Enzyme Assisted Biodegradation of Direct Red 81 By Micrococcus Glutamicus NCIM 2168

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Abstract:

Azo dyes have been extensively used in textile, pharmaceutical, paper, paint industries. The industries manufacturing dyes generate a large volume of water. Wastewater containing dyes in most of the cases is discharged into water bodies without any treatment or impartial treatment. This hampers not only flora and fauna of the aquatic ecosystem but showed adverse effects on human beings. Existing physical and chemical methods have their advantages and disadvantages. Biodegradation of dyes finds an eco- friendly process. In the present study, *Micrococcus glutamicus* NCIM 2168 was used for decolourization of the dye Direct Red 81. The isolate decolourized 98.54% of the dye at pH 6 and 28°C in 9 hours. Degradation of the dye was confirmed by the change in λ_{max} of the decolorized sample. Confirmation of the degradation was done by HPLC and GCMS studies. Degradation was brought about by Oxidoreductases. Toxicity studies revealed nontoxic nature of the product. The culture was found to decolourise mixture of five dyes. Hence, the selected bacterial culture can be successfully used for the treatment of dye containing wastewater.

Keywords: Azo Dyes, Decolorization, Biodegrdation, Oxidoreductases

1. Introduction:

Textile and dye industries are the major industries consuming a large amount of water. During the processing about 10-15% of dyes enter into water bodies (Nimkar, 2018)). Almost 80000 tons of dyes are manufactured per year. Despite the stringent measures, many industries discharge either untreated or partially treated wastewater into nearby water bodies. This affects the aquatic environment resulting in poor light penetration. This hampers the photosynthetic ability of algae and affects flora and fauna of the aquatic habitat (Solis et al., 2012). About 60-70% of the manufactured dyes are azo dyes. They contain azo bond (N=N) and are recalcitrant. The azo group is rare amongst natural products and confer xenobiotic property to the dyes. These dyes are found to be toxigenic, carcinogenic, mutagenic and also induce allergic reactions in human beings (Imran, et al., 2015; Brüschweiler et al., 2017; Dehghani et al., 2018). These dyes are difficult to treat because of synthetic origin and more complex aromatic structure hence resistant to degradation. These dyes were treated by physicochemical methods. But these methods were found too costly and are less efficient. Various microorganisms are reported by several researchers for biodecolourization and biodegradation of the dyes (Kumar et al., 2006). This ecofriendly approach of using microorganisms for treating dyes was found to be cheaper, efficient and results in mineralization of the dyes as reported by Gul, 2018. In the present study, *Micrococcus glutamicus* NCIM 2168 was used for detecting the ability of the bacterium to degrade the selected azo dye Direct Red 81.

2. Material and Methods:

2.1 Culture used:

The culture of *Micrococcus glutamicus* NCIM 2168 was obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The culture was selected for its known property of dye degradation.

2.2 Chemicals and reagents:

All chemicals and reagents used were of high purity. Direct Red 81 was procured supplied by Spectrum dyes and chemicals Pvt Ltd., Surat, India

2.3 Optimization of process parameters:

2.3.1 Decolourization experiment:

Decolourization experiment was carried out with the initial concentration of 50 mg/L of the dye Direct Red 81 under static condition at pH 7and 28°C. Aliquot of inoculated broth was taken after every two hours, subjected to centrifugation at 10000g for 10 min and the supernatant was used for absorption studies by spectrophotometer at the λ_{max} 511 nm of the dye. The experiment was carried out in triplicate. Control tubes without inoculated culture were kept throughout the experiments. Percentage decolourization was calculated from initial (A_0) and final(A_t) absorbance by using the formula as stated in Sartale et al. 2009. % Decolorization = A0 – At/ A0 X 100.

2.3.2 Effect of pH:

The ability of the selected culture to decolourize the dye was studied by using basal nutrient medium Nutrient broth (M 002) Hi- Media of pH ranging from 3 to 8. The mean of the three readings was taken.

2.3.3 Effect of temperature:

The temperature plays a key role in decolourization and degradation of the dye. Hence, the decolourization was carried out at temperature ranging from 28 to 50°C under static condition.

2.3.4 Effect of dye concentration:

The ability of the selected culture to decolourize the increasing concentration of the dye was studied by using the dye concentration from 50-500 mg/L.

2.4 Analysis of the degradation products:

The decolourized broth was subjected to centrifugation for separation of the bacterial cells. Metabolites formed during the degradation were extracted in twice the amount of dicholorometahne . Then it was evaporated and the residue was dissolved in small quantity of HPLC grade methanol. The same is used for analyses.

2.4.1 UV absorbance study

The extracted metabolites were scanned from 200-1000 nm using SL 244 Elico double beam spectrophotometer at room temperature.

2.4.2 TLC

To confirm degradation of the dye Thin layer chromatographic separation of the degraded products was carried out. The separation was done on precoated silica gel 60 F_{254} plates 'Merck' 20 X 20 cm aluminum plates. TLC plate were developed using iodine. The mobile phase used for TLC of the dye and metabolites was composed of methanol: ethyl acetate: n-propanol: water: acetic acid in proportion of 1:2:3:1:0.2 (v/v) (Kalyani et al., 2008).

2.4.3 HPLC

HPLC analysis was performed in an isocratic system (Shimadzu SCL 10 AVP) and 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 at λ_{max} of the respective dye (Sartale et al., 2009).

2.4.4 Enzymes involved in degradation

Cell free extract was obtained by centrifugation followed by sonication. The homogenate was subjected to centrifugation, supernatant was used as a source of enzymes for carrying out assay of oxidoreductase enzymes as per Kalyani et al., 2008.

2.4.5 Toxicity studies

For confirmation of nontoxic nature of the metabolites, toxicity assay was carried out using *Sorghum vulgare Phaseolus mungo*. Ten seeds were irrigated by water as a control, the dye and the metabolites of degradation respectively. Three sets of the same experiment were kept

2.4.6 Statistical analysis was done by ANOVA

3 Results and Discussion:

3.1 Optimization of process parameters:

3.1.1 Decolourization experiment:

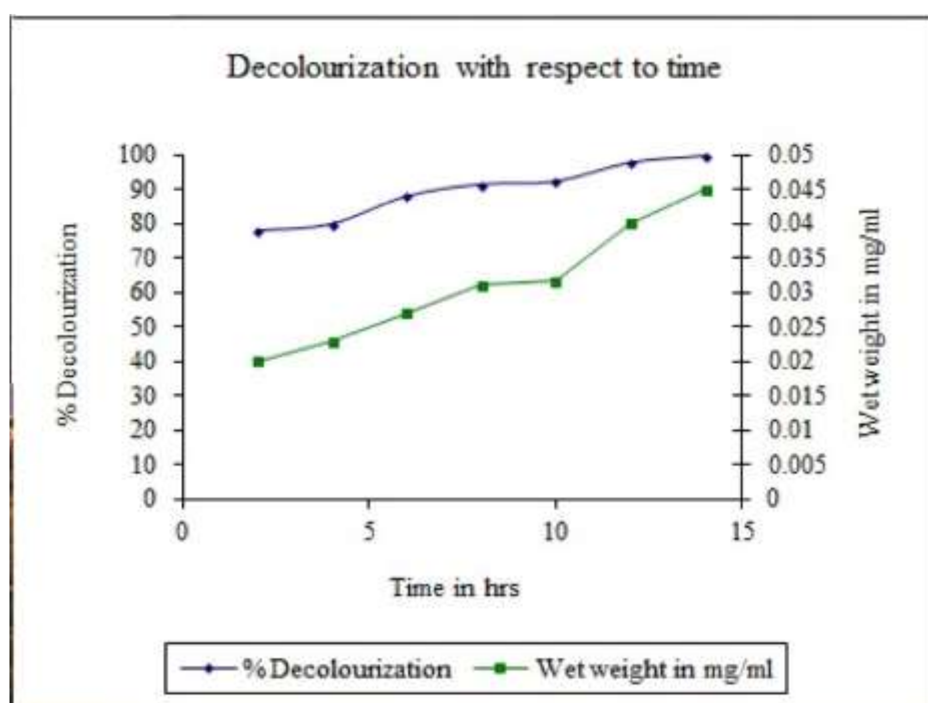

Fig 1 Decolourization with respect to time

The decolourization experiment showed 98.06% of the dye decolourization at pH 6 and 28°C in 12 hours under static anoxic condition. Decolourization efficiency is higher under static condition. This is because oxygen is essential for the growth of the organism but adversely affects the activity of azoreductase enzyme (Ogugbue et al., 2012). This enzyme plays a key role in degradation of the azo bond. Decolourization was associated with increase in wet weight indicating growth of the culture in presence of the selected dye. The selected culture was found to decolourize mixture of five azo dyes as well as dye industry effluent.

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3.1.2 Effect of pH:

The pH of the medium has significant impact on decolourization. Hence, decolorization was studied at pH ranging from 3 to 8. It was observed that percentage decolourization was low in acidic pH . At pH 5 to 8, on an average 95% of decolourization was observed. Highest decolourization was noted at pH 6. Therefore, pH 6 was used in further study. Bheemaraddi et al 2014. reported 100% decolourization of the azo dye Reactive violet 5 by *Paracoccus* sp**.** Decolourization of the dyes is favoured at neutral pH (Chan J. and Kuo, 2000).

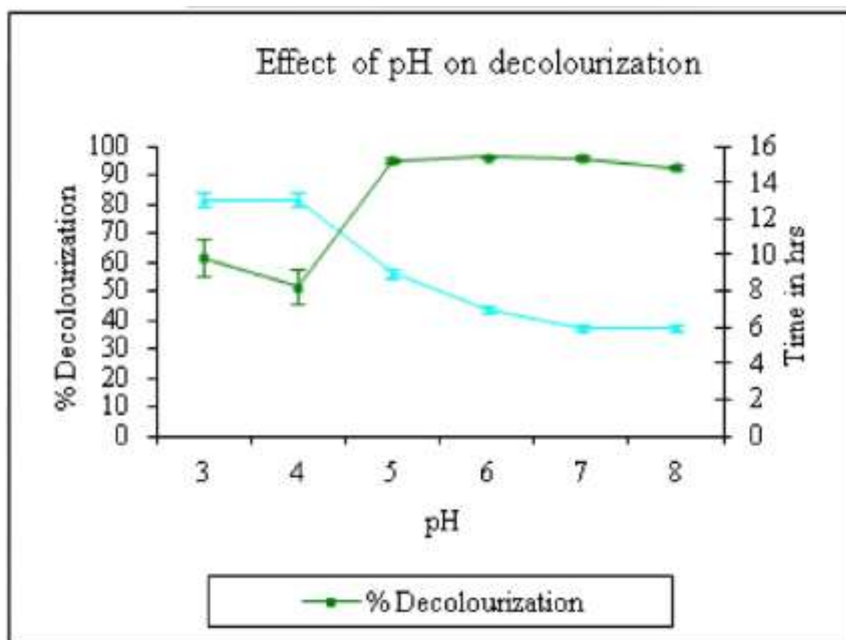

Fig 2 Effect of pH on decolourization

3.1.3 Effect of temperature:

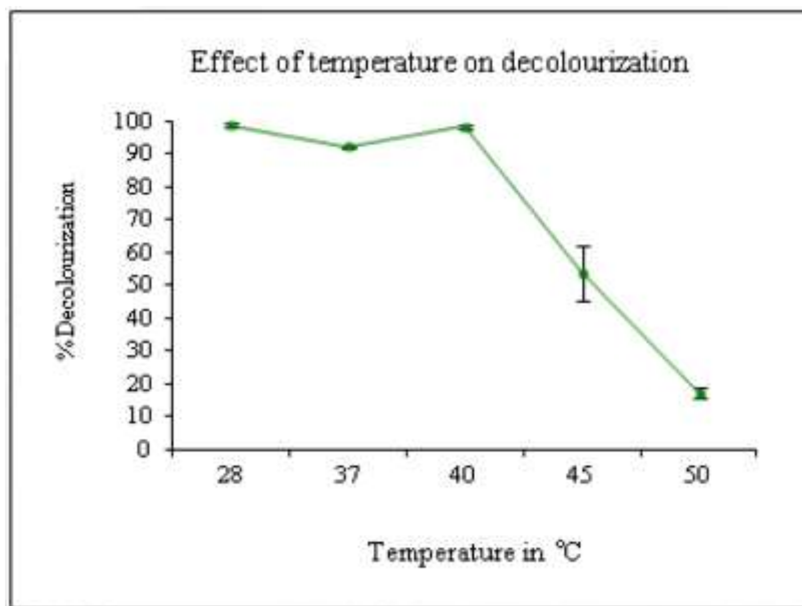

Fig 3 Effect of temperature on decolourization

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The bacterium was able to decolourize the dye within a temperature range of 28-40°C. Maximum decolourization of 98.54% was observed at 28°C in 9 hours. At 45°C decolourization was 53.71% in 13 h whereas lowest decolourization of 17.07% was noted at 50°C.

This may be due to enzyme denaturation at higher temperatures. Bacillus cohnii RAPT1 decolourized eactive Red 120 optimaly at 35° C (Padmanaban et al., 2016).

3.1.4 Effect of dye concentration:

As dye concentration increased, the rate of decolourization was increased up to 350mg/L, above which the efficiency of decolourization at increased dye concentration was decreased remarkably. It was observed that the culture could decolourize 88.11% of the dye at 500mg/L in 72 h.

This may be due to the toxicity exerted by the increased dye concentration on the organism. Bheemaraddi et al ,2014 reported that decolourization decreased slowly with increase in dye concentration. Same is also reported by Parshetti et al 2009.

3.2 Analysis of the degradation products:

3.2.1 UV absorbance study:

To confirm biodegradation of the dye, the degradation product was subjected to Ultraviolet spectrophotometric analysis. The dye DR 81 gave maximum absorption at 511 nm. The degradation product showed a sharp peak at 240nm. Absence of the peak at λ_{max} of the dye confirms degradation of the dye.

3.2.2 TLC: TLC analysis revealed a sharp spot at $R_{f\ value}$ 0.97 of DR 81 . Degradation products showed two spots at $R_{f\ 0.67}$ and 0.54

3.2.3 HPLC

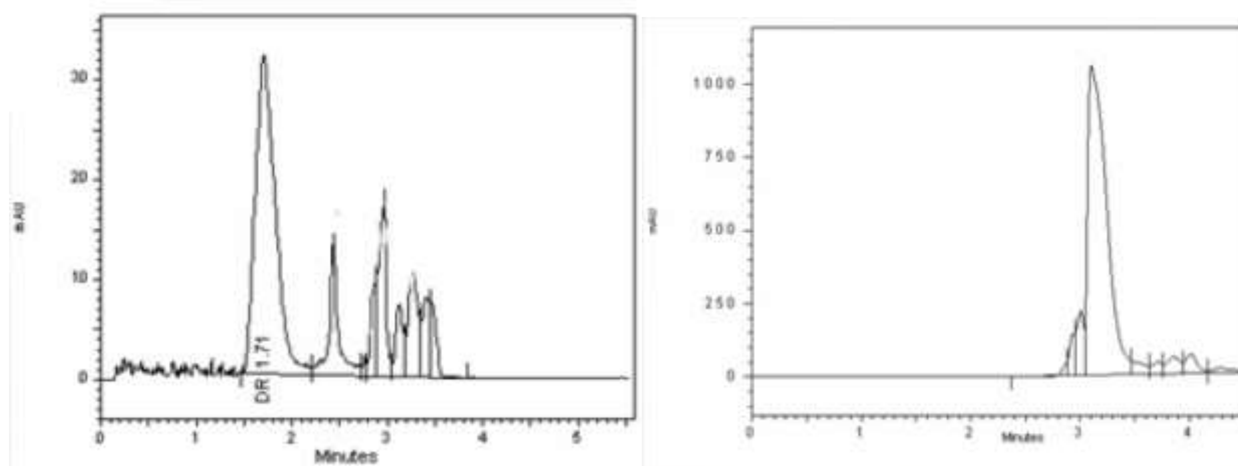

Fig 4A HPLC of DR 81 **Fig4B HPLC of the degradation products**

HPLC of DR 81 gave sharp peak at 1.71 minutes while the degradation products showed major peak at 3.01 minutes followed by small peak at 4 minutes. Disappearance of characteristic sharp peak of DR 81 confirms biodegradation of the dye.

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3.2.4 Enzymes involved in degradation

Table 1 Enzymes in uninduced and induced cells

Sr. No	Lignin peroxidase	Laccase	NADH-DCIP reducatse	Azoreductase
Uninduced	1.641±0.081	0.8991±0.032	0.5068±0.002	0.077±0.15
Induced	2.0925±0.02781	2.12±0.0075	1.438±0.0127	0.148±0.016

Decolourization depends on the type of enzymes produced by the organism. The dye and the metabolites formed are responsible for induction or inhibition of the enzymes involved in the process. The cell free extract used in the present study is a crude enzyme which is the mixture of oxidoreductive enzymes. Enzyme analysis unfolded remarkable increase in the amount of lignin peroxidase, laccase, NADH-DCIP reductase and azoreductase. This confirms enzymatic degaradtion of the dye by oxidoreductase enzymes. The significant increase was observed in lignin peroxidase enzyme in induced cells obtained after decolourization as compared to uninduced cells in absence of the dye (Parshetti et al.,2009).

3.2.5 Toxicity studies

The water from water bodies in which dye wastewater is disposed of is many times used for agricultural purposes hence it was essential to study phytotoxicity.

Table 2 Toxicity studies

Table 2A Sorghum Vulgare L

	Root length	Shoot length
Control	6.64	10.46
DR 81	5.74	8.31
Degradation product	6.95	10.51

Table 2B Phaseolus mungo L

	Root length	Shoot length
Control	5.11	10.3
DR 81	4.72	8.31
Degradation product	5.99	10.12

The results of the phytotoxicity revealed that there is no significant difference in the root and shoot length in case of wheat and mung irrigated with the dye but in case of metabolite irrigated wheat and mung the root and shoot length was significantly increased ($p \leq 0.001$) as compared to control. Percentage germination in control, DR 81 and degradation products was 100, 50 and 90, respectively. Thus, the degradation products formed by the activity of the culture exhibited nontoxic nature. Similar results were reorted by Ogugbue et al. in 2012 while studying degradation of triphenylmethane dyes by *Aeromonas hydrophilia.*

4 Conclusion:

The present study revealed that Micrococcus *glutamicus* NCIM 2168 decolourized 98.54% of a complex diazo dye Direct Red 81 at pH 6 and 28°C. The culture could decolourize upto 500 mg/L of the dye which is much above than the dye found in wastewater, Decolourization was brought about by enzymatic degradation of dye as shown in significant increase in oxidoreductase enzymes. Phytotoxicity studies demonstrated nontoxic

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nature of the degradation products. Hence, *Micrococcus glutamicus* NCIM 2168 can be a potential bacterium for the treatment of the dye containing wastewater.

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