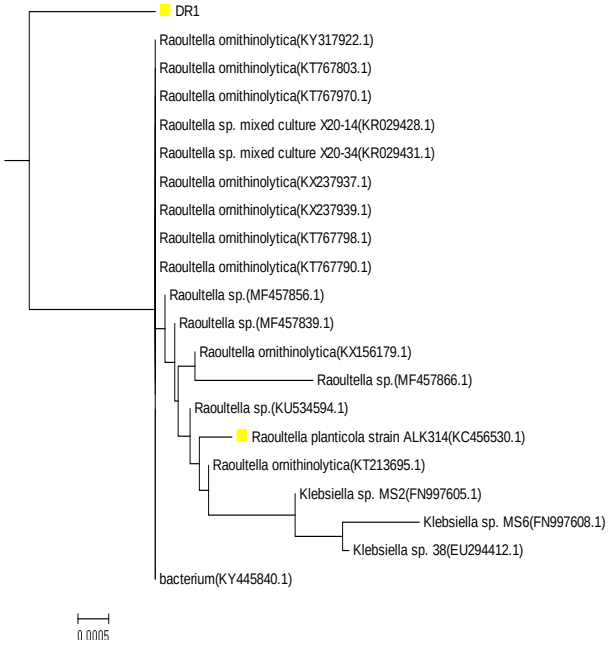
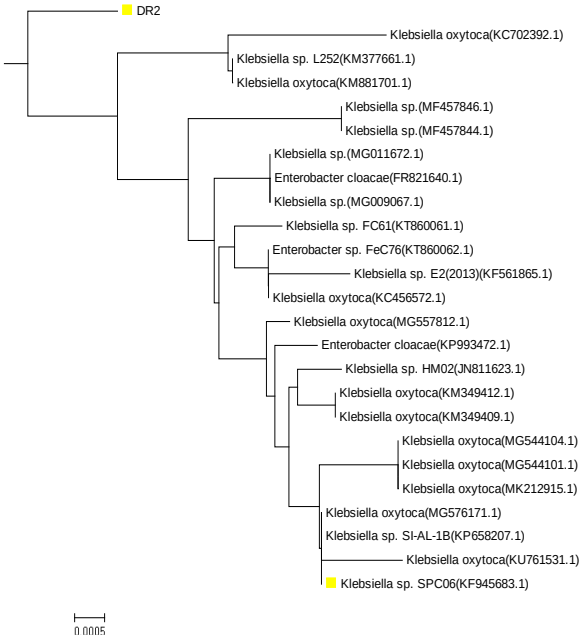


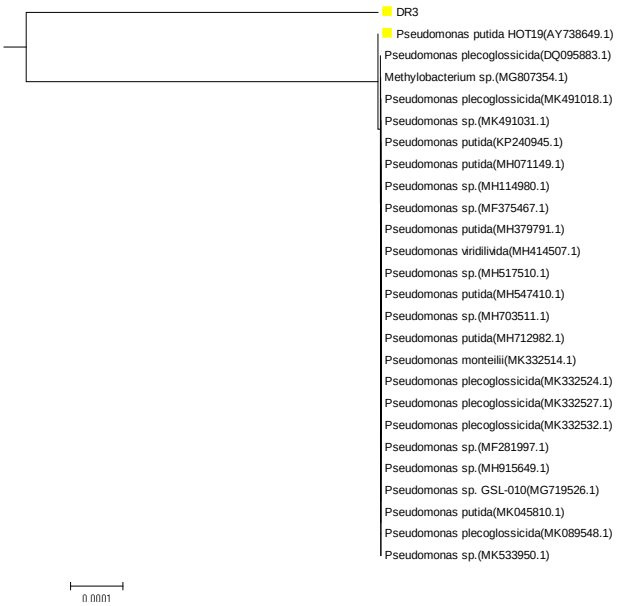
**Fig.5** Phylogenetic tree showing the genetic relationship among the cultivated bacteria and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. (a) Scale bar 0.0005 = 0.05%, (b) Scale bar 0.0005 = 0.05% , (c) Scale bar 0.0001 = 0.01% and (d) . Scale bar 0.0005 = 0.05% difference among nucleotide sequences



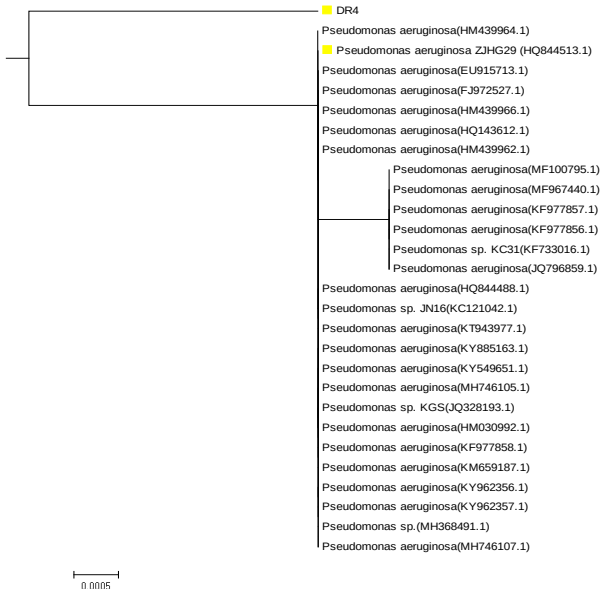

**(a)**




**(b)**




**(c)**




**(d)**

In conclusion, the textile dye (Direct Red 81) is degradable under aerobic conditions with a concerted effort of bacteria isolated from textile dye effluent. Nutrients (carbon and nitrogen sources) and physical parameters (*p*H and temperature) had significant effect on dye decolourization of Direct Red 81 dye effectively during optimization and more interesting DR4 isolate (*Pseudomonas aeruginosa* strain ZJHG29) showed consistent decolourization of textile dye (Direct Red 81) throughout the study.

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**How to cite this article:**

Sk. Md. Atiqur Rahman, Ananda Kumar Saha, Rokshana Ara Ruhi, Md. Fazlul Haque and Moni Krishno Mohanta. 2019. Decolourization of Textile Azo Dye Direct Red 81 by Bacteria from Textile Industry Effluent. *Int.J.Curr.Microbiol.App.Sci.* 8(04): 1742-1754.
doi: https://doi.org/10.20546/ijcmas.2019.804.203