

## **Detoxification and color removal of Congo red by a novel *Dietzia* sp. (DTS26) - A microcosm approach**

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### **Abstract**

The present study deals with the decolorization and detoxification of Congo red (CR) by a novel marine bacterium *Dietzia* sp. (DTS26) isolated from Divar Island, Goa, India. The maximum decolorization of 94.5 % (100 mg L<sup>-1</sup>) was observed under static condition within 30 h at pH 8 and temperature 32 ± 2 °C. Bacterially treated samples could enhance the light intensity by 38 % and the primary production levels 5 times higher than the untreated. The strain was also able to reduce COD by 86.4 % within 30 h at 100 mg L<sup>-1</sup> of CR dye. The degraded metabolites of CR dye were analyzed by FTIR, HPLC, GC-MS and the end product closely matches with 4-amino-3-naphthol-1-sulfonate which is comparatively less toxic than CR. Bioassay experiments conducted in treated samples for *Artemia franciscana* showed better survival rates (after 72 h) at higher concentration of CR (500 mg L<sup>-1</sup>). This work suggests the potential application of DTS26 in bioremediation of dye wastes and its safe disposal into coastal environment.

**Keywords:** Congo red. *Dietzia* sp. Light intensity. COD. Toxicity removal. Biodegradation

## 1. Introduction

Congo red (CR) ( $C_{32}H_{22}N_6Na_2O_6S_2$ ; molecular weight:  $696.66 \text{ g mol}^{-1}$ ) is a sodium salt of benzidinediazo-bis-1-naphthylamine-4-sulfonic acid which is known to be carcinogenic because of the presence of the aromatic amine group (Zollinger, 2003; Jalandoni-Buan et al., 2010). It is a diazo dye commonly used in paper, textile and wood industries. The effluents containing CR dye are highly dense and colored in nature; their disposal in to the coastal waters significantly reduces the photosynthetic activity by lowering the light penetration and is also toxic to many aquatic animals (Anjaneya et al., 2011). Therefore it is necessary to treat the dyeing wastewater before its final discharge into the environment (Purkait et al., 2004).

Several physicochemical decolorization methods such as adsorption, chemical precipitation, photolysis, advanced oxidation processes have been reported (El Gaini et al., 2009). The physicochemical methods have some significant drawbacks, such as: highly expensive; generate high levels of sludge, unable to remove the recalcitrant azo and other organic dyes completely (Champagne and Ramsay, 2010). Therefore biological treatments are recommended (O'Neill et al. 2000). Several studies have been reported for the decolorization of CR and other textile dyes by various microorganisms i.e., *Rhizobium radiobacter* (Telke et al., 2008), *Aspergillus ochraceus* (Humnabadkar et al., 2008), *Penicillium ochrochloron* (Shedbalkar and Jadhav, 2011), *Candida oleophila* (Lucas et al., 2006), *Trametes villosa* (Machado et al., 2006), and *Cosmarium* sp. (Daeshwar et al., 2007). Synthetic dyes are mainly degraded by enzymatic action (Saratale et al., 2011) and the enzymes Azoreductase, laccase and lignin peroxidase are reported to attack the aromatic amine structure of the dyes (Chen et al., 2003; Niladevi and Prema, 2008).

Although the various microorganisms are reported in degradation of various textile dyes in literature, the intermittent products are often toxic and carcinogenic. In recent years, a number of treatment methods have proven the removal of color in the effluent, but their toxicity reduction from the decolorized metabolites has not been given much attention (Silva et al., 2012). In many cases dye degraded metabolites are more toxic than the parent dye (Solis et al., 2012). This explains the need for isolating potent strains which could degrade complex mixtures of dyes in to less toxic metabolites after the treatment.

The aim of the present work was to investigate the potential of a novel marine bacterium DTS26 for decolorization and detoxification of CR dye. The effects of different parameters on decolorization process and the degraded metabolites were characterized by using various analytical methods. The study was also aimed to assess the toxicity of the metabolites formed after the degradation of CR dye by DTS26. To our knowledge, this forms first comprehensive report on decolorization and detoxification of CR dye by marine DTS26 strain under various environmental conditions.

## **2. Materials and Methods**

### *2.1. Dye and Chemicals*

The CR dye ( $\lambda_{\text{max}} = 495\text{nm}$ ) used in this study was purchased from Hi-Media India, Pvt. Ltd, Mumbai. The color index and the structure of the dye are shown in **Table S1**. The  $1000\text{ mg L}^{-1}$  of dye was prepared in double distilled water and used as a stock. Solutions of desired concentration were obtained by successive dilution. Chemicals related to enzymatic analysis i.e., Tartaric acid, n-Propanol and Catechol were purchased from Merck-Germany. Nicotinamide adenine dinucleotide (NADH), mono sodium phosphate, di sodium phosphate, 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) were purchased from Sigma-Aldrich USA. Hydrogen peroxide and calcium chloride was purchased from S.D fine chem ltd., Mumbai, India. All chemicals used were of highest purity available and were of analytical grade.

### *2.2. Isolation, screening and maintenance of dye decolorizing bacteria*

Sediment samples were collected from the Divar Island, Goa. (Once collected, the samples were stored at  $4\text{ }^{\circ}\text{C}$  until further analyses were carried out). The samples were serially diluted from  $10^{-2}$ – $10^{-7}$  and then plated on 50% sea water nutrient agar (SWNA) (M001– Hi-Media). Isolated colonies were tested for dye removal capability by exposing them in to  $25$ – $150\text{ mg L}^{-1}$  of CR added with glucose and Yeast extract  $1.0\text{ g L}^{-1}$  (Decolorized media (DM) and incubated for 3–4 days in static condition (Babu et al., 2013). Color intensity of the dye was measured at 12 h intervals. The bacterial isolates showing positive indications of growth and decolorization were gradually raised to high concentration of the dye ( $150$ – $500\text{ mg L}^{-1}$ ). Potential cultures were sub - cultured frequently and maintained in nutrient agar slants and stored in sealed tubes at  $4\text{ }^{\circ}\text{C}$ . The same bacterial isolate were also stored in glycerol vials at  $-80\text{ }^{\circ}\text{C}$ .

### 2.3. Molecular identification of dye degrading strain

The genomic DNA was extracted from the isolate by using DNeasy kit (Qiagen). 16S rRNA genes of the bacterial isolates were amplified using universal forward and reverse 16S rDNA primers 27F [5'-AGA GTT TGA TCC TGG CTC AG-3'] and 1492R [5'-GGT TAC CTT GTT ACG ACT T-3'] (Lane, 1991). Nucleotide sequencing for these samples was performed using a 3130xl genetic analyzer (Applied Biosystems- Foster City, USA) at National Institute of Oceanography, Goa, India. The nucleotide sequences of the isolates obtained were compared with the sequences available in public database using BLAST software ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Neighbor-joining method was employed to construct the phylogenetic tree using MEGA 5 software (Tamura et al., 2011) and the maximum likelihood method was adopted for calculating the evolutionary distance.

### 2.4. Decolorization assay

A loopful of microbial culture was inoculated in 100 mL of nutrient broth (NB) (M088-Hi-Media) and incubated on rotary shaker for 30 h at 125 rpm at  $33 \pm 2^\circ\text{C}$ . After 30 h the culture was centrifuged at 10,000 rpm for 10 min at  $4^\circ\text{C}$ . Supernatant was discarded. The cell pellet was washed and resuspended in 0.85 % of NaCl and used for further studies.

One mL of the bacterial inoculums prepared as mentioned above (approximately  $1.2 \times 10^6$  cells  $\text{mL}^{-1}$ ) was inoculated in to a 250 mL Erlenmeyer flask containing DM with  $100 \text{ mg L}^{-1}$  (100 mL) of CR dye. The same composition of DM was used for all decolorization experiments except for optimization studies. The flasks were incubated in the dark at  $33 \pm 2^\circ\text{C}$  for 36 h under static condition. Every 6 h an aliquot of 4 mL was removed from the flask centrifuged at 10,000 rpm for 10 min at  $20^\circ\text{C}$  and the absorbance values of supernatants were measured using UV-Vis Spectrophotometer (Agilent-Cary 300, Singapore) at 495 nm. All the experiments were performed in triplicate and the decolorization activity was measured (Babu et al., 2013).

Decolorization percentage was calculated using the formula:

$$\text{Decolorization (\%)} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

### 2.5. Optimization of process parameters

The decolorization of CR ( $100 \text{ mg L}^{-1}$ ) by DTS26 was studied using DM with respect to various incubation times (0–36 h) with an interval of 6 h, temperature from 10–60 °C with an interval of 10 °C, pH 4–10. All the experiments were carried out in static condition.

### 2.6. Transmittance of light

The DM containing different concentrations of CR dye ( $100\text{-}500 \text{ mg L}^{-1}$ ), inoculated with 1mL of bacterial cultures ( $10^6 \text{ cells mL}^{-1}$ ) were incubated at room temperature (RT) for 30 h and centrifuged as mentioned in section 2.4. The supernatants were measured for their light transmittance using premier color scanner (SS5100A, India). This Instrument measures the transmittance of light from 400 - 700 nm with every 10 nm difference and gives RFL values (Reflectance values). Samples without bacterial cultures were used as control.

### 2.7. Primary productivity (PP)

The effect of dye disposal on primary production was studied by setting up a small microcosm experiment. The bacterially treated and non treated dyes (300 mL; concentration  $100 \text{ mg L}^{-1}$ ) at the laboratory scale were transferred into 1200 mL of freshly collected seawater from the Donapaula coastal region. Samples were mixed well and left in a tub for 30 min; from this 300 mL was taken in polycarbonate bottles (Nalgene, Germany) in 4 numbers. Each bottle was inoculated with one ampoule of radio-labelled  $^{14}\text{C}$  vials (Board of Radiation and Isotope Technology, Mumbai; Specific activity 185 kBq). Two bottles were used for light and the other two were used for dark. Neutral density screens (black bags) were employed to control the light in the dark bottles. These samples were further incubated in a tub containing freshly collected seawater for a period of 12 h. The temperature of the sea water in the tub was checked periodically, and fresh seawater was added to maintain the temperature. The incubated samples were filtered through GF/F filters (47 mm diameter,  $0.7 \mu\text{m}$  pore size, Whatman, USA) and the filter papers were exposed to concentrated HCl fumes to drive off any inorganic  $^{14}\text{C}$  adhering to samples. The filter paper was kept in a scintillation vial and stored at  $-20 \text{ }^\circ\text{C}$ . Ten mL liquid scintillation cocktail (Spectrochem Pvt Ltd, Mumbai) was added to each vial and incubated overnight; the radioactivity was measured by scintillation counter (Wallac 1409DSA, Perkin Elmer, USA). PP rate was expressed in  $\text{mgC}^{-1}\text{m}^{-3}\text{day}^{-1}$  as suggested by Knap et al. (1994).

## 2.8. Enzyme activities

### 2.8.1. Preparation of cell free extracts

The bacterial isolate were grown in NB at  $33 \pm 2$  °C for 30 h and centrifuged at 7,000 rpm for 15 min at 4 °C. The clear supernatant was used as an extracellular enzyme. The cells were suspended in 50 mM potassium phosphate buffer (pH 7.4) and sonicated (Ultrasonicator: Model No. SKL 250D) by keeping the sonicator as the output of 70A, and giving 16 strokes each of 9s, with 6s interval for 4 min at 4°C. This homogenate was centrifuged at 8000 rpm for 20 min at 4 °C and the supernatant was used as a source of intracellular enzyme (Joshi et al., 2010).

### 2.8.2. Enzyme assays

Lignin peroxidase activity was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture (pH 3.5) containing 2.5 mL 100 mM n-propanol, 250 mM tartaric acid, 10 mM H<sub>2</sub>O<sub>2</sub> (Kalyani et al., 2008). Tyrosinase activity was determined in a reaction mixture of 2 mL, containing 0.01 % catechol in 0.1 M phosphate buffer (pH 7.4) at 495 nm (Kalme et al., 2007). Azoreductase activities were measured according to the method of Ozdemir et al. (2008). One unit of enzyme activity was defined as the amount of enzyme that catalyses the oxidation of 1  $\mu$ mol of NADH min<sup>-1</sup>. Laccase activity was determined using ABTS as the substrate. The reaction mixture consisted of 0.5 mL ABTS (5 mM) dissolved in 100 mM acetate buffer (pH= 4.5) and 0.5 mL of enzyme solution or culture supernatant (at desired dilution). One unit of enzyme activity was defined as a change in absorbance unit per minute per mg protein of the enzyme (Saratale et al., 2010). All the enzymatic reaction was carried out at  $31 \pm 2$  °C where reference blanks contained all components except the enzyme. All enzyme assays were run in triplicate and the average values were taken to calculate unit of enzyme activity. The protein content was determined by Lowry et al. (1951) with bovine serum albumin as the standard.

## 2.9. Detoxification Experiments

### 2.9.1. Chemical oxygen demand (COD) Removal

The COD was characterized in the DM after the end of 30 h. The COD removal rate was calculated for treated CR at different concentration varying from 100-500 mg L<sup>-1</sup>. The treated CR dye was centrifuged at 10,000 rpm for 10 min at 20 °C then the supernatant was extracted for the analysis of COD. The same

procedure was followed for untreated samples to obtain the initial value. The supernatant was refluxed with potassium dichromate in the presence of silver sulfate, mercury sulfate and concentrated H<sub>2</sub>SO<sub>4</sub> which was titrated with Ferrous Ammonium Sulfate (FAS) using ferroin as an indicator (APHA, 2005).

$$\text{COD Removal (\%)} = \frac{\text{Initial COD} - \text{Final COD}}{\text{Initial COD}} \times 100$$

### 2.9.2. Toxicity assay

Toxicity assay was done by using *Artemia franciscana*. The cysts of *A. franciscana* were hatched out in sterilized sea water (35 ppt) with aeration for 48 h to nauplii stage under light condition (1000 lux). Twenty four-multiwell plates (Costar USA, 3 mL capacity) were used for this experiment. The volume of the well was 3 mL and the width 106mm. Thirty eight nauplii were transferred to each well containing 1.5 mL of CR dye prepared in seawater with different concentrations (100-500 mg L<sup>-1</sup>) for treated and non treated samples. Bacterially treated dye was used as treated samples. Control wells contained normal seawater. The multi-well plate was incubated at RT (30 ± 2 °C) for 72 h. After incubation, the number of dead and live nauplii in each well were counted by using an inverted trinocular microscope (Nikon Eclipse TS100F) with different time intervals of 24, 48, 72 h respectively (Silva et al., 2010). All the samples were run in triplicate. The percentage mortality of treated and untreated CR was calculated by dividing the number of dead *A. franciscana* by the initial number in each well (Singh et al., 2011). The toxicity of untreated dye was also assessed by the same procedure.

### 2.10. Biodegradation analysis of CR

The degraded metabolites were analyzed by UV-Vis spectroscopy (Shimadzu/Japan), Fourier Transform Infrared Spectroscopy (FTIR) and High performance liquid chromatography (HPLC). Biodegraded metabolites of CR were extracted from the culture supernatant with an equal volume of ethyl acetate. The same samples were used for both UV-Vis and FTIR; the analysis was carried out as per the methodology suggested by Babu et al. (2013). For HPLC analysis biodegraded metabolites of CR were extracted from the culture supernatant with an equal volume of ethyl acetate and methanol (1:5 v/v). The solvent was evaporated completely and re-dissolved in 200µl of HPLC grade methanol, filtered through 0.22µm membrane filter. For analysis of biodegradation and determination of metabolites the sample was injected into a HPLC system equipped with SPD 10A VP UV-visible detector (Shimadzu Corp.,

Kyoto, Japan) using C<sub>18</sub> waters column (4.6 mm X 250 mm; particle size 5 µm, Waters Corporation, 34 Maples Street, Milford, MA 01757-3696, USA). Isocratic elution was performed with mobile phase of methanol: water (90:10 v/v) at a flow rate of 1 mL min<sup>-1</sup>. An aliquot of 20 µl was injected and analyzed by UV–visible detector at wavelength of 518 nm. Gas chromatography–mass spectrometry (GC–MS) analysis of degraded metabolites was carried out using a Perkin Elmer, Turbo Mass Gold, MS system, USA. GC was conducted in the temperature programming mode with a Restek column (30.0m x 250µm). The initial column temperature was 80 °C for 2 min, and increased linearly at 10 °C/min to 280°C, which was held for 10 min. The ionization voltage was 70 eV. The temperature of the injection port was 280 °C and the GC-MS interface was maintained at 290 °C. The helium carrier gas flow rate was 1.0 ml min<sup>-1</sup>. Degradation products were identified by comparison of fragmentation pattern, as well as with the fragmentation products detected in CR degradation process (Li, 2013; Telke et al., 2010).

### *2.11. Statistical analysis*

Data were analyzed by one-way ANOVA with a turkey scheffe alpha multiple comparison test. Readings were considered significant when P was <0.05 by using Predicted Analytical Software 18.0 (PASW).

## **3. Results and discussion**

### *3.1. Molecular identity of the potential strain*

A total of 137 strains were initially screened in 50 mg L<sup>-1</sup> CR concentration. The isolate (DTS26) showed maximum decolorization in a shorter residence time of 72 h. Thus the strain was selected and identified as *Dietzia* sp. based on 16S rRNA gene sequence. The sequence was submitted to GenBank with an accession number of KJ396270. Phylogenetic relationship of the isolate DTS26 and its closest neighboring sequences in GenBank database is given in **Fig. S1**.

### *3.2. Effects of process parameters on decolorization*

The decolorization efficiency of CR dye was studied by different agitation and various time intervals by DTS26. The maximum decolorization was 93.10 and 28.56% within 30 h under static and shaking conditions respectively. The decolorization activity was found to increase when the flasks were kept in static condition; meanwhile the decolorization activity decreased under shaking condition (**Fig.1a and**



b). It was found that under shaking conditions, the presence of oxygen deprives the azoreductase from obtaining the electrons needed for cleavage of azo dyes. Whereas under static conditions, these electrons are available to azoreductase from NADH to decolorize azo dyes (Tripathi and Srivastava, 2011).

Effects of various pH and temperatures on decolorization of CR are shown in **Fig. 1c and d**. The maximum decolorization (90.81%) was achieved at pH 8 within 30 h. The pH tolerance is relatively important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions (Sandrin and Maier, 2003). Similar studies by Anjaneya et al. (2011) reported that maximum decolorization (84%) of Metanil Yellow by *Lycinibacillus* sp. was observed at pH 8. Decolorization of synthetic dyes by microorganisms was mainly affected by the environmental factors such as pH and temperature (Pearcea et al., 2003). The maximum decolorization (94.52 %) was observed at  $32 \pm 2$  °C and it significantly decreased at higher temperatures. This might be due to the reduction of cell viability at higher temperatures or inactivation of the enzymes which are responsible for decolorization of CR dye (Selvakumar et al., 2013). Compared to previous reports, our strain (DTS26) yielded better decolorization in a shorter residence time and showed higher activity at alkaline pH. This suggests that DTS26 has potential application for the treatment of industrial wastewater which is more alkaline.

### 3.3. Light intensity and Primary productivity analysis

The light intensity tested for treated samples gave higher RFL values (59.67, 51.32, 52.90, 41.13 and 29.07 for 100-500 mg L<sup>-1</sup> concentrations respectively) when compared with the non treated (**Fig.2a**). Treated dyes were able to enhance the transmittance level up to  $\approx 38\%$ . Transmittance levels were decreased with increased concentrations of CR dye. Light intensity was reduced by 28 % for 400 and 6% for 500 mg L<sup>-1</sup> (**Fig.2a**). Primary production data indicates nearly five times (4.69 mgC<sup>-1</sup>m<sup>-3</sup>day<sup>-1</sup>) higher in the treated samples when compared with non treated samples (1.07 mgC<sup>-1</sup>m<sup>-3</sup>day<sup>-1</sup>) shown in **Fig.2b**.

Most of the industrial discharges were generally into estuaries, rivers or in and around coastal regions (Matthiessen and Law, 2002). Disposal of dyes into the aquatic environment causes toxicity due to their long residence time and inhibit the light intensity (Carmen and Daniela, 2012; Gattuso et al., 2006). Light intensity plays a major role in photosynthesis of living organisms in marine environment. The disposal of untreated textile effluent into the aquatic system reduces the light penetration and forms

turbidity in the marine environment; it also inhibits the photosynthetic activity of aquatic organisms. Thus the marine food chain is altered due to its disposal (Zaharia et al., 2009).

However, PP was mainly controlled by abiotic factors, mostly irradiance of light. Phytoplankton plays a vital role in primary production and it utilizes maximum light for this purpose (Salinas and Larson, 2000). PP significantly affected by the decreased level of light penetration due to the disposal of industrial effluent (Levings et al., 2005; Smith, 2007). It also reduces the level of chlorophyll content in marine ecosystems which is mainly responsible for PP (Singh and Singh, 2006). In this study we could not measure the chlorophyll and phaeopigments to support and quantify the PP because of the colored dye, which interfered with the estimations. This study indicates the enhancement of light intensity by 38% subsequently which leads to fivefold increase in PP.

### 3.4. Detoxification Experiments

#### 3.4.1. Bioassay on *A. franciscana*

Toxicity rates were estimated by studying the mortality rate of *A. franciscana*. The mortality rates in 100 mg L<sup>-1</sup> of CR dye was close to 9, 30 and 38 % followed by 200 mg L<sup>-1</sup> which showed 15, 36 and 53% after 24, 48 and 72 h respectively (**Fig.3a, b and c**). Whereas the untreated dye of similar concentrations showed 29, 55, 57 as well as 39, 63, and 68 % respectively. Bacterially treated samples were able to provide high level of survival. After 24 h the mortality percentage in control was three times higher compared to treated samples. Higher concentrations of the dyes (300-500 mg L<sup>-1</sup>) lead to more mortality and the survival rates also decreased accordingly.

Textile industries use various groups of dyes, 70% of them were azo dyes which are known to be toxic in nature (Ogunjobi et al., 2012). When the color content was removed by the treatment process, it also reduced the toxicity of the textile effluent generated at the end of the process (Furlan et al., 2010). *Artemia* sp. could uptake the dye which accumulated in mid-gut regions, thus increasing mortality rates when the dye concentrations increased from 300-500 mg L<sup>-1</sup> (Prasad and Rao, 2013). The present toxicity study revealed that the mortality rates of treated dye were much less than with untreated ones. It is further concluded that the treated dyes were less toxic to aquatic organisms and could be discharged with less harm to any aquatic ecosystem.

### *3.4.2. Chemical Oxygen Demand*

The COD level was found to increase when the dye concentrations are increased. COD removal rates showed maximum 86.36% at 100 mg L<sup>-1</sup> (**Fig.4**). The removal rate was found to decrease as the dye concentration increased from 100-500 mg L<sup>-1</sup>. A minimum of 36.95% could be removed by 500 mg L<sup>-1</sup> concentrations of dye. Earlier studies by Isik and Sponza (2003) reported that 77% of COD was removed at 100 mg L<sup>-1</sup> of CR dye by using USAB/CSTR reactor. COD plays a major role in aquatic pollution and it's also considered as a pollution indicator. The microorganisms continued to ingest the obtained organic compounds until near-complete removal of COD in the dye concentration (He et al., 2004). The higher reduction of COD in the CR dye (100 mg L<sup>-1</sup>) indicates the mineralization and also the detoxifying nature of dye (Phugare et al., 2011), because COD removal rate and color removal is a co-metabolic process (Lu et al., 2008). The present study suggested that removal of COD from decolorized supernatant of CR dye could be less toxic to aquatic organisms.

The textile effluent contains a high level of COD (642 mg L<sup>-1</sup>) therefore, the direct discharge of this effluent into the aquatic ecosystem results in oxygen depletion with significant detrimental effects on aquatic biota especially fish (Morrison et al., 2001). Moreover, the high organic content could lead to the growth of blue-green algae, which could release toxic substances (cyanotoxins) into the water (Ramavandi and Farjadfard, 2014). The initial COD concentration was high when compared to the permissible limit prescribed by the Central Pollution Control Board. The present results showed that COD level was reduced from 1760 to 240 mg L<sup>-1</sup> (86.36%) treated by DTS26 at CR concentration of 100 mg L<sup>-1</sup>. Hence the present bacterial strain would be helpful for the reduction of pollution levels in marine environment.

### *3.5. Mechanism of Degradation*

#### *3.5.1. Enzyme studies*

The induction of extra and intracellular enzyme activities was tested for four different enzymes which were responsible for the dye degradation. Extra and intracellular synthesis of azo reductase and tyrosinase in treated dye shows more activity when compared with enzyme activities in NB (**Table 1**). Enzyme activities of lignin peroxidase were not observed with DTS26. Laccase and lignin peroxidase were statistically non significant against decolorization of CR. In contrast to Telke et al. (2010) who

reported that the decolorization activity of CR might be due to the intracellular laccase activities. The decolorization of CR was mainly due to the induction of azo reductase which involves the reductive cleavage of azo bonds ( $-N=N-$ ). Further it represents formation of corresponding amines (intermediate metabolites) resulting in color removal of azo dyes (Saratale et al., 2011). The reductive biodegradation of azo dyes takes place upon action of enzymes such as azo reductase and tyrosinase. An intracellular azoreductase is responsible for the decolorization activity, as previously shown for other azo dye-degrading microorganisms (Leelakriangsak and Borisut, 2012; Tamboli et al., 2010 ). Tyrosinase was observed during the decolorization of sulfonated azo dyes by the bacteria *P. desmolyticum* and *Exiguobacterium* sp. (Kalme et al., 2007; Dhanve et al., 2008). *Providencia* sp. SDS showed the presence of laccase, and azo reductase for decolorization and detoxification of textile effluents by a developed bacterial consortium (Phugare et al., 2011). The biodegradation of synthetic azo dyes was mainly because of their biotransformation by various oxidative and reductive enzymes (Raghukumar et al., 1996).

### 3.5.2. UV-Vis spectral analysis for color removal

UV-Vis spectral analysis (200–800 nm), carried out for the decolorization analysis of CR treated by DTS26 at various time intervals, are presented in **Fig. S2**. The major peaks observed between 480-505 nm at 0 h were found to reduce their peak area after 18, 24 and 30 h respectively and new peaks were also observed for 18 and 30 h. Maximum decolorization by complete reduction in the peak area could be observed at the end of 30 h. If the decolorization process is attributed to biodegradation, either the maximum absorbance peak will completely disappear or it will shift to another wavelength, resulting in the formation of new peaks (Chen et al., 2003). The reduction in absorbance peaks area confirms the decolorization of CR dye by the process of biodegradation. Further it indicates the bacterial action and its metabolic process over the CR dye.

### 3.5.3. FT-IR analysis

The FTIR spectrum of control CR showed peaks in the region for unsubstituted and multi-substituted benzene rings. The peaks were observed at  $642\text{ cm}^{-1}$  for C–C bending vibrations,  $696\text{ cm}^{-1}$  for C–H stretching vibrations for disubstituted aromatic compound,  $832\text{ cm}^{-1}$  for p-disubstituted ring vibrations,  $1,061\text{ cm}^{-1}$  for S=O stretching vibrations of sulfonic acid,  $1,225\text{ cm}^{-1}$  for C–N stretching vibrations,  $1,352\text{ cm}^{-1}$  for C–N bending vibrations,  $1,447\text{ cm}^{-1}$  for aromatic C=C stretching vibrations,  $1,584\text{ cm}^{-1}$

for N=N stretching vibrations, 3,066  $\text{cm}^{-1}$  for O-H stretching vibrations and 3,469  $\text{cm}^{-1}$  for N-H stretching vibrations of aromatic primary amine (**Fig.S3a**). The FTIR spectrum of CR sample obtained after treatment with bacterium showed peaks at 618  $\text{cm}^{-1}$  for ring bending vibrations, 814  $\text{cm}^{-1}$  for p-disubstituted benzene ring vibrations, 1,137  $\text{cm}^{-1}$  aromatic C=C stretching vibrations, 2921  $\text{cm}^{-1}$  C-H stretching vibrations and 3,333  $\text{cm}^{-1}$  for N-H stretching vibrations of primary amide. The cleavage of azo bond was confirmed by the absence of peak at 1,584  $\text{cm}^{-1}$  for N=N stretching vibrations in the FTIR spectrum (**Fig. S3b**) of CR sample obtained after treatment. The same results were supported by Telke et al. (2010) who observed that the absence of peak at 1,587  $\text{cm}^{-1}$  corresponds to N=N stretching vibrations in the FTIR spectrum for degraded metabolites of CR. Absence of N=N indicates the cleavage of azo bond by DTS26.

#### 3.5.4. HPLC and GC-MS analysis

The HPLC elution profile of standard CR showed retention time at 1.779 min compared to the treated sample (**Fig. S4a**). The treated sample showed prominent peaks at retention time of 1.728 (CR), 2.263, 2.420 and 2.516 min when metabolites separated from the sample obtained after decolorization (**Fig. S4b**). The sharp decrease in the concentration of CR was confirmed by the analysis. Several peaks were also observed in the chromatogram of treated sample indicating the production of metabolites by the bacterium. These results confirmed the degradation of CR by bacterium. In the present study, the generation of prominent peaks in HPLC chromatogram may be the degraded products of CR. The appearance of new peaks in the degraded metabolites formed after decolorization of CR by DTS26 support the production of different intermediate metabolites (Lade et al., 2012).

GC-MS profile and proposed the degradation pathway of CR by DTS26 are shown in and **Fig 5** and **Fig S5**. The lignolytic enzymes are known for their immense roles in the biodegradation of pollutants from the textile industry and paper whitening (Wesenberg et al., 2003). The lignin-degrading enzyme produced by certain bacteria can degrade polymeric dyes through oxidative cleavage (Kalme et al., 2006). The specific and nonspecific reduction of the azo bond in CR is due to the presence of azoreductase and laccase activities. In the presence of molecular oxygen laccase is known to attack aromatic rings via two steps of electron transfer (Nagai, 2002) and veratryl alcohol oxidase has been implicated in the prevention of polymerization of reactive rings produced by laccase (Marzullo et al., 1995), thereby making laccase products susceptible to ring cleavage. The apparently, 4-amino-3-

naphthol-1-sulfonate a degraded product was produced by the action of laccase enzyme on CR and the 3-hydroperoxy 8-nitrosonephthol is produced by the desulfonation and amine oxidation of the 4-amino-3-naphthol-1-sulfonate.

#### 4. Conclusions

*Dietzia* sp. (DTS26) a newly isolated actinobacterial strain showed a greater potential in color removal and detoxification of textile dye CR. This strain produces extra and intracellular enzymes, specifically azoreductase for decolorization of CR and laccase is responsible for degradation. Results obtained through this study significantly reveal that the turbidity and the color of the dye could be decreased for its safe disposal into the coastal waters with increased rates of productivity. Reduction of COD and enhanced survival rates of *A. franciscana* in the treated samples also supports detoxification of CR dye thus helping biological organism for better survival. The present study suggests that the bacteria DTS26 could be a good bioremediator for the treatment of effluents containing CR dye.

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## Figure captions

**Fig.1.** Effects of (a) agitation, (b) incubation time, (c) pH and (d) temperature on decolorization of CR dye.

**Fig. 2(a)** Light intensity values for different dye concentrations in comparison with treated and non treated samples. **2(b)** Transmittance of light and primary productivity for control and treated samples of CR dye concentration ( $100 \text{ mg L}^{-1}$ ).

**Fig. 3.** ((a) 24 h (b) 48 h (c) 72) Toxicity levels tested with *A.franciscana* for its survival rates in treated and untreated dyes at various time intervals.

**Fig. 4.** COD removal rates in various dye concentrations compared with bacterially treated samples.

**Fig. 5.** Proposed degradation pathway of CR by *Dietzia* sp.

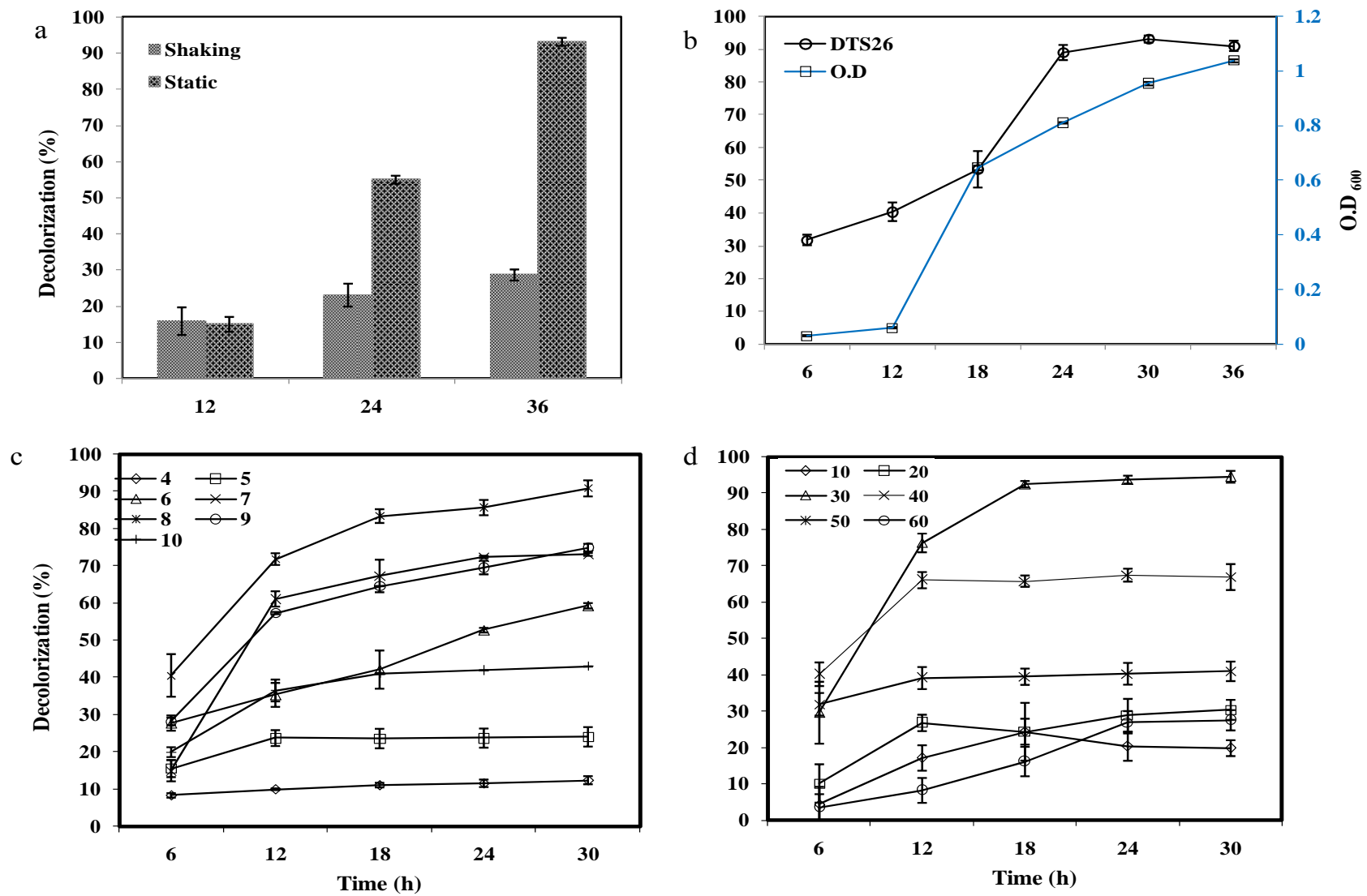


Fig. 1.

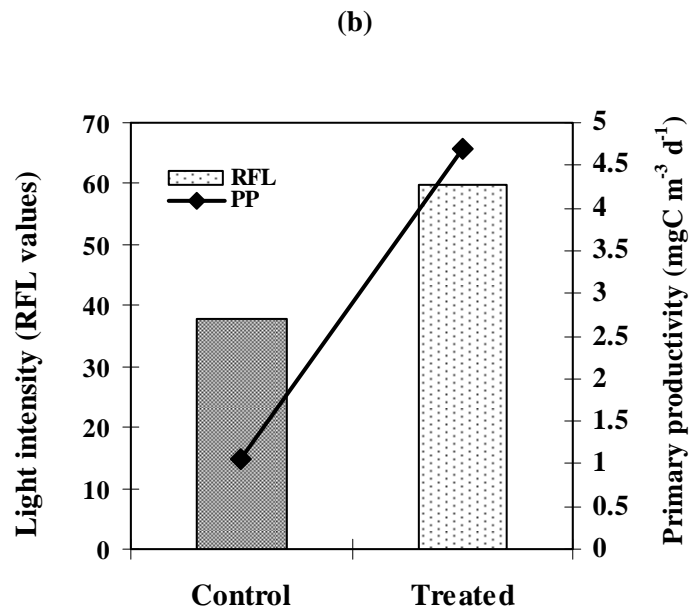
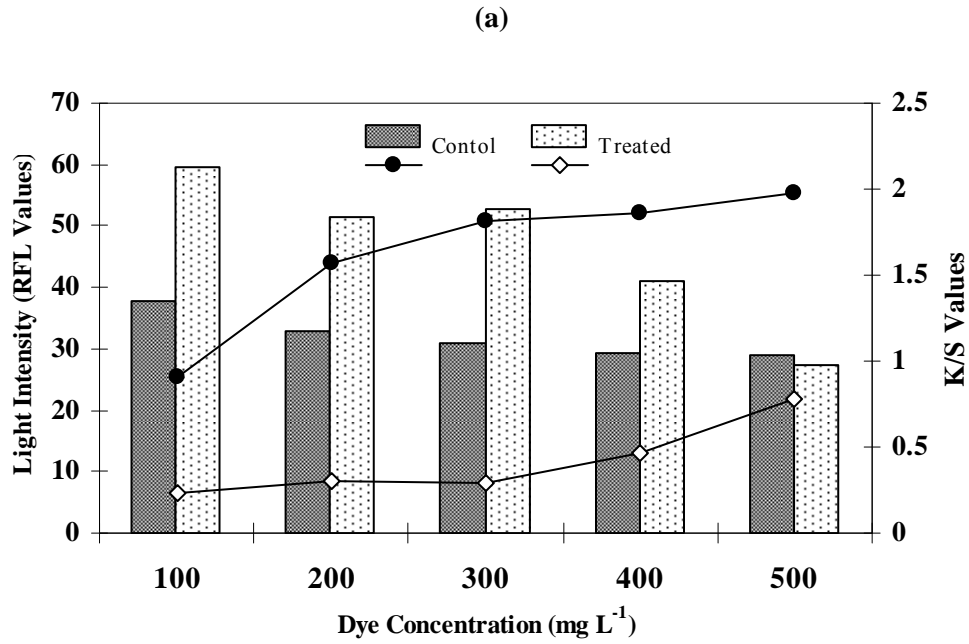
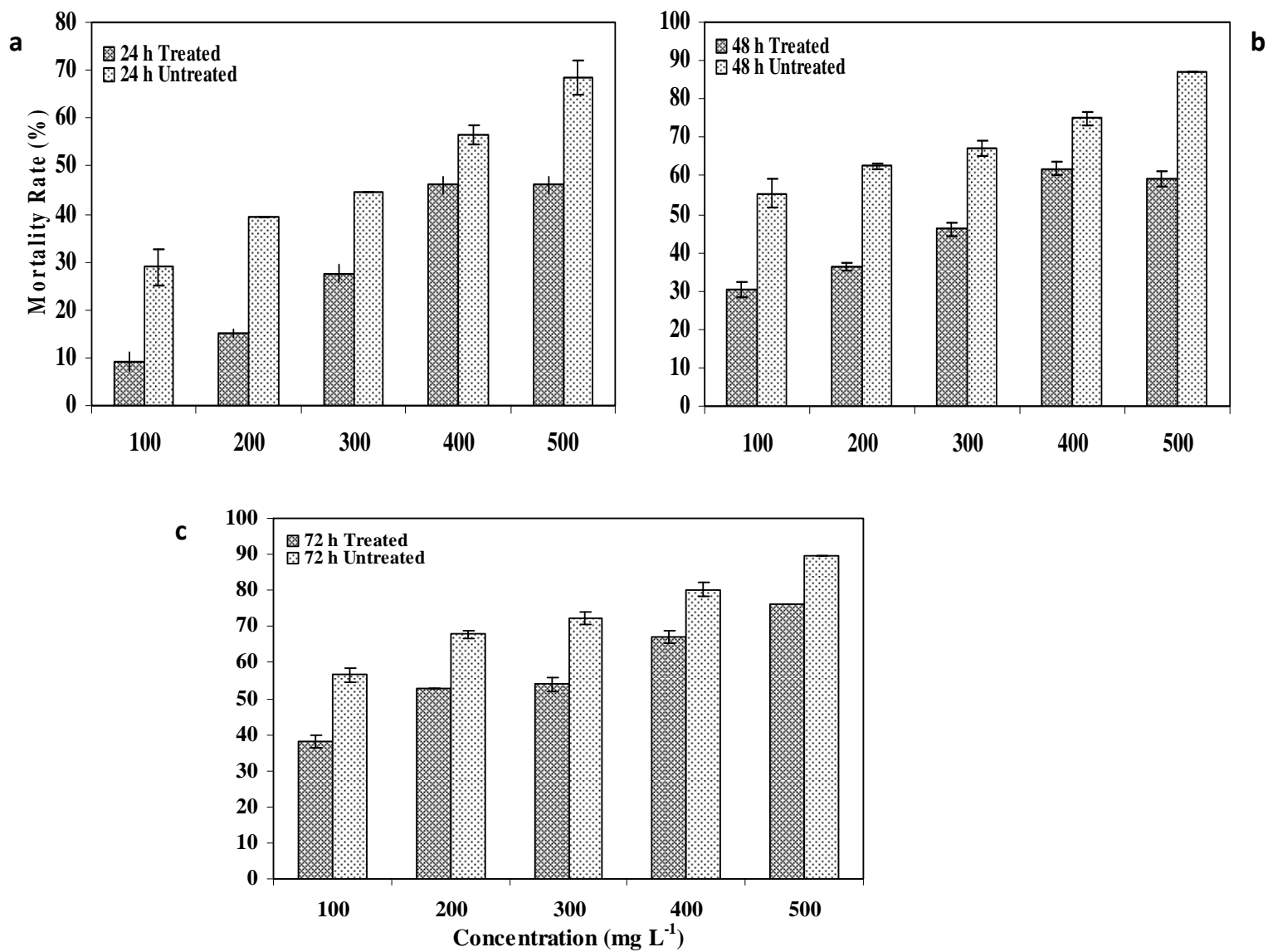


Fig. 2.



**Fig. 3.**

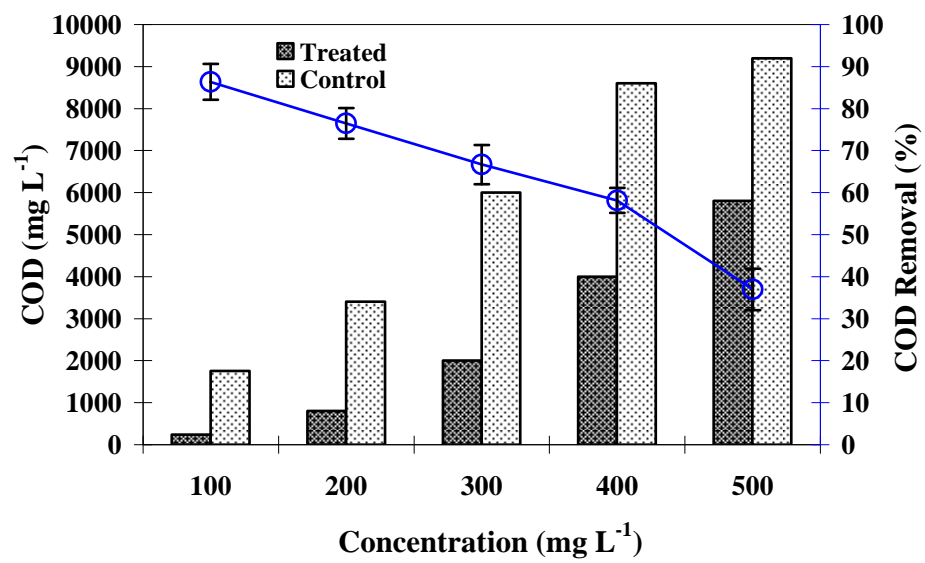
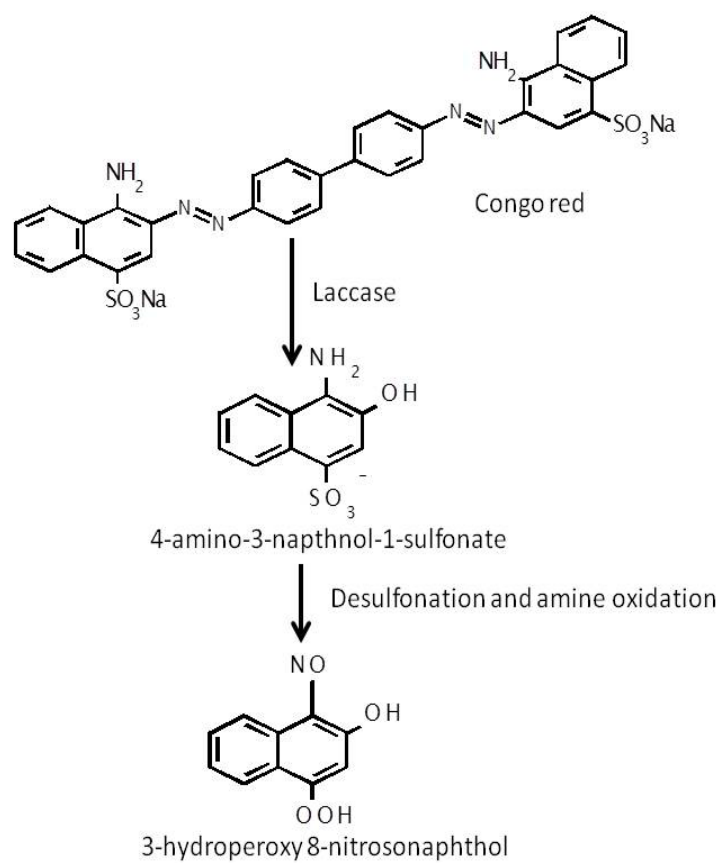


Fig. 4.



**Fig. 5.**



**Table 1**

Assay for different types of enzymes during decolorization

Enzyme	Enzyme production in NB		Enzyme production in DM	
	Ext	Int	Ext	Int
Laccase <sup>a</sup>	0.0185±0.0007	0.0443±0.0003	0.0067±0.001*	0.0337±0.0013*
Lignin peroxidase <sup>b</sup>	BDL	BDL	BDL	BDL
Tyrosinase <sup>b</sup>	0.0078±0.0001	0.0138±0.0001	0.0403±0.0005***	0.0719±0.0007***
Azo reductase <sup>c</sup>	0.1577±0.000	0.1576±0.000	0.2501±0.0001***	0.1897±0.0004***

Ext-Extracellular, Int-Intracellular.

± Mean, Standard Deviation of triplicates

BDL- Below Detectable Level

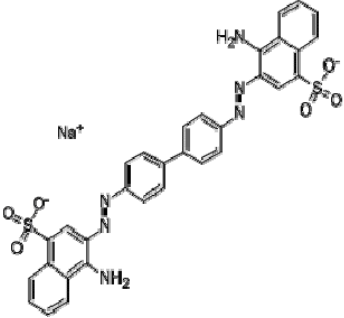
<sup>a</sup> - Enzyme unit's min<sup>-1</sup> mg protein<sup>-1</sup><sup>b</sup> - Enzyme unit's mg<sup>-1</sup> min<sup>-1</sup><sup>c</sup> - Enzyme catalyses the oxidation of 1 μmol of NADH min<sup>-1</sup>

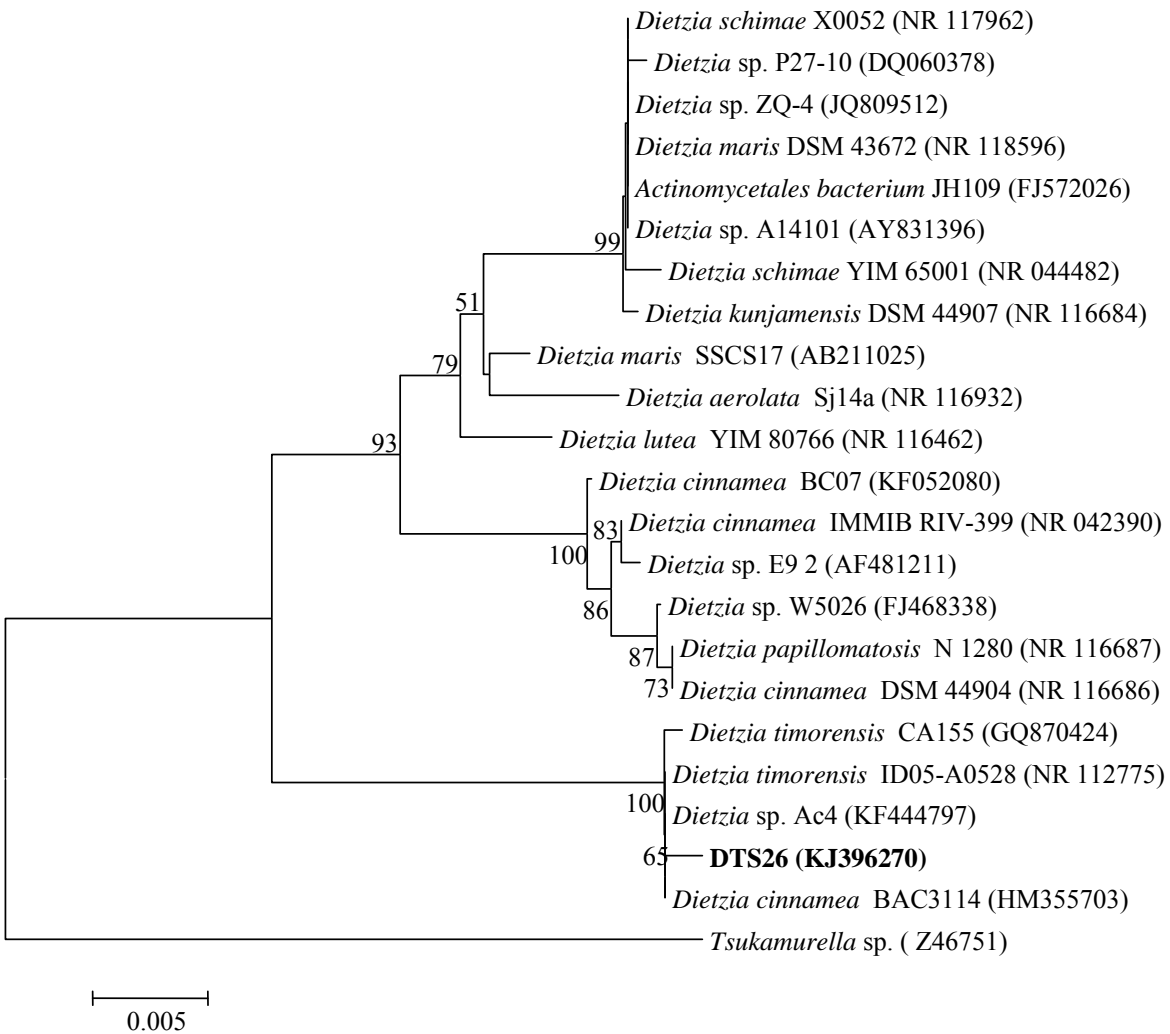
\*\*\* Significant at the level of P &lt; 0.05,

\*Not significant by One-way analysis of variance (ANOVA).

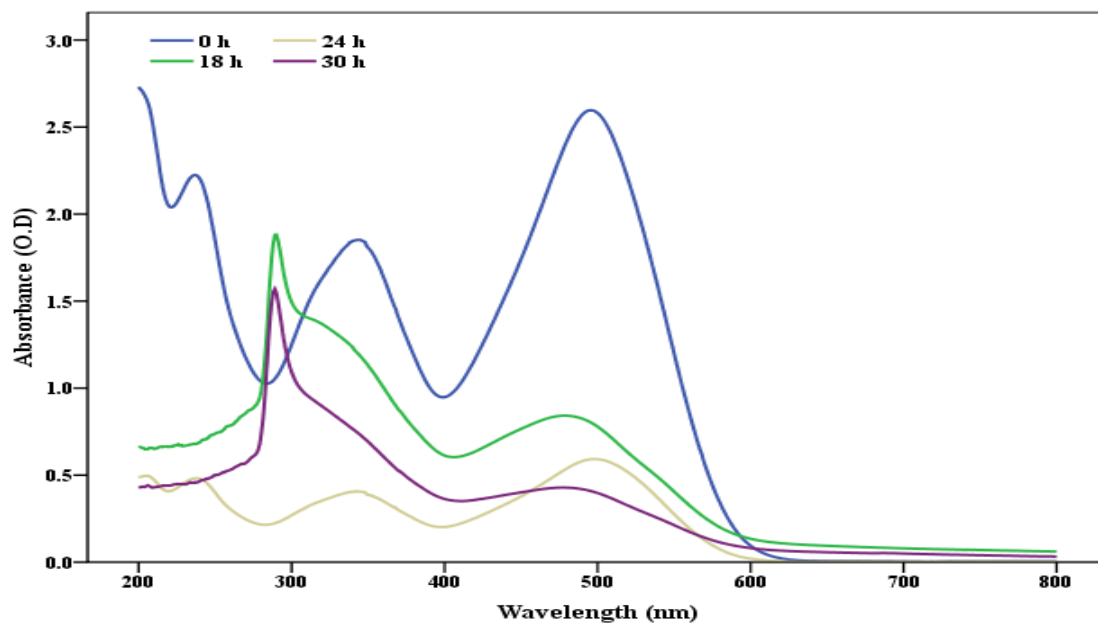
**Table S1**

Characteristics of the dye used in this study.

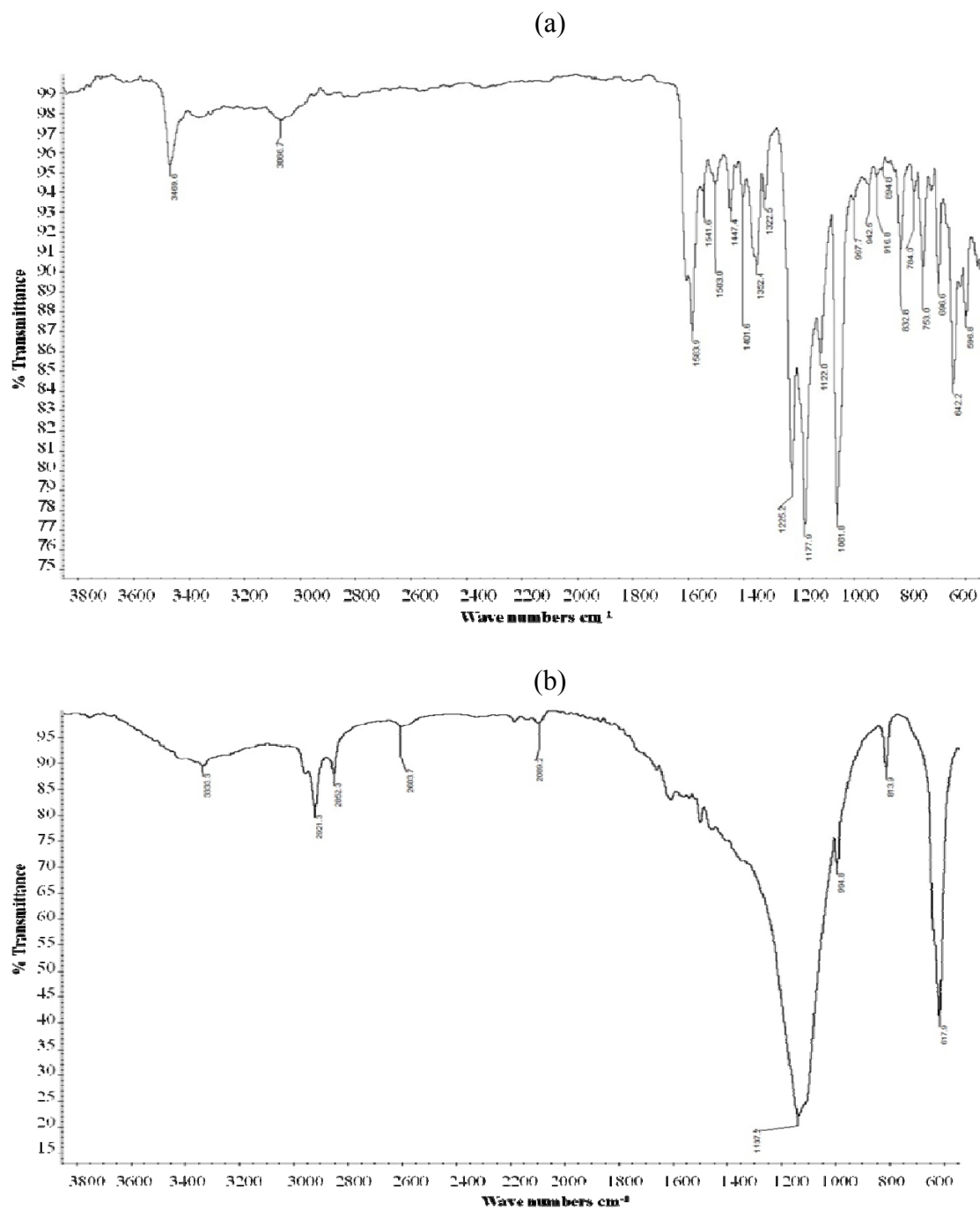
Dye	C.I. name	Chemical structure	C.I. number	Class	λ <sub>max</sub>
Congo Red	Direct red 28		Na 22120	Diazo	495



**Fig. S1.** Neighbour-joining tree showing the phylogenetic relationship of strain **DTS26** and phylogenetically related species based on 16S rRNA gene sequences. The neighbouring sequences were retrieved from GenBank database. Bootstrap percentages (1,000 replications) are shown at branching points and the values >50 % are shown. *Tsukamurella* sp. (Z46751) was used as an out group.

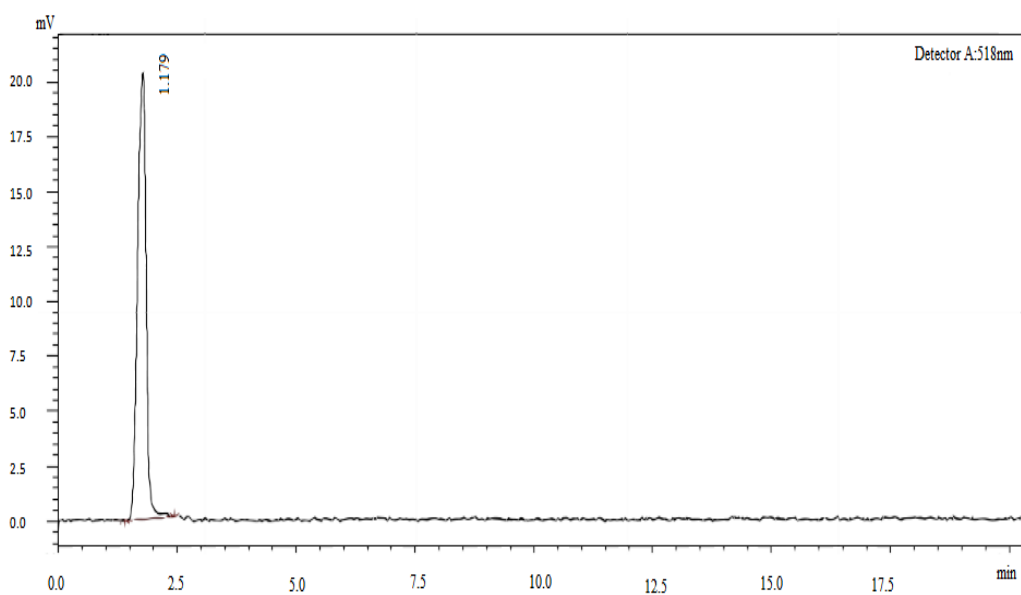


**Fig. S2.** UV-Visible spectral decolorization analysis of CR by DTS26.

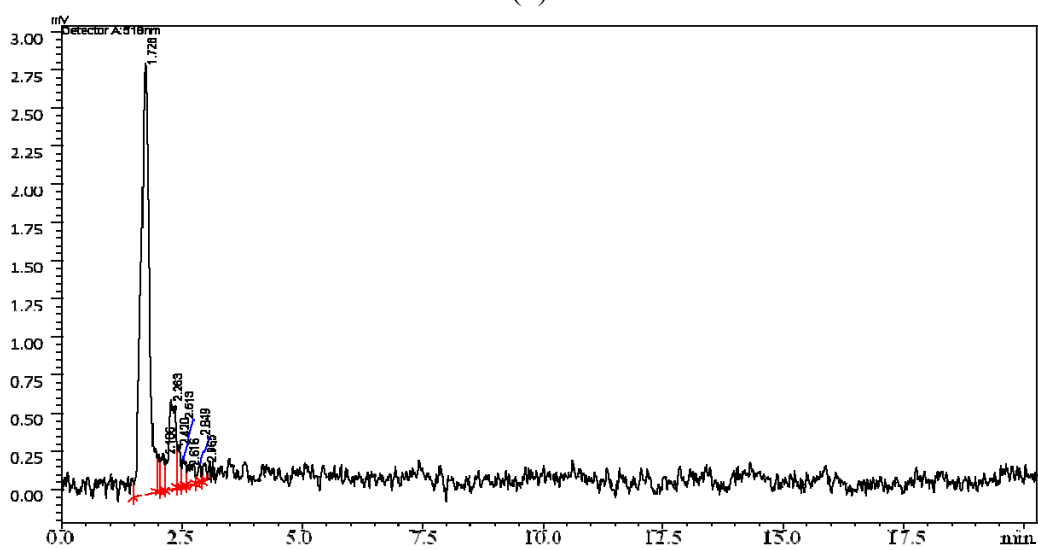


**Fig. S3.** The FTIR spectra of Congo red before (control-a) and after treatment (b) with bacterium indicated biodegradation.

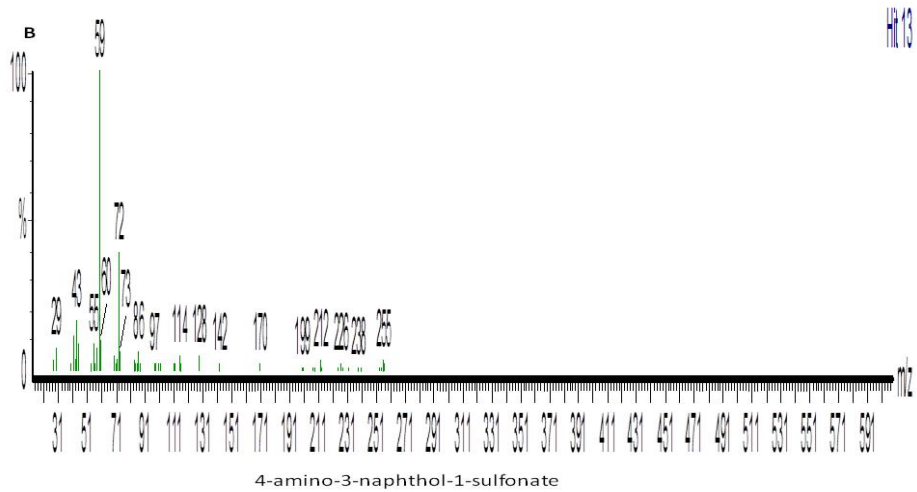
(a)



(b)



**Fig.S4.** HPLC profile of Congo red before (a) and after treatment (b) with bacterium showing products formed after biodegradation along with other metabolites produced by the bacterium.



**Fig.S5.** GC-MS profile obtained after biodegradation.