International Journal of Research in BioSciences
Vol. 1 Issue 1, pp. (29-41), July 2012
Available online at http://www.ijrbs.in
ISSN 2319-2844

Research Paper

Decolorization, degradation and azo-reductase study by bacterial transformation of reactive red HE8b

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(Received 18 April, 2012, Accepted 20 July, 2012)

Abstract

To achieve this goal (remediation of dyes) decolorization and degradation was proved by our identified culture DN1 by performing various process parameter. Among them optimized results was dye concentration 200mg.L^{-1}(86.2%), temperature 40^{0}C (91.5%), inoculum size 2.5 mL (92.0%) and pH 6.5 (89.5%). Various types of carbon and nitrogen sources are supplemented into medium to enhance the decolorization. Among them lactose (0.6%) (94.1%) and yeast extract (0.5g%) (96.5%) was optimum as carbon and nitrogen source respectively. Furthermore, degradation was also proved by performing UV-Vis, HPTLC and FTIR analysis. Azo-reductase study was also carried out from DN1. Total protein determination was performed for crude and partially purified enzyme, followed by protein determination activity of Azo-reductase was checked in both acetate buffer (30.68±0.05) and phosphate buffer (12.64±0.09). Acetate buffer gave higher activity rather than phosphate buffer, so characterization was performed from acetate buffer. Among them methyl red was used as a fixed substrate, optimum pH was 5.5 and optimized temperature was.

Keywords: Reactive dye, Azo-reductase, Decolorization, Biodegradation, HPTLC, FTIR

Introduction

Rapid urbanization and industrialization has lead to a vast release of waste to the environment adding to the pollution load. Majority of colored effluents contains dyes released from textile, dyestuff and dyeing industries. India's dye industries produces every type of dyes and pigments. Production of dyestuff and pigments in India is close to 80,000 tones [10]. The textile industry is one of the greatest generators of liquid effluent pollutants dye to the high quantities of water used in the dyeing processes [3]. The traditional textile finishing industry consumes about 100 L of water to process about 1 Kg of textile materials. The chemical structure of dyes in general is comprised of a conjugated system of double bonds and aromatic rings. The major classes of dyes have anthroquinoid, indigoid and azo aromatic structures [2] . Several methods were adapted for the reduction of azo dyes to achieve decolorization like physical, chemical and biological. Physical treatment methods such as screening, sedimentation and skimming remove floating objects. Chemical treatment methods such as Precipitation, pH adjustment, Coagulation etc., to remove toxic materials and colloidal impurities [5]. In case of enzymatic remediation of azo dyes, azo reductases and laccases seem to be the most promising enzymes. Azo-reductases catalyze the reaction only in presence of reducing equivalents like FADH and NADH. Most of the Azo dye have sulphonate substituent groups and a high molecular weight and they are unlikely to pass through cell membranes [7].

Materials and Methods

Sample collection and preservation

Samples were collected from contaminated soil near Naroda G.I.D.C., Phase-IV, Ahmedabad, and Gujarat, India. The samples were collected in plastic bag and cane. The samples were collected on 19th Dec.2011 the collected samples were stored at 4^{0}C.

Dyes and Media

Different dyes like reactive red HE8b, reactive red M8b, reactive blue RGB, reactive yellow HE49, reactive violet 5R, direct black 13, disperse black, red CD dark and many more were used for the study.

The Bushnell Haas Medium (BHM) in (g.L^{-1}) magnesium sulphate 0.2, calcium chloride 0.02, monopotassium phosphate 1.0, dipotassium phosphate 1.0, ammonium nitrate 1.0, ferric chloride 0.05, supplemented with 0.5% yeast extract (YE) and 100 mg.L^{-1} reactive dyes were employed for enrichment study.

Isolation and evaluation of dye decolorizers

A loopful suspension from 24 h incubated enriched flask was streaked on BHM containing 0.5% YE. Separate colonies of the predominant types of microorganisms were purified by re-streaking on the plate and applied on the BHM with 0.5% YE and 100 mg.L^{-1} reactive dye. Further it was checked for its ability to decolorization. The colony characteristics and microscopic study was also performed to obtain single pure isolate.

Identification of isolate DN1

Identification was based on Gram staining, motility and various biochemical test as outlined in Bergey's Manual of Systematic Bacteriology.

Identification of bacteria using 16S rDNA sequencing

A 16S rDNA analysis method was used to identify the selected bacterial strains. The nearly full -length 16S rDNA gene was amplified by PCR with forward and reversed primal universal. Purified PCR products were sequenced. The sequences were finally assembled to produce nearly full- length sequence and the sequences available in the National Centre Biotechnology Institute. (NCBI) Gene bank data base. The probable identify of the bacterial strain was thus determined. Identification to the species level was defined as a 16S rDNA sequence similarity of ≥ 99 % with that of the prototype strain in Gene bank: identification at the genus level was defined as a 16S rDNA sequence similarity of ≥97 % with that of the prototype strains in Gene bank. A failure to identify was defined as a 16S rDNA sequence similarity score lower than 97 % with those deposited in gene bank at the time of analysis. (April, 2012). Sequencing of the bacterial strain was conducted by the Gujarat State Biotechnology Mission GSBTM, Gandhinagar Government of Gujarat, India.

Determination of optical density (OD) of culture supernant

Samples from experimental and control flasks were clarified. The OD of the supernant was determined with spectrophotometer at (λmax540). % dye decolorization was calculated:

$$\% \text{ Decolorization} = \frac{\text{initial absorbance-observed absorbance} \times 100}{\text{Initial absorbance}}$$

Effect of process parameters on RRHE8b decolorization

The physical and chemical condition affects the dye decolorization and degradation activity of potent pure culture. Due to this the suitable optimal condition in which the culture show its optimum activity was examined. The optimization of physicochemical parameters such as dye concentration, pH, temperature, inoculum size, carbon sources and nitrogen sources ones at a time.

Analytical Methods

Decolorization and Degradation of model dye was proved by Spectrophotometric analysis, HPTLC method and FTIR analysis respectively.

Azo-reductase study

Preparation of cell free extract

The bacterial strain DN1 was grown in BHM medium at 37ºC for 24 hrs the cells were harvested by centrifugation at 17,000 rpm (4ºC) for 15 min. the cell pellet were resuspended into 10 mL acetate buffer (0.1 M, pH 7) and was subjected to ultrasonication. Ultrasonication was carried out using sonicator (Sonics- Vibracell Ultrasonic Processor) at sonifier output of 70% amplitude for 8 cycles, each cycle was for 20 second and 1 minute interval was kept between each cycle at 4ºC.

Partial purification of azo-reductase

The crude enzyme extract was precipitated by solid $(NH_4)_2SO_4$ at 80% saturation at 4ºC with continuous stirring and left for overnight. The precipitated enzyme was collected by centrifugation (17,000×g, 15 min) and dissolved in 0.1 M acetate buffer pH 7.0 and dialyzed against 0.01 M acetate buffer. The dialysed sample was then used for azo-reductase assy.

Protein determination

Protein concentration in crude extract and in dialyzed was measured by the method of Lowry *et al*. using BSA as a standard protein.

Azo-reductase assay

Azo-reductase activity was assayed using methyl red as the dye substrate. the assay mixture contained 0.5 mL of 200 mM acetate buffer (pH 7.0) with 0.2 mL of 2 mM of the dye methyl red, 0.2 ml of 10 mM NADH and 0.1 mL of enzyme in 2 mL of reaction mixture. The reaction mixture without NADH was pre incubated for 10 minute at 30ºC and the reaction was started by the addition of NADH. Dye decolorization was followed by monitoring the decrease in color intensity at 430 nm. One unit of (U) azo-reductase was defined as the amount of enzyme required to reduce 1 µmoL of dye $min^{-1}mL^{-1}$ under the assay conditions.

Characterization of azo-reductase

Characterization of azo-reductase were done under various process parameter such as substrate (Methyl Red) concentration (µL) 20, 40, 60, 80, 120, 140, 160, 180, 200, 240, 280, 320, 360 and 400, pH 5.0 and 5.5 (acetate buffer using Na acetate and acetic acid), 6.0, 6.5, 7.0 and 7.5 (phosphate buffer using K_2HPO_4 and KH_2PO_4), 8.5, 9.0 and 9.5 (tris buffer using tris base and HCl).

Results and Discussion

Investigations were carried out to isolate and screen microorganisms capable of decolorizing medium containing RRHE8b. Various process parameters were optimized for maximum decolorization by the efficient isolates. Attempts were also made to quantify the degradation of individual colorants present in medium containing RRHE8b by the promising microbial isolates.

Isolation of dye decolorizing bacterial culture

Screening was carried out using enrichment culture technique. Bushnell Haas Medium (BHM) along with 0.5% yeast extract and 100 $mg.L^{-1}$ RRHE8b were used for screening purpose. Total 6 bacterial cultures were isolated from contaminated site, which has ability to decolorize 60 to 86% of dyes within 9 to 48 h (Table 1).

Identification of bacterial culture

Identification based on morphological and cultural characteristics

The primary identification of selected bacterial isolate on the basis of gram's reaction, morphological characteristics.

Effect of process parameters on RRHE8b decolorization

The factor such as dye concentration, pH, temperature, inoculum size, carbon source and nitrogen source were optimized by varying parameters one at a time. The experiments were conducted in 250 mL Erlenmeyer flask.

Effect of dye concentration on RRHE8b decolorization

The Reactive dye molecule is a complex structure, keeping this point in mind, the influence of dye concentration on the decolorization ability of the organism was investigated. The maximum decolorization was observed at 200 $mg.L^{-1}$ dye concentration in terms of 86.2±0.23% (with 11.85±0.23 $mgL^{-1}h^{-1}$ average rate of decolorization) (Graph: 1) [9]. Observed results are strongly supported to our results using 200 $mg.L^{-1}$ optimum dye concentrations during their study on biodegradation of textile dyes. It may be due to bacterial isolate has the ability to degrade higher dye concentration of textile wastewater.

Effect of pH on RRHE8b decolorization

pH of the medium greatly affect the percentage of decolorization. Bacterial culture generally exhibit maximum decolorization at pH near 7.0. The pH range of our culture is from 6.0-9.0. Potent bacterial culture DN1 gave maximum decolorization at pH 6.5 (89.5±0.36%)(with decolorization rate 17.99±0.12$mgL^{-1}h^{-1}$) (Graph: 2) [10], showed pH 6.9 for biodegradation of orange G by a novel bacterial isolated bacterial strain *Bacillus megaterium* ITBHU01,the obtained data are very similar to our results.

Effect of temperature on RRHE8b decolorization

Temperature is the key factor, which affect the cell and its metabolic reaction during breakdown or utilization of complex carbon compound in form of reactive dye. So as to investigate effect of temperature ranging from 30 to 50˚C and checked the percentage of decolorization. Maximum percentage of decolorization was observed at 40ºC (91.5±0.45%) (with decolorization rate 20.96±0.36$mgL^{-1}h^{-1}$) (Graph: 3). Our results are very similar to [11] Wang *et al*., (2011) they suggested optimum temperature at 40˚C during their study on decolorization of the azo dye by bacterial isolate. At higher temperature percentage of decolorization was rapidly decreased [8], reported efficient decolorization at 40˚C. This may be owing to a greater synthesis of enzymes and optimal growth condition of the isolate for its dye decolorizing ability.

Effect of inoculum size on RRHE8b decolorization

Optimization study was carried out with the selected potent culture DN1, inoculum size plays an important role because of sufficient cell biomass required to decolorize and degrade substrate in form of dye to product. The effect of inoculum size on dye decolorization was studied with the addition of different inoculum size 1.0-4.0% (vv^{-1}) and observed the percentage of decolorization. %. Optimum Inoculum size for decolorization was 2.5% (vv^{-1}) with 92.0±0.23%decolorization and 19.69±0.09$mgL^{-1}h^{-1}$ average rate of decolorization. (Graph 4) **[4]** Nermeen *et al., (*2011) results are supporting our work by using 1 to 4% inoculum size during their work on bioremediation of red dye by *Streptomyces globosus* under static and shaking condition.

Effect of different carbon source on RRHE8b decolorization

To find out the effect of different carbon source on model dye, different carbon sources such as, galactose, fructose, lactose, dextrose, maltose, starch and xylose added. Among them lactose gave maximum percentage of decolorization (94.1±0.56%) (with 26.85±0.23 $mgL^{-1}h^{-1}$ average rate of decolorization) Graph: 5. Maximum decolorization was reported in presence of lactose was also suggested by Sahasrabubhe *et al.,* (2011). Carbon sources seemed to be effective to promote the

decolorization probably due to the preference of the cells in assilimilating the added carbon sources over using the dye compound as the carbon source [1] Chaube *et al*., (2010) proposed lactose as optimum carbon source during their study on biodegradation and decolorization of food azo dye.

Effect of different nitrogen sources on RRHE8b decolorization

Various nitrogen sources such as yeast extract, peptone, urea and meat extract were studied to achieve maximum percentage of decolorization. Among these yeast extract gave higher percentage of decolorization (96.5±0.65%) (with decolorization rate 19.03±0.23mg$L^{-1}h^{-1}$) (Graph: 6) [6] Ponraj *et al*., (2011) reported optimum nitrogen source yeast extract during their findings on isolation and optimization of culture condition for decolorization of true blue using dye decolorizing organism.

Degradation analysis of RRHE8b

Spectrophotometric method

The UV-Vis spectrum of control and test of the model dye showed different peaks as shown in Graph: 7. UV–Vis scan (400–800 nm) of supernatant of different time intervals showed decolorization and decrease in dye concentration from Batch culture peak observed at 540 nm (0 h) was decreased without any shiftin up to complete decolorization of medium(6 h).

HPTLC method

In order to confirm the biodegradation further investigations were supported and studied by HPTLC analysis. The control and test samples were scanned at different wave lengths (254 and 366 nm). The HPTLC analysis, revealed that both the samples showed the different R_f values it indicates that new metabolic products were formed Results were shown in Graph 8 and 9.

FTIR method

FTIR spectra of control DN1 showed specific peaks in the finger printing region 1600-600 cm^{-1}. The first peak was displayed at 3436.57 indicates N-H stretching,2360.31 cm^{-1} represents R^2N+H_2 and 2341.82 cm^{-1} represents R^3N^+H. the peak displayed at 1636.28 cm^{-1} shows shift bases, imines and oximes. The displayed peak at 1067.01 cm^{-1} indicate presence of acid anhydrides, the last peak displayed at 677.50 cm^{-1} shows C-Cl stretching. The FTIR spectrum of decolorized sample containing metabolites revealed that different peaks were recorded. The fist spectrum observed at 3236.31 indicates N-H stretching. The two nearest peaks at 2360.21 and 2341.69 cm^{-1} shows N^+H_2 and N^+H stretching respectively. The recorded spectrum at 2078.05 cm^{-1} indicates R-N≡C. and N≡C stretching. While S≡O stretching observed at 1087.56 cm^{-1}. The last spectrum at 678.67 cm^{-1} was due to C-Br stretching. By observing our present data it can be said that primary and secondary amines may be preset in degraded form of the dye.

Purified of Azo reductase

Protein determination: The unknown protein content can be quantified by plotting the optical density values in the bovine serum albumin(BSA) standard graph (data not shown) with respect to the concentration of the protein were measured using UV spectrophotometer at 430 nm (Table 3).

Azo-reductase assay

In present study, azo-reductase assay initially determined using acetate buffer (pH 5.4) and phosphate buffer (pH 5.5). Among both of them induction of enzyme activity was observed at acetate buffer (Table: 4 A&B).

Characterization of enzyme azo-reductase

Effect of substrate

Enzymatic reaction were carried out by varying the concentration of methyl red at the same time. This might be due to electron transport chain functions are carried out by methyl red as a electron donor Graph 12.

Vmax= 208.15

Km= 104.075

Effect of pH

To fulfill the objective of enzyme study was characterize with different buffer solutions such as acetate(pH 5.0 and 5.5) tris(pH 8.5, 9.0 and 9.5)and phosphate (6.0, 6.5, 7.0 and 7.5) buffer. The maximum activity was noticed at pH 6.0 in presence of methyl red as a substrate (Graph 13).

Vmax= 303.1

Km= 151.55

Effect of temperature

Enzyme activities were measured under different temperature from 30 to 45℃. the optimum temperature of the enzyme was found at 30˚C (Graph 14).

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Table 1: Isolation of dye decolorizing bacterial culture

S. No.	Isolation	Time (h)	Decolorization (%)
1	DN1	9	86
2	DN2	24	60
3	DN3	48	68
4	DN4	48	73
5	DN5	24	65
6	DN6	48	68

Table: 2 Protein determination

S. No.	Sample	Enzymatic activity (Umg^{-1})
1	Crude	9.48
2	Partially purified enzyme	1.02

Table 3: Enzymatic activity of crude and partially purified enzyme in both acetate and phosphate buffer

Table A: Activity in phosphate buffer

S. No.	Phosphate buffer	NADH 10 mM (µL)	Methyl red 2mM (µL)	Enzymatic activity (Umg^{-1})
Crude	500	200	200	11.22±0.05
Partially purified enzyme	500	200	200	12.64±0.09

Table: B Activity in acetate buffer

S. No.	Phosphate buffer	NADH 10 mM (µL)	Methyl red 2mM (µL)	Enzymatic activity (Umg^{-1})
Crude	500	200	200	30.56±0.06
Partially purified enzyme	500	200	200	30.68±0.05

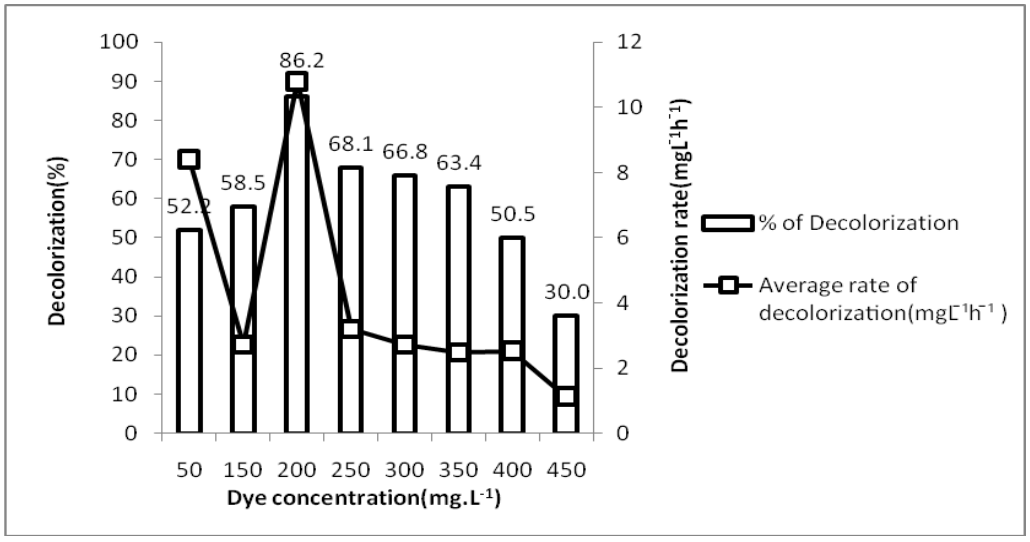


Figure 1: Effect of dye concentration on decolorization of RRHE8b by DN1

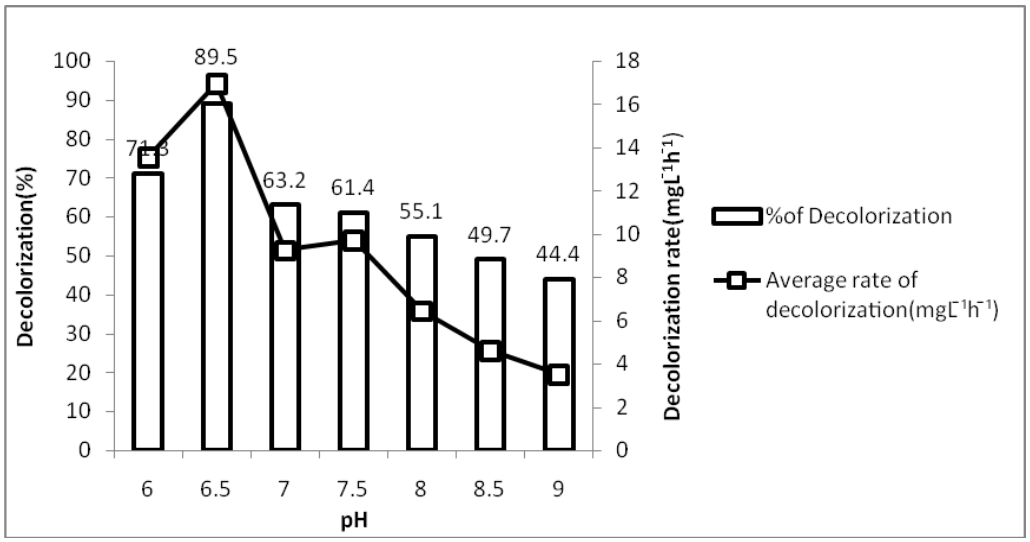


Figure 2: Effect of pH on decolorization of RRHE8b by DN1

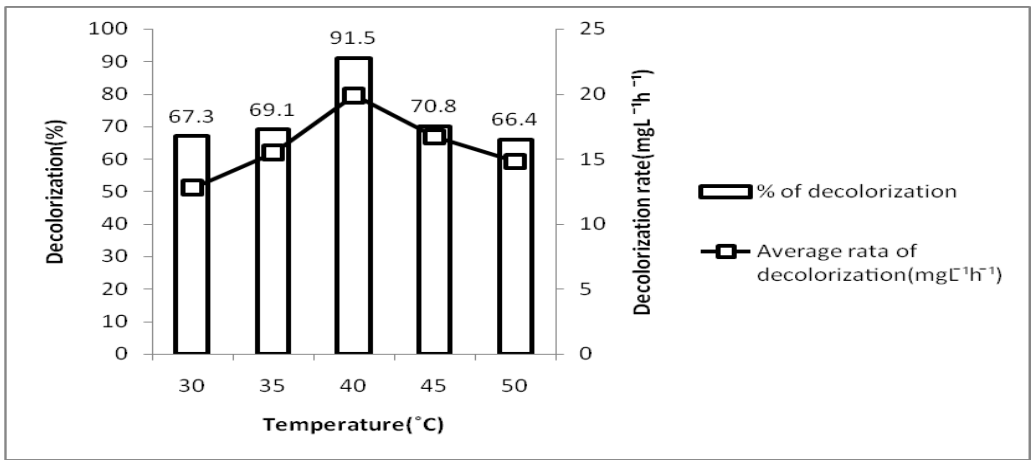


Figure 3: Effect of temperature on delocolorization of RRHE8b by DN1

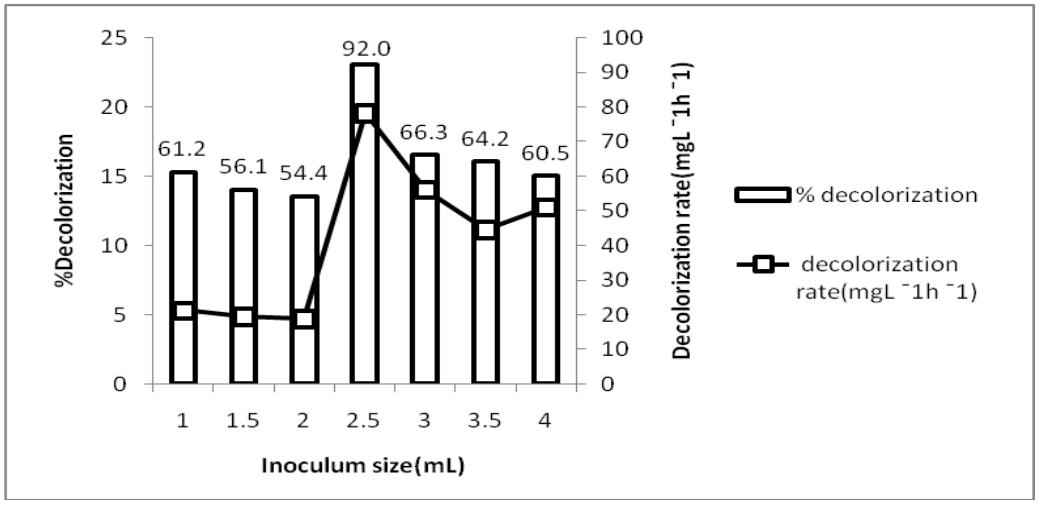
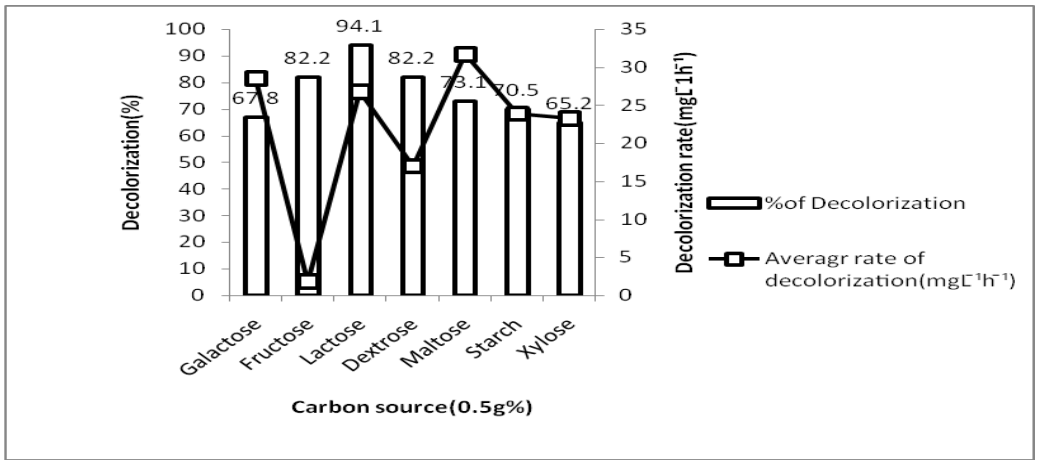
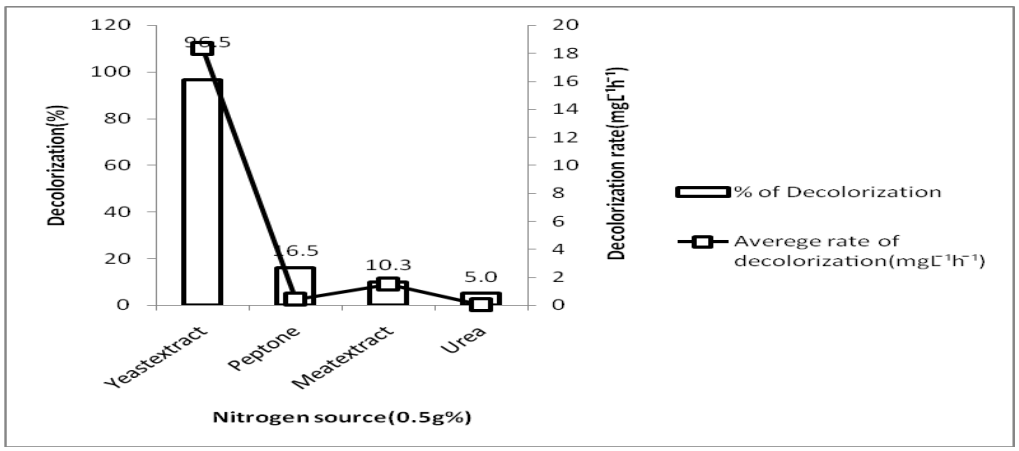


Figure 4: Effect of inoculum size on decolorization of RRHE8b by DN1



Graph: 5 Effect of carbon source on decolorization of RRHE8b by DN1



Graph: 6 Effect of nitrogen source on decolorization of RRHE8b by DN1

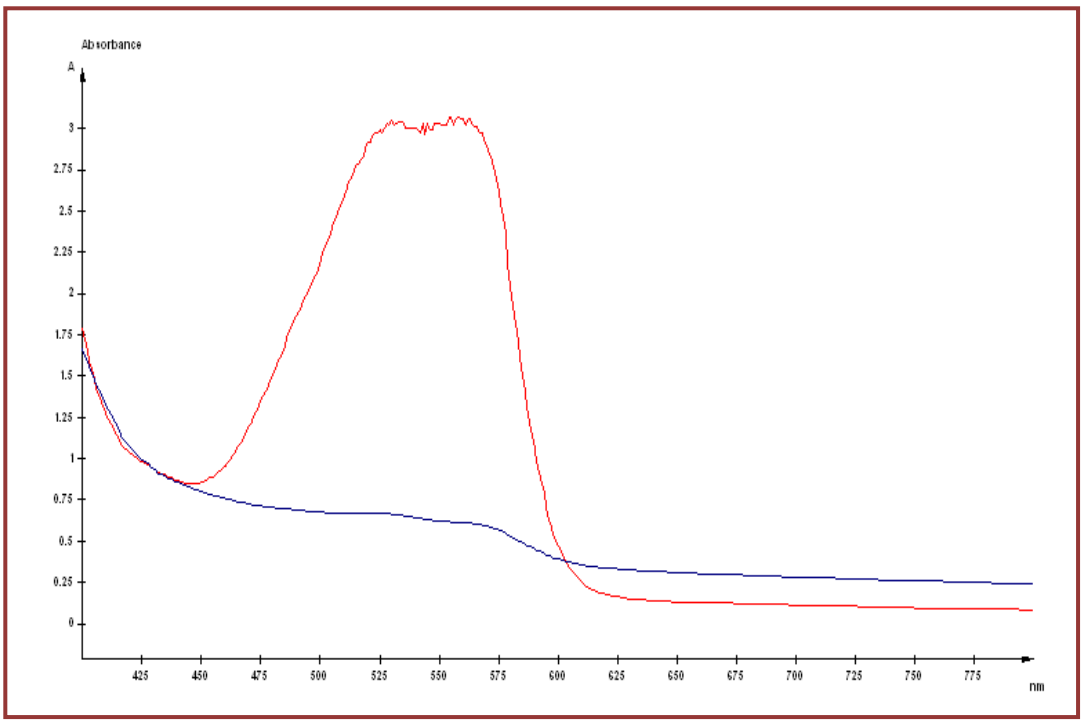


Figure 7: Spectrophotometric analysis control (red) test (blue) of RRHE8b

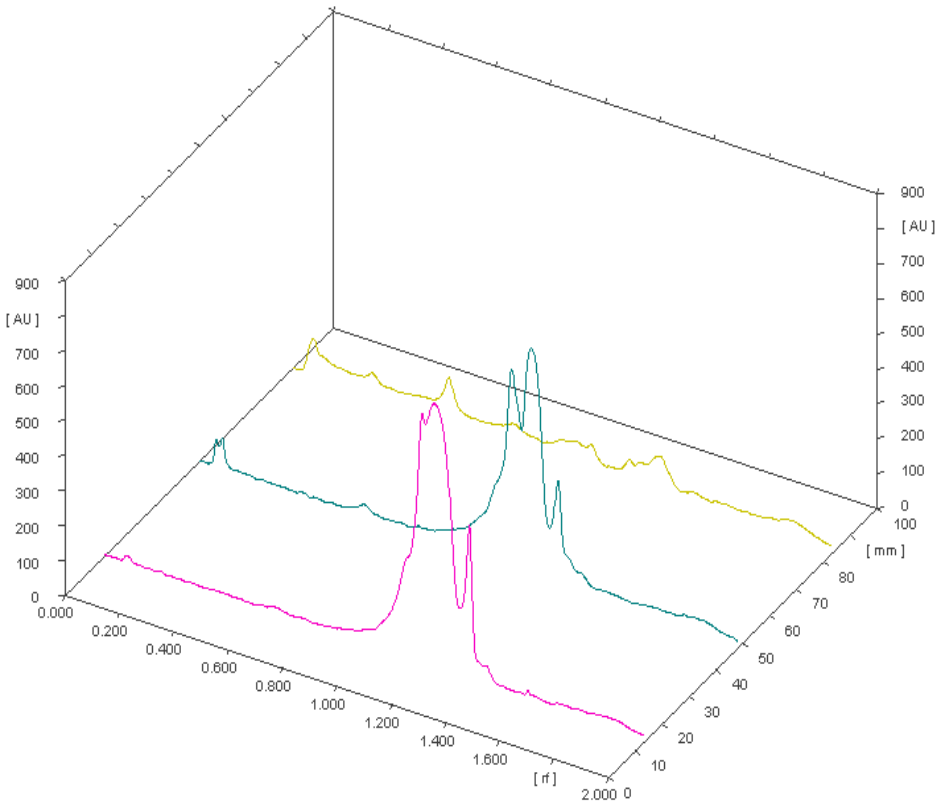


Figure 8: HPTLC analysis by visible light (366nm)

Lane no.	color	sample
1	Pink	1%dye solution
2	Light Blue	control
3	Light Green	test

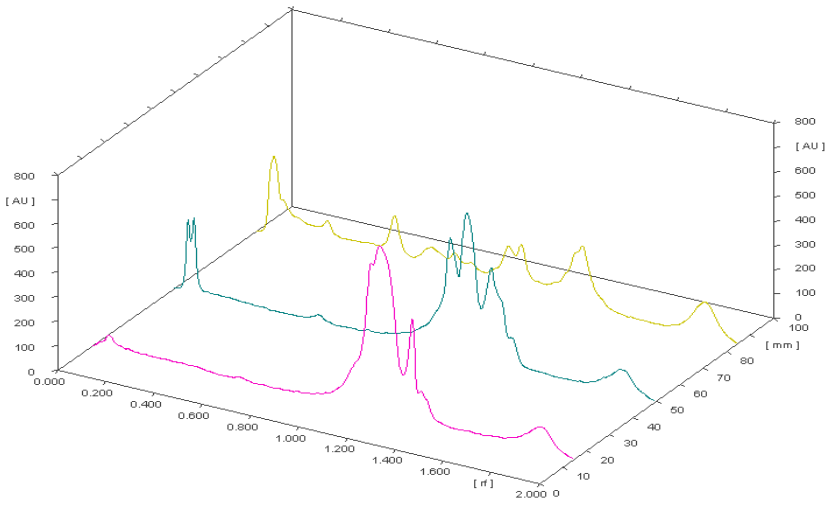


Figure 9: HPTLC analysis by UV light (254nm)

Lane no.	color	sample
1	Pink	1%dye solution
2	Light Blue	control
3	Light Green	test

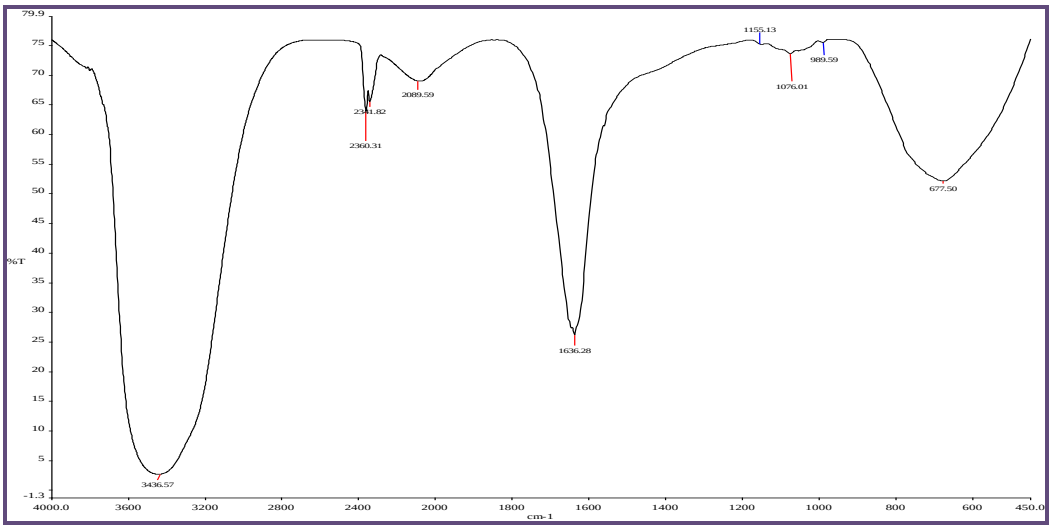


Figure 10: FTIR analysis of DN1 (control)

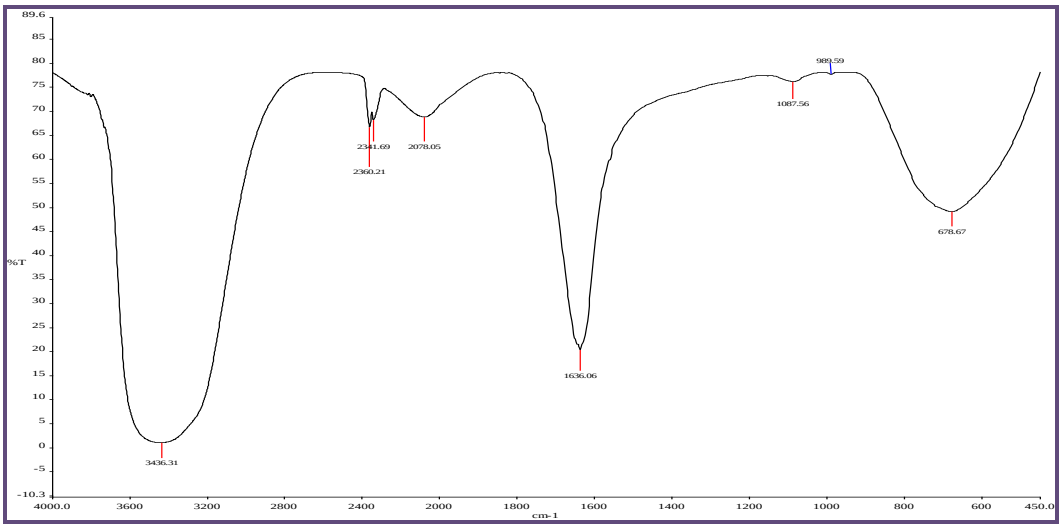


Figure 11: FTIR analysis of DN1 (test)

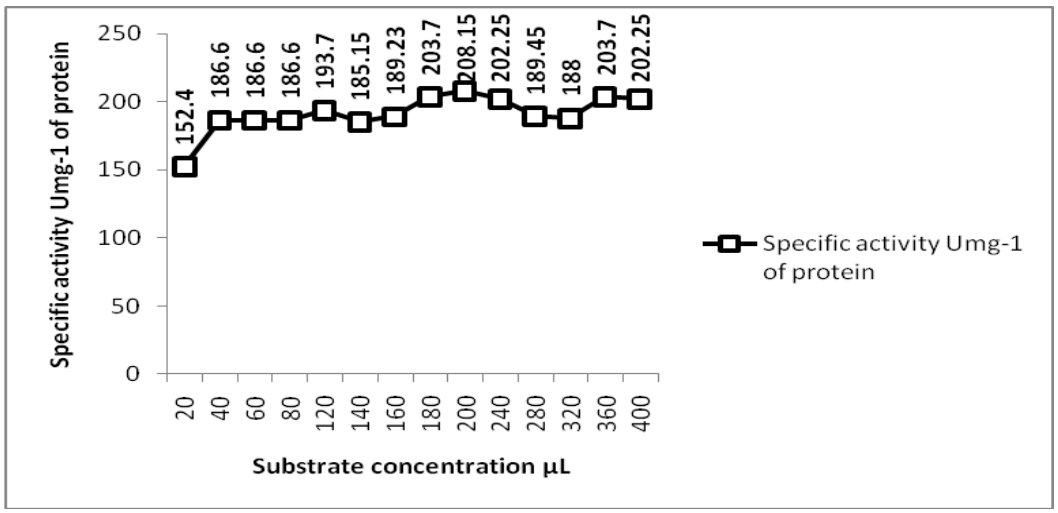


Figure 12: Effect of substrate on azo reductase

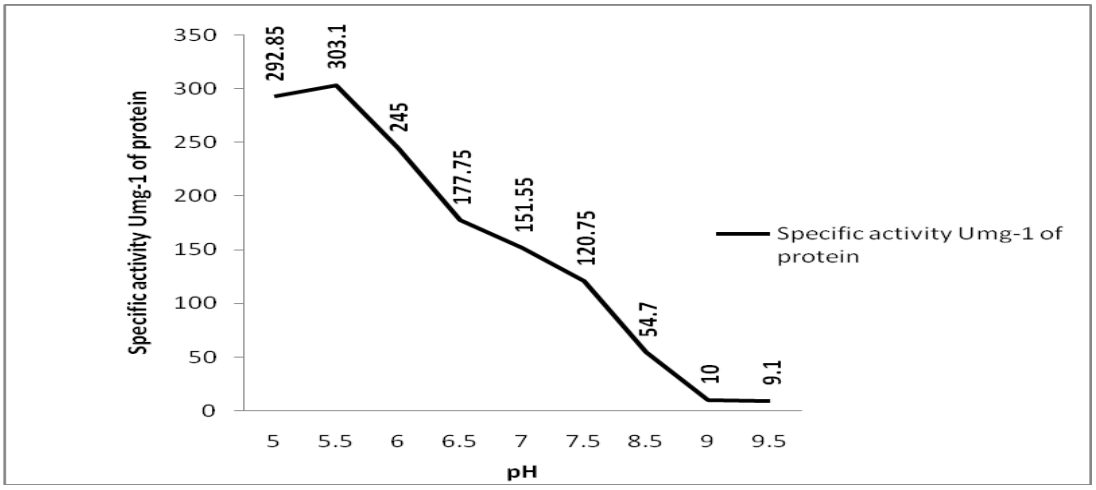


Figure 13: Effect of pH on azo reductase

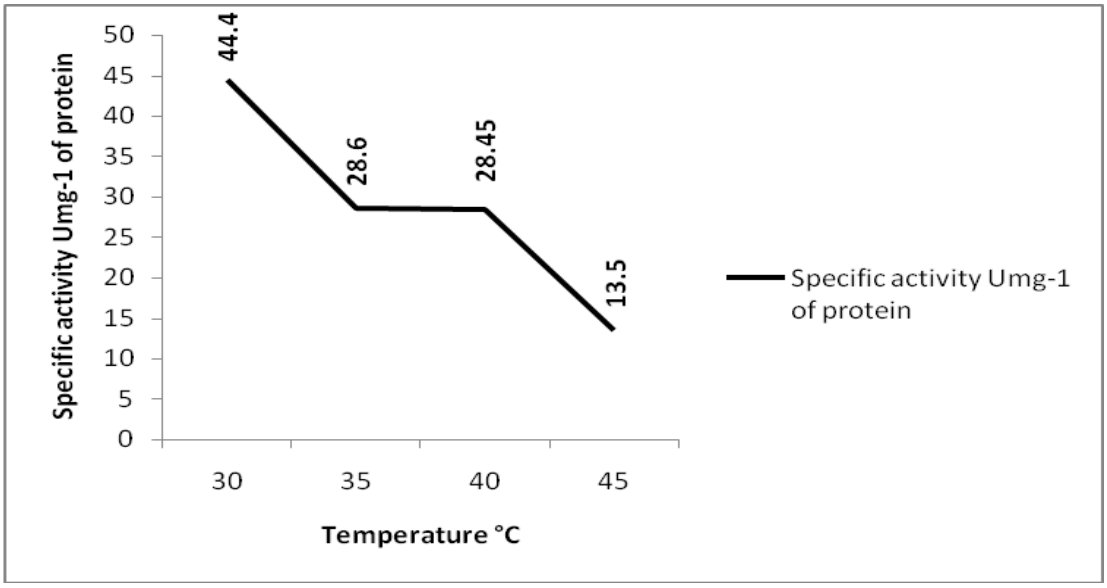


Figure 14: Effect of temperature on azo-reductase