Isolation and Characterization of Bacteria from Dye Wastewater Treating Down Flow Fixed Film Reactor (DFFR)

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Abstract

The effluent collected from an ongoing Down Flow Fixed Film Reactor (DFFR), containing Reactive violet 5 (V5R) dye manufacturing industry's wastewater, was studied for screening and isolation of organisms capable of decolourizing textile dyes. A bacterial consortium containing Raoultella terrigena, Enterobacter aerogenes and isolate B were isolated on the basis of dye decolourization. The consortium exhibited 94±2 % decolourization ability within 5 to 96 h under a wide pH range from 4 to 10, temperature 25 to 50°C, inoculum size 5 to 25 % (v/v) and 100 to 1000 mg/L dye concentration. The consortium was able decolourize Reactive Violet 5 R (V5R) in presence of peptone (0.25 to 3.0 g %), meat extract (0.25 to 10 g %) and sodium chloride (0.5 to 10 g %). The isolates were identified by Biolog® software. Selected bacterial consortium had the ability to decolourize five different Reactive dyes. A down flow fixed film bioreactor was developed using consortium which exhibited 96±2 %, 89 % and 97.88 % decolourization, COD reduction and copper remediation of 200 mg/L V5R respectively. A dyeing unit's waste containing Reactive Violet 5, Reactive blue BB and Reactive Red 5 B, was effectively decolourized to 90±2 % within 6±2 h. The developed bioreactor was successfully operated for more than 200 d.

Key words- *Bacterial consortium, Downflow Fixed Film reactor, Reactive Violet 5, Decolourization*

1. Introduction

Azo dyes are the largest class of synthetic dyes because of the ease and cost effectiveness of their synthesis and the greatest variety of colours (Sartale *et al*., 2010). Synthetic dyes are extensively used in textile dyeing, paper printing, colour photography, food, pharmaceuticals, cosmetics and other industries (Sheth and Dave, 2009). Approximately 10,000 different dyes and pigments are used industrially and over 7 x 10^5 metric tons of synthetic dyes are produced annually worldwide (McMullan *et al*.,2001). The Reactive group of azo dyes exhibit low levels of fixation with the textile fiber viz., up to 50 % of the initial dye. The unfixed dye is lost in the spent dye bath in its hydrolysed form which no longer has affinity for the fabric and therefore cannot be reused in the dyeing process (Watanapokasin *et al*., 2009). Very small quantity of reactive azo dyes show their visual effect with adverse impact in terms of total organic carbon (TOC) and chemical oxygen demand (COD) which cause severe environmental problems worldwide (Jirasripongpun *et al.*,2007). The possible contamination of drinking water supplies is of concern because azo dyes are known to be enzymatically degraded in the human digestive system, producing carcinogenic substances (Kirk and Othmer, 1993). There conventional methods for dye effluent treatment are physical, chemical and biological. The biological treatment methods have proved to be cheaper and effective in the treatment of wastewater containing recalcitrant chemicals including azo dyes (Ibrahim *et al.,* 1996). A large number of microorganisms have been isolated in recent years that are able to degrade dyes. Azo dye waste water treatment has been studied in aerobic as well as anaerobic treatment processes. In recent years some fundamental work has revealed the

existence of a wide variety of microorganisms capable of decolourizing a wide range of dyes (Banat *et al*., 1996; Kanekar *et al*.,1996; Dave and Dave, 2009).

Materials and Methods

Materials

The dyes Reactive Violet 5 (V5R), Reactive Black WNN, Reactive Orange W3R, Reactive Red ME6BL and Reactive Red BS used in this study were obtained from Ganesh Dye Chem., Vatva, G.I.D.C., Ahmedabad, Gujarat, India. Nutrient broth (NB) (containing g/L: Peptone, 5.0; NaCl, 2.5; Meat extract, 1.5; Distilled water, 1000 mL; pH 7.0 ± 7.2), Glucose phosphate broth (GPB) (containing g/L: Glucose, 5; K_2HPO_4, 5; Peptone, 5; Distilled water 1000 mL; pH, 7.0 ± 7.2), 3 % KOH, Vancomycin discs, oxidase discs etc. were used from Hi Media Laboratories Pvt. Ltd., Mumbai, India. All chemicals used were of analytical grade.

Microorganisms and Cultural Conditions

The Reactive dye decolourizing bacteria were isolated from the ongoing DFFR. The isolated pure cultures were maintained on Nutrient Agar slant at 4 – 8 °C.

Development of Consortium for Decolourization of V5R

All the four isolates (A, B, C and D) with their combinations (AB, AC, AD, BC, BD, CD, ABC, BCD, DAB and ABCD) were actively transferred to NB and GPB supplemented with 100 mg/L V5R. After 15 successive cycles, DAB showed maximum decolourization (96 %) within 7- 9 h, so DAB consortium was selected for further study.

Characterization and Identification of the isolates

The isolates were grown on nutrient agar slants. The Gram staining characteristics were further confirmed by Vancomycin and KOH test. The Gram negative bacterial isolates were characterized into GN – NENT (Gram negative non enteric) and GN – ENT (Gram negative enteric) using TSI (triple Sugar Iron) agar slants.The cultures were tested for the possession of cytochrome oxidase or indophenol oxidase using the Oxidase test. The selected isolates were transferred on BUG medium (Biolog®, USA) and prepared suspensions were inoculated in Gram negative and Gram Positive Biolog plates as per the standard protocol provided (Biolog®, 2001). All the media and Biolog® plates were incubated for 6 to 24 h at 32 ± 2^{o}C. Results were recorded and interpreted for identification using Biolog® software (Biolog Inc., USA).

Study of various physicochemical parameters and medium optimization

Decolourization of V5R (100 mg/L) by selected DAB consortium was studied under different physicochemical parameters, such as, temperature (25, 30, 35, 40, 45, 50ºC), shaking and static conditions, pH (4, 5, 6, 7, 8, 9 and 10), dye concentrations (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mg/L), inoculum size (5 %, 10 %, 15 %, 20 % and 25 %; v/v), time intervals (1- 10h at the interval of one hour) and continuous dye addition. Nutrient optimization was done by altering the concentration of media components viz., peptone (0.25 %, 0.5 %, 1.0 %, 1.5 %, 2.0 %, 2.5 % and 3.0 %), meat extract (0.25 %, 0.3 %, 0.5 % and 1.0 %) and NaCl (0.5 %, 1.0 %, 2.0 %, 3.0 %, 4.0 %, 5.0 %, 6.0 %, 7.0 %, 8.0 %, 9.0 % and 10.0 %).

Analytical Techniques

Aliquots of decolourized broth were scanned through a wavelength range of 200–800 nm using spectrophotometer (Jasco V – 530, Japan). Aliquots of 2 mL of the inoculated and uninoculated culture media were withdrawn after decolourization and centrifuged at 6000 rpm for 15 min using REMI R-24 centrifuge.

Decolourization was quantitatively analyzed by measuring the absorbance of the supernatant using spectrophotometer (Systronics 169) at maximum wavelength, λ_{max}, of 558 nm for V5R. The percent decolourization and rate was calculated according to equation described by (Sheth and Dave 2009).

Detection of degradation by TLC

The decolourized broth was collected in microfuge tubes, centrifuged at 6000 g for 25 minutes and 15µL of the sample was spotted on TLC plates. Degradation of the dye was monitored on precoated TLC silica gel plate $60F_{254}$ (200 mm X 200 mm) plates supplied by Merck, Germany. The solvent system used was ammonia: acetone: propanol (6:6:6). The dye chromatogram was observed under visible and long wave ultra violet light.

Detection of Copper removal

For assessing the ability of the consortium to remove copper from V5R, Atomic Absorption Spectrophotometeric analysis was performed using Elico SL 194 (India).

Spectrum of decolourization

The consortium was also tested for the decolourization of Reactive Black WNN, Reactive Orange W3R, Reactive Red ME6BL, Reactive Blue RGB and Reactive Red BS.

Bioreactor study

The specially designed reactor was constructed from glass column, which was operated in batch mode. The details of the reactor are given in the **Table 1**. Two columns were utilized, one of them named as control (C), which remained uninoculated and the second named as test (T), which was inoculated with the selected bacterial consortium. For the development of the biofilm, 200 mL of the decolourized broth (90-95% decolourization of 100 mg/L V5R in NB) was added to (T). After 4 days of incubation, 95 mL (50% of the working volume) was removed and replaced with 95 mL sterile NB containing 50 mg/L V5R. This procedure was continued for 16 days. After 16 days of reactor operation, the entire working volume (190±10 mL) was replaced by NB containing 50 mg/L V5R. This procedure was repeated three times. On 24th day of reactor operation, dye concentration of the influent was increased to 100mg/L. This was repeated for three consecutive days. From the 27th day, the Test column was fed with 190±10 mL of medium containing g/L: peptone, 5; NaCl, 5 supplemented with 100 mg/L of V5R, whereas meat extract supplementation was stopped. The biologically treated V5R containing medium, in Test column was assessed for decolourization, degradation and copper removal.

Table 1 Reactor design

Specification	Dimension/ quantity
Outer diameter (cm)	3.8
Inner diameter (cm)	3.4
Height (cm)	54
Total volume (mL)	380
Working volume (mL)	190±10
Packing material	Furnace charcoal
Size (cm)/ No. of pieces	1.0±0.2/889
Height occupied by packing material (cm)	37
Weight of packing material (g)	177±10

Results and Discussion

Isolation and Identification of Microorganisms

Four isolates viz. A, B, C and D were obtained from ongoing down flow fixed film reactor remediating V5R manufacturing industry's wastewater. The isolates were studied colonial and morphological characteristics. Isolate A; small, round, smooth convex colonies, Gram negative short rods. Isolate B; big, irregular, rough, flat colonies, Gram positive big rods. Isolate C; intermediate, irregular moist, greenish colonies, Gram negative short rods. Isolate D; small, round, entire opaque, Gram negative short rods. A, B and C were KOH test positive where as B was negative, Isolate B gave Vancomycine test positive and remaining three were produced negative results conforming isolate B as Gram positive and isolates A, B and C Gram negative bacteria. On the basis of oxidase test and TSI agar slant observation A and D were identified as GN–ENT and C as GN–NENT. The identification by Biolog® software showed that A, C, and D were *Enterobacter aerogenes, Pseudomonas aurantiaca* and *Raoultella terrigena* respectively. The identity of isolate B was not confirmed.

Effect of Physicochemical Parameters and Media on Dye colorization

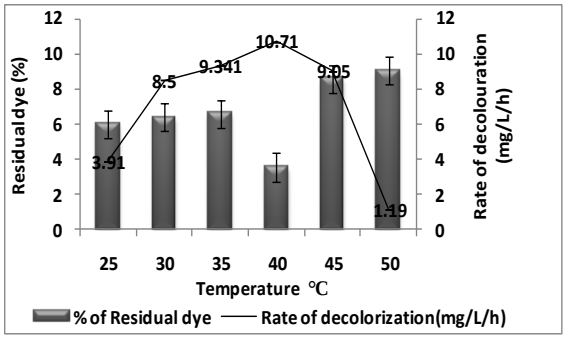
Shaking and Static Condition

Consortium containing isolates DAB exhibited 92 ± 1 % decolourization when incubated under static condition with a dye removal rate of 9.2 mg/L/h whereas under agitated conditions 28 % decolourization was obtained with a dye removal rate of 0.291 mg/L/h . The results suggest that microaerophilic conditions favour the decolourization of V5R. Microbial reduction of azo dyes is an enzymatic reaction linked to anaerobiosis because it is inhibited by oxygen. Therefore, facultative or obligate anaerobes are necessary for azo dye reduction. Similar observations have been made by (Dawkar e*t al*.,2010; Sartale *et al*., 2010), for the reactive dye RHE7B and other reactive dyes supplemented to NB using bacterial consortium GR of *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168 respectively.

Temperature

It was noticed that the consortium DAB showed active decolourization over a broad range of temperature and the dye removal rate was in the range of 1.19 mg/L/h – 10.71 mg/L/h **(Fig. 1)**. The dye removal rate of culture increased by 2.7 folds, on raising the incubation temperature from 25 to 40°C; whereas further increase in incubation temperature to 50°C decreased the removal rate by 9 folds. The maximum removal rate was attained at 40°C. The decrease in the removal rate at temperature higher than 40°C could be due to the denaturation of the dye decolourizing enzymes. Similar results were obtained by Waghmode et al., (2010), during their study of Rubine GLF by microbial consortium GG-BL consortium containing *Galactomyces geotrichum* MTCC 1360 and *Brevibacillus laterosporus* MTCC 2298). Similarly the isolate *Enterococcus faecalis* strain YZ66 showed the maximum decolourization at the similar temperature. This may be due to a greater production of enzymes and optimal growth conditions of the isolate for its dye decolourizing ability. The decolourization at this optimum temperature may be owing to higher respiration and substrate metabolism [13].

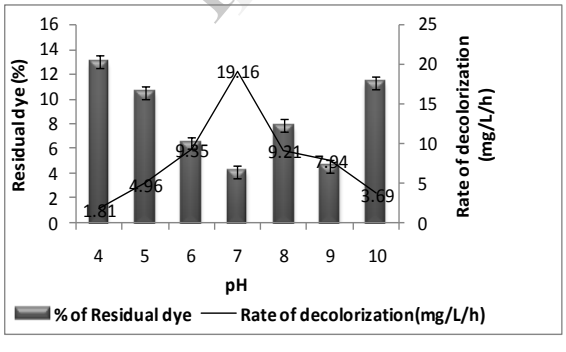
Fig. 1 Decolourization of V5R at different incubation temperature



pH

The Reactive V5R dye manufacturing industry discharges effluent having a pH in the range of 4.0 to 6.0, whereas the textile dyeing unit discharges effluent within a pH range of 8.5 to 10.5. Due to this, the bio-decolourization of the consortium DAB was studied over pH 4.0 to 10.0 (**Fig. 2)**. At pH 4.0, the dye removal rate was 1.81 mg/L/h with 87 % decolourization and 3.69 mg/L/h with 88.66 % at pH 10. The optimum pH range was found to be 6 - 8 with a decolourization of 92 ± 3 %. At pH 7.0, maximum dye removal rate of 19.16 mg/L/h was obtained. Similar results have been reported by (Junnarkar *et al*., 2006; Mate and Pathade, 2012). Our study showed that maximum decolourization percentage was obtained at neutral pH to slightly acidic/alkaline pH. The enzyme functions are based on the conformation of the enzyme. Acidic and alkaline conditions may lead to the breakage of ionic bonds on the active sites of some enzymes and this ionic group must be in suitable form. The variations in pH of the medium might have resulted in changes in the ionic form of the active site and thereby affect the decolourization by enzymes possessed by the consortium DAB.

Fig. 2 Effect of pH on decolourization

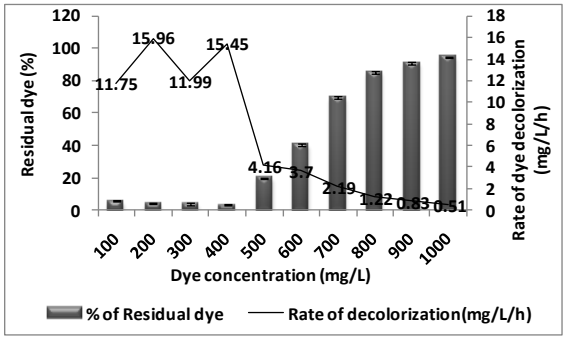


Dye concentration

The Reactive dye molecule is a complex structure; and about 12% of the synthetic textile dyes are lost in the wastewater systems. Varying amounts of these dyes are present in the effluents. Keeping this point in mind, the influence of dye concentration on the decolourization ability of the consortium was investigated. The time required for the decolourization of the dye was directly proportional to the concentration of the dye in the system **(Fig. 3)**. The highest decolourization rate 15.96 mg/L/h were obtained at 200 mg/L dye. The developed microbial consortium resulted in increased decolourization ability even at 400 mg/L. Dye concentration above 400mg/L showed gradual inhibition of decolourization ability of the organism. Similar observations have been recorded earlier for decolourization of AR 119 dye by (Dave and Dave, 2009). Moreover, (O'Neill *et al*., 1999) reported that the dye concentration in the reactive dye bath effluent was observed within narrow range of 100- 200 mg/L; whereas consortium DAB exhibited decolourization of V5R

dye at concentrations much above those reported in wastewaters and thus it can be successfully employed for treatment of dye-bearing industrial wastewaters.

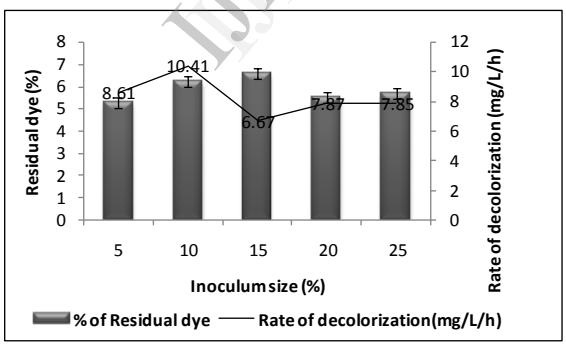
Fig. 3 Effect of increasing dye concentration



Inoculum size

The effect of inoculum size for decolourization was evaluated with the addition of different inoculum ranging from 5 to 25 % (v/v). The obtained results **(Fig. 4)** showed maximum decolourization (93.76 ± 0.50) with 10.41 ± .050 mg/L/h rate of dye removal was observed at 10 % (v/v) inoculum size, further increases in inoculum size did not affect the decolourization. Increasing the inoculum volume from 20 – 25 % (v/v), no significant increase in decolourization rate was observed. The optimum dye removal rate was obtained (10.41 mg/L/h) at 10 % inoculum size with 93.76 % decolourization within 9 h.

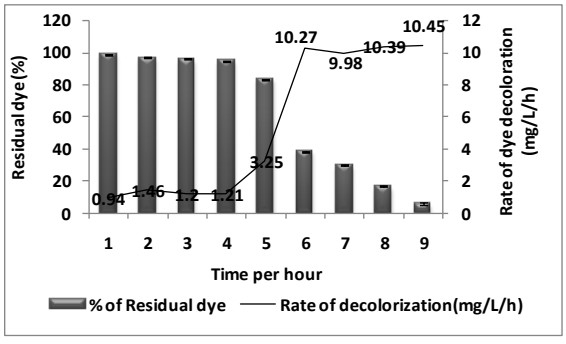
Fig. 4 Effect of inoculum size



Decolourization at different time intervals

The V5R dye decolourization was seen in gradually increasing manner with reference to time by the used DAB consortium **(Fig. 5)**. During the first 4 hours of incubation, 1.2 ± 0.25 mg/L/h V5R removal rate was observed. This may be the time required for the synthesis of the dye decolourizing enzymes possessed by the Consortium DAB. During the 5th hour, the exponential growth phase of the Consortium DAB started with a 2 fold increase in the V5R removal rate. The dye removal rate showed an exponential rise during the 6th hour with 10.26 mg/L/h, which was steadily maintained till the 9th hour of incubation. After 9h incubation 94.11 % dye decolourization was observed with 10.45 mg/L/h dye removal rate. The rapid decolourization activity was seen at the 6th hour of incubation with 61.63% dye decolourization having 10.27 mg/L/h dye removal rate. At the end of the 9th hour 94.11 % of decolourization.

Fig. 5 Time scale study



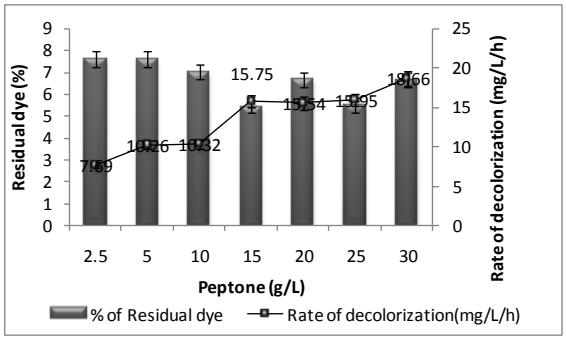
Effect of repeated addition of dye aliquots

The effluent is continuously discharged from the textile dyeing units and the concentration of dye keeps on fluctuating in the effluent. Keeping this fact in mind, the decolourization of repeated addition of dye was done. It was observed that on achieving 98.24 % decolourization of first dye aliquot (100 mg/L) within 5h a second aliquot (100 mg/L) was added, which was decolourized 98.39% within next 12h. The third aliquot of 100 mg/L was decolourized within next 28h (Ghodake e*t al*., 2011) reported the effect of continuous dye addition with Amarath dye by repeated addition of amaranth (100 mg/l) was studied for the utilization of same biomass for more times. First two cycles showed 90 % reduction in colour in 48 h; however, third cycle took 72 h for 50 % removal of the colour. The results clearly suggested that increase in number of cycles of dye addition caused decrease in decolourization. It might be due to nutrition depletion and accumulation of aromatic amines, which inhibit microbial activity or enzyme production (Saratale *et al.*, 2009).

Effect of peptone

Influence of media components on biodecolourization ability of selected consortium DAB was studied. Decolourization performance of selected bacterial consortium was studied at different concentration of peptone in the range of (0.25 to 3.0 g/L) **(Fig. 6)**. Maximum decolourization (94.55 ± 0.50 %) was observed at 1.5 g% with 15.4 ± .005 mg/L/h dye removal rate. Further increase or decrease in concentration did not show significant difference in dye decolourization percentages. Jirasripongpun (2007) showed 91 % decolourization of Reactive Red 195 by *Enterobacter sp*. when 1.0 g% peptone was used in the medium.

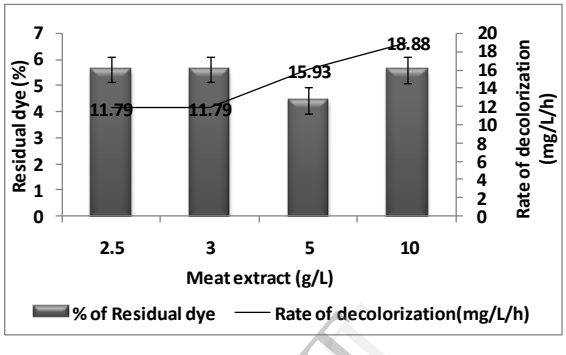
Fig. 6 Effect of peptone concentration



Effect of meat extract

The effect of meat extract concentration was evaluated in the range of (0.25 to 1.0 g/L) **(Fig. 7)**. Maximum decolourization (95.58 ± 0.30) was observed at 0.5g/L with 15.93 ± 0.020 mg/L/h rate of decolourization. Decrease in concentration from 0.5g/L steady type of decolourization and dye removal rate was observed. On the other hand by increasing the concentration of meat extract from 0.5 g/L, 18.88 ± 0.020 mg/L/h dye removal rates was observed with 94.42 % decolourization rate. Sahasrabudhe and Pathade (2011) showed nearly 89% decolourization of Reactive yellow 145 by *Enterococcus faecalis* strain YZ66, by supplementing 1 % meat extract to the synthetic medium.

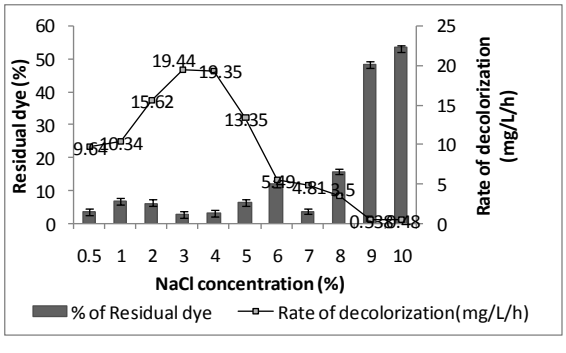
Fig. 7 Effect of meat extracts concentration



Effect of Salt (NaCl) concentration

One of the interesting finding useful in dye decolourization study was the resistance of the culture to NaCl concentration in the medium. The NaCl concentration used for the study was ranging from 0.5 to 10 %. **Figure 8** clearly indicates that bacterial consortium DAB gave maximum decolourization (97.23 ± 0.020) with 3 % NaCl concentration with 19.35 ± 0.010 mg/L/h rate of decolourization. After checking the whole optimum conditions together with both 100 mg/L and 200 mg/L dye concentrations, 100 mg/L dye removed from the system within less time (4-5 h) as compare to 200 mg/L (12h). Therefore 100 mg/L was selected as the favorable concentration for reactor set up.

Fig. 8 Effect of NaCl on decolourization



Spectrum of dyes decolourized by the bacterial consortium

The bacterial consortium DAB showed rapid decolourization of all dyes within 12 h at 36 ± 2°C **(Table: 2)**. Culture could decolourize 100 mg/L of Reactive Black WNN, Orange W3R, Red ME6BL, Blue RGB, and Blue RGB within 10-12 h at 36 ± 2°C in the range of 85 to 94 %. Similar observations were also made by [12].

Table 2 Spectrum of decolourization

No.	Name of dye	Incubation time	Decolourization (%)
1.	Reactive Black WNN	10	90.13
2.	Orange W3R	11	86.45
3.	Red ME6BL	10	88.39
4.	Blue RGB	10	94.61
5.	Blue RGB	9	84.94

Degradation and Copper Removal

The TLC plates exhibited spots with different R_f compared to the abiotic control, suggesting the degradation of dye. The abiotic control (100 mg/L V5R) contained 5.17 mg/L Cu where as the consortium treated sample contained 0.11 mg/L Cu, suggesting 97.88 % Cu removal.

Performance of Bioreactor:

The performance of bioreactor was studied after 15 days for their decolourization. The concentration of V5R used for bioreactor setup was initially 50 mg/L which showed 96 % of decolourization within 18±2h. After 20 days of reactor startup, the concentration of the dye in the influent was increased to100 mg/L which also showed nearly 98 % of decolourization within 10±2h. Initially the working volume of the test column was 190±10 mL but after 30d the volume of effluent decreased to 165mL, which may be due to the formation of biofilm on the packing material. The considerable decrease in the time of decolourization could be attributed to the formation of the active biofilm on the packing material. The UV-Vis spectrum shows a shift in the absorption maximum towards UV region, suggesting the breakdown of the dye **(Fig. 9)**. The wavelengths of absorption peaks can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups within a molecule. DAB decolourized the model dye by reducing the azo bond as observed from the UV-Visible spectrum. The degradation of V5R by DAB was confirmed using TLC. When the spots of the control column were compared with the test column, the spots of the test column had different R_f values, suggesting the degradation of the dye by the developed fixed film in the test column. After 30 days of reactor startup, the concentration of the dye in the reactor was increased to 200 mg/L, which was decolourized to 94±2 % within 6±2h. The COD of the treated effluent was 696 mg O_2/L and the COD of the control column was 6340 mg O_2/L suggesting 89 % decrease in COD after treatment. The TS obtained was 6800 mg/L for test and 14400 mg/L for control. After 35d, the influent was replaced with actual dyeing unit's wastewater containing three different dyes (Reactive Violet 5, Reactive blue BB and Reactive Red 5 B and 1 % peptone), which showed up to 90 % of dye decolourization within 6±2 h.

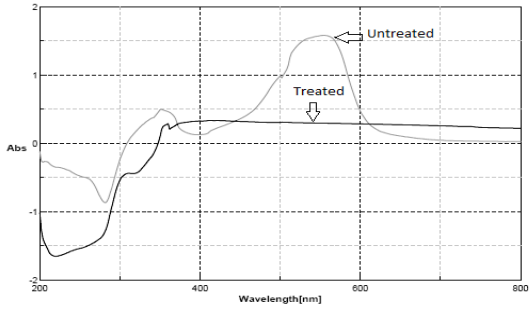


Fig. 8 UV-Visible spectrum of untreated and treated Reactive Violet 5R (Bioreactor).

Conclusions

In the present study, the four isolated bacteria and their combinations gave good decolourization and among them DAB containing *Enterobacter aerogenes*, *Raoultella terrigena* and one was unidentified Gram positive bacteria proved best decolourizer at the optimum physicochemical conditions such as static condition having 7 pH, temperature 40 °C, with inoculum size 10% (v/v), 100 mg/L dye (V5R) concentration. The copper removal rate was also high at 100 mg/L concentration.

Acknowledgement

The authors are thankful to Prof. S. R. Dave and Dr. Devayani Tipre (Department of Microbiology, Gujarat University) for their support in identification (Biolog®) of isolates.

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