Biotransformation of diazo dye Direct Red 81 by Georgenia sp. CC-NM PT-T3
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ABSTRACT

Biotransformation of Direct Red 81, a diazo dye by Georgenia sp. CC-NM PT-T3 was studied under static anoxic condition. It was able to decolourize 97.56% of Direct Red 81 within 4 h at 50mg/L dye concentration. The selected isolate showed dye decolourization over a wide range of pH from 5-8 and temperature of 28±2°C - 45°C. The biodegradation was confirmed by Uv-Visible spectrophotometry, TLC, HPLC. Toxicity test demonstrated no toxicity of the degraded product. The isolate was also able to decolourize mixture of five azo dyes. Hence, this isolate can be effectively used for bioremediation of azo dye containing effluent.

Key words: Decolourization, Biodegradation, Azo dyes, Toxicity, Direct Red 81

INTRODUCTION

Rapid industrialization has necessitated the manufacture and use of different chemicals in day-to-day life. The dye manufacturing and textile industry extensively uses synthetic chemicals. Wastewaters from these industries pose a threat to the environment, as large amount of chemically different dyes are used. A significant percentage of these dyes are disposed into the environment via wastewater. Approximately 10,000 different dyes and pigments are used industrially, and over 0.7 million tons of synthetic dyes are produced annually, worldwide. It is very difficult to treat effluents of these industries because of their high BOD, chemical oxygen demand (COD), heat, colour, pH and the presence of metal ions( Shah et al 2013). The industries generate a large amount of wastewater containing dyes and represent one of the largest causes of water pollution, as 20–40% of dyes are lost in the effluent depending on the type of the dye to be
Azo dyes have been used increasingly in industries because of their ease and cost-effectiveness in synthesis compared with natural dyes. However, most azo dyes are toxic, carcinogenic and mutagenic. Azo bonds present in these compounds are resistant to breakdown, with the potential for persistence and accumulation in the environment. However, they can be degraded by several bacteria under aerobic and anaerobic condition. Attempts have been made to treat the effluent by different physical and chemical processes. These processes were found to be costly, less effective and lead to production of large amount of sludge causing secondary pollution (Shah et al 2013). Biodegradation of dye by microorganisms is found to cost effective and environment friendly hence in the present study evaluate the potential of the isolate Georgenia sp. CC-NM PT-T3 to biotransform the diazo dye Direct Red 81.

MATERIAL AND METHODS

Microorganism and culture conditions:

The bacterium in the present study was isolated by enrichment culture technique by gradually adapting the isolate to the increased concentration of Direct Red 81 (DR 81) in a basal nutrient medium (Sahasrabudhe and Pathade, 2012) from dye industry effluent contaminated soil, sewage, dung and dye waste. Pure culture was maintained on the nutrient agar slants. Composition of nutrient broth and agar used for decolourization is (g/L) Peptic digest of animal tissue 5, NaCl 5, Beef extract 1.5, Yeast extract 1.5 and pH 7.4±0.2

Dyestuff and chemicals:

All chemicals used were of the highest purity and of analytical grade. Nutrient broth dehydrated was purchased from Hi-Media, Mumbai, India. The textile dye Direct Red 81 (DR 81) was obtained from Spectrum dyes, Surat, India.
Identification of the culture:

The isolate is an actinobacterium which shows rod and coccus cycle. 16s r-RNA sequencing of the isolated organism was done in GeneOmbio Technologies Pvt. Ltd., Pune, India.

Decolourization studies:

*Georgenia* sp.CC-NMPT-T3 was grown for 24 hrs at 37°C on nutrient agar.10% inoculum of O.D<sub>600</sub> 1.0(Mathew et al.,2004; Parshetti et al., 2009)was used throughout the study. Decolourization studies were carried out in nutrient broth. The dye was filter sterilized by using 0.2 µm cellulose acetate paper filter, Sartorius Biolab, Germany and added after sterilization of medium throughout the study. The dye (50mg/L) was added immediately and incubated at static condition at 37°C. The aliquot (3ml) of culture media was withdrawn at different time intervals and centrifuged at 6000g for 20 min. Decolourization with respect to time was monitored by measuring the absorbance of the culture at λ<sub>max</sub> of the dye at 511 nm. In order to detect pH and
temperature optima of decolourization of the dye by the isolate, nutrient broth with different pH ranging from 3-8 was inoculated with 10% inoculum at 50mg/L dye concentration and was incubated at temperatures ranging from 25-50°C. Abiotic control was always kept in each study.

**Decolourization performance at different dye concentration:**

Once decolourization ability of the isolate was confirmed at 50mg/L concentration, the decolourization performance of the isolate was studied at different dye concentrations from 50-500mg/L under the same experimental setup. The % decolourization was measured after every hour. All decolourization experiments were performed in three sets. Abiotic controls (without microorganism) were always included. The % decolourization was calculated as (Sartale et al., 2009; Sahasrabudhe and Pathade, 2012).

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% \text{Decolourization} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100
\]

**Decolourization performance at different pH and temperatures:**

Sterile nutrient broth of different pH 3, 4, 5, 6, 7 and 8 was inoculated with 10% inoculum and incubated at 37°C under static condition. The dye concentration was 50 mg/L. For temperature studies sterile nutrient broth of pH 7.0 was inoculated with 10% inoculum and filter sterilized dye at 50 mg/L was added aseptically. The broth was incubated at 25°C, 30°C, 37°C, 40°C, 45°C and 50°C. All decolorization experiments were performed in triplicates. Abiotic control (without microorganism) was always included in each study.

**Analytical procedure:**

The metabolites produced during the biodegradation of DR 81 at 4 hrs i.e. after decolourisation of the medium were extracted twice with equal volume of dichloromethane (DCM). The DCM extracts were pooled and evaporated at 40°C in a rotary evaporator and then transferred to a test tube (Moutaoukkil et al., 2003). The extracted residue was dissolved in small volume of HPLC grade methanol and the same sample was used for analysis. During UV visible spectral analysis, changes in absorption spectrum in the decolourized medium (400-800nm) were recorded in comparison with the results from the control runs (Sartale et al., 2009). The mobile phase used for TLC was composed of methanol, ethyl acetate, n-propanol, water and acetic acid (1:2:3:1:0.2 v/v) and silica gel plates ‘Merck’ was used for separation. TLC plate was developed using iodine.
HPLC analysis was performed in an isocratic system Shimadzu (SCL 10 AVP) equipped with dual absorbance detector using C 18 column with HPLC grade methanol as mobile phase at the flow rate of 1.0 ml / min for 10 min (Telke et al., 2009).

Toxicity study:
Phytotoxicity tests were carried out in order to assess the toxicity of DR 81 and metabolites formed after decolourization of DR 81. Phytotoxicity tests were carried out at a final concentration of 500 ppm on two kinds of seeds. One from grains *Sorghum vulgare* (monocot) and second from pulses *Phaseolus mungo* (dicot), commonly cultivated. Phytotoxicity was conducted at room temperature (10 seeds of each) by watering separately 5ml sample of control DR 81 and its degradation products per day. Control set was carried out using distilled water at the same time. Germination% as well as the length of plumule and radical was recorded after 7 days (Sartale et al., 2009).

Statistical analysis:
Data was analyzed by one way analysis of variance (ANOVA) with Turkey-Kramer multiple comparison test. Readings were considered significant when P was ≤0.05.

RESULTS AND DISCUSSION:

Isolation and identification of dye decolourizing bacteria:
Isolation of bacteria was carried out by the enrichment technique using nutrient broth and DR 81 as source of carbon and nitrogen that has rapid decolourization capacity. Decolourization occurred only when a carbon and nitrogen sources were available for growth. Isolated bacterium was Gram positive facultative anaerobic motile coccus. 16sr-RNA analysis done by geneOmbio Technologies, Pune. The nucleotide alignment of this strain showed it was most phylogenetically similar to the *Georgenia* sp.CC-NMPT-T3 (Sahasrabudhe et al., 2012).

Decolourisation studies:
Dye decolourisation can be judged clearly by observing the spectrum changes of dyes. Dye adsorption to cell reveals the approximate decrease of all peaks in proportion to each other, whereas dye removal by biodegradation shows the complete reduction of the major peaks and the production of new peaks at the same time.
The isolate showed ability to decolourize 97.56% of DR 81 within 4 hrs at a dye concentration 50 mg/L. The absorbance peaks in the visible region disappeared indicating complete decolourization (Elisangela et al., 2009). In the UV spectra the peak at 511 nm was replaced by new peak at 304 nm.

There was no abiotic loss of DR 81 within 24 h incubation indicating that the decolourization of DR 81 was due to biological mechanism rather than adsorption. To confirm whether this decolourization was due to the bacterial action or variation in pH, change in pH was recorded in the range of 7.1±0.2.

**Effect of physiochemical conditions on the decolourization performance:**

The effect of various physiochemical conditions such as pH, temperature, dye concentration, effect of carbon and nitrogen sources on decolourization of DR 81 by the isolate were studied in detail. All parameters were studied at 37°C under static condition. 10% inoculum with O.D$_{600}$ 1.0 was used at a dye concentration 50 mg/L.

**Effect of pH:**

![Effect of pH on decolourization](image)

*Fig 1 Effect of pH on decolourization*

Bacterial cultures generally exhibit maximum decolourization at pH values near 7.0, our culture exhibited decolourization activity in the range of pH 5-8(Fig 1). At pH 3 and 4, decolourization
observed was on an average 53.87± 4.77 %. The isolate showed more or less constant decolourization from pH 5 to 8, maximum 97.58% being at pH 6. Generally, bacteria show better decolourization and biodegradation activities at neutral or basic pH (Ali, 2010). Wang et al., 2009 studied decolourization of Reactive Black 5 by Enterobacter EC3.

Effect of Temperature:

The isolate showed 97.68 ± 1.30% decolourization from 28-45°C but at 50°C there was 16.37% decolourization (Fig. 2). The temperature ranging between 30-40°C was found to be suitable for the decolorization by S.saprophyticus strain AUCASVE3 and a further increase in the temperature gradually reduced decolorization activity of bacterial culture. This might be due to decrease in bacterial number above the optimal growth temperature (Hakim et al., 2013).

Effect of initial dye concentration:

Actual concentrations of reactive dyes in dye house effluent have been reported to range from 60-250mg/L (Bhatt et al., 2005).
The isolate showed decolourizing ability up to 350 mg/L at a faster rate after which the rate began decreasing. It has been proposed that dye concentration can influence the efficiency of microbial decolourization through a combination of factors including the toxicity imposed by dye at higher concentration (Bhatt et al.2005). The isolate could decolourize 97.56% of 50 mg/L of the dye in 4 hrs whereas it took 66 hrs to decolourize 85.56% of 500 mg/L (Fig 3). Thus, the isolate which could decolourize dye up to the reported dye concentration in wastewater, can be successfully employed for treatment of dye bearing industrial wastewater.

Analysis of metabolites resulting from decolourization and biodegradation of DR 81 by Georgenia sp.CC-NMPT-T3:

In order to understand the possible mechanism of the dye decolourization, we also analyzed the products of degradation of DR 81 by UV visible spectral analysis, TLC and HPLC.

UV visible scan (400-800nm) of the culture supernatant withdrawn at different time intervals indicated the decolourization and decrease in dye concentration from batch culture. Peak obtained at 511 nm decreased at complete decolourization. The absorbance peak in the
visible region disappeared indicating complete decolourization. In the UV spectra, the peak at 511 nm was replaced by new peak at 304 nm.

TLC analysis showed the appearance of one spot in the sample containing the extracted metabolites of completely decolourized medium with Rf value 0.82 whereas Rf value of DR 81 was noted as 0.97 confirming the biodegradation of DR 81 by Georgenia sp.CC-NMPT-T3

**HPLC:**

![HPLC of DR 81](image1)

![HPLC of degraded products](image2)

**Fig 5 A HPLC of DR 81**  
**Fig 5B HPLC of degraded products**

HPLC elution profile of DR 81 showed prominent peak at retention time 1.71 min. while degradation products by Georgenia sp. Show 2.891, 3.08, 3.488 and 3.861 min, respectively. The analysis showed the presence of new peak with disappearance of the peaks of DR 81 confirming the degradation of the dye by Georgenia sp.CC-NMPT-T3. (Fig 5 A and Fig 5 B)
Phytotoxicity studies:

There is no significant difference in the root and shoot length in case of *Sorghum vulgare* irrigated with the dye but in case of metabolite irrigated, the root and shoot length was significantly increased ($p \leq 0.05$) as compared to control.

There is no significant difference in the root and shoot length in case of *Phaseolus mungo* irrigated with the dye and the root length of mung seeds irrigated with metabolite but in case of metabolite irrigated, shoot length was significantly increased ($p \leq 0.05$) as compared to control mung seed (Fig 6).

Phytotoxicity study showed good germination rate as well as significant growth in the plumule and radical for both the selected plants ($P \leq 0.05$) in the metabolites extracted after decolourization as compared to dye sample. This indicates the detoxification of DR 81 by the isolated bacterial culture.

**CONCLUSIONS:**

The selected organism is able to degrade 97.56% Direct Red 81 at 50mg/L in 4 h. The isolate was able to decolourize the dye up to 500mg/L. The isolate can degrade the dye in wide range of pH from 5.0 to 8.0. The selected organism shows decolourization and degradation of the dye at a temperature range of $28^\circ$C to $45^\circ$C. UV-Vis spectrophotometry, TLC and HPLC studies confirm the degradation of the dye.
Phytotoxicity studies show nontoxic nature of the formed metabolites. Taking into consideration all the results, *Georgenia* CCNM-PT-T3 can be used for treatment of dye wastewater containing azo dyes.

References:


