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RESEARCH ARTICLE

BIOLOGICAL DECOLORIZATION OF REACTIVE RED 31 AND REACTIVE YELLOW 81 DYES BY NOVEL ISOLATED BACTERIAL STRAIN *STREPTOCOCCUS* SP. VBH1

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ABSTRACT

The present study emphasizes on the decolorization of reactive dyes by the bacteria isolated from paper industry waste water. The isolate VBH1 was selected based on its maximum decolorization of 150mg/l of Reactive red 31and Reactive yellow 81. Based on phenotypic characterization and phylogenetic analysis of 16S rDNA sequence, the strain VBH1 belongs to the genus *Streptococcus*. The optimal conditions for the decolorization of dyes by the isolate VBH1 for 150 mg/l of dyes remained shaker with glucose as carbon supplement and tryptone as nitrogen supplement at pH 8.0 and temperature 35ºC. On physiochemical parameters optimization, 100% decolorization of Reactive red 31 and Reactive yellow 81 was achieved within 22h. Although the degraded product formed was non-toxic as compared to the parent dyes, scanning micrographs taken after dye decolorization revealed drastic changes on the bacterial cell morphology.

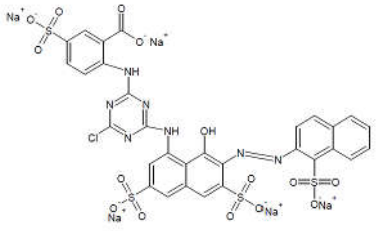
INTRODUCTION

Small-scale industries in India contribute 3900 million liters wastewater per day (Agarwal, 2001). Presence of very low concentrations of dyes in effluents is highly visible and undesirable (Kilic et al., 2007). Some of these dyes are potentially mutagenic (Mathur et al., 2005), carcinogenic and toxic (Atkins, 2000). Several methods are used in the treatment of textile effluents to achieve decolorization include physiochemical methods such as filtration, specific coagulation, use of activated carbon and chemical flocculation. Some of these methods are effective but quite expensive and have many disadvantages and limitations (Do et al., 2000; Maier et al., 2004). It is, therefore, important to develop efficient and cost-effective methods for the decolorization and degradation of dyes in industrial effluents and contaminated soil (Bhatt et al., 2000). Bioremediation offers a cheaper and environmental friendly alternative method for color removal in textile effluents. Several reports have been published on bacterial azo dye reduction under different conditions (Hu, 2003; McMullan et al., 2001; Stolz, 2001). Azo dyes generally resist aerobic microbial degradation, only organisms with specialized azo dye reducing enzymes were found to degrade azo dyes under fully aerobic conditions (Ganesh, 1994). Aerobic metabolism of dyes by *Pseudomonas mendocrina* M2M B-404 and *Sphingomonas xenophaga* BN6 had been studied by Sarnaik and Kanekar (1999) and Stolz (1999), respectively. Also study reported by Buitron et al. (2004) with azo dye Acid red 151 under aerobic condition using a microbial consortium led to 99% color removal. The present work was done on the isolation and identification of potential dye degrading bacteria isolated from local paper industry waste water. Furthermore, the studies were conducted to determine the effect of optimized physiochemical parameters with respect to time on the decolorization efficiency of the isolate for the dyes Reactive red 31and Reactive yellow 81.

MATERIALS AND METHODS

Dyes and chemicals

The dyes; Procion red H8B (CI-Reactive red31) and Reactive yellow HE4G (CI-Reactive yellow 81) as shown in Fig.1(A, B) used in the study were of textile grade and procured from Karnataka Khadi Gram Udyog Samyukta (KHDC), Bengeri, Hubli (Karnataka). All the other analytical grade reagents were purchased from HiMedia and Sigma Aldrich, Bengaluru, India.



A

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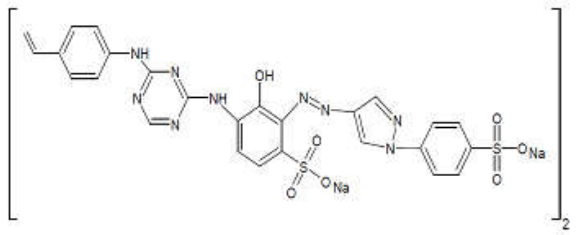


B

Fig.1: Molecular structure of Reactive red 31 (A) and Reactive yellow 81 (B)

Isolation of dye degrading bacteria

The waste water sample was collected from the local paper industry at Meerut, Uttar Pradesh, India. The isolation and routine sub culturing of dye decolorizing bacteria was carried out on nutrient media (in g/L: Peptone, 10; Sodium chloride, 5; Yeast extract, 5 and Agar, 15) at pH 7.0 at 35ºC. The waste water sample was serially diluted and 100µl of 10^{-5} dilution was spread on the nutrient agar plates and morphological distinct pure colonies isolated were further transferred to nutrient agar slants and broth as stock. The 150mg/l of each dye amended in nutrient broth were inoculated with the isolated bacterial strains (1% v/v) further incubated at 35ºC. After every 10h interval, aliquot of 1ml was withdrawn and centrifuged at 10,000 rpm for 10minutes and clear supernatant obtained was taken to measure the dyes decolorization at their respective absorbance maxima (λmax) using the spectrophotometer up to 50h where a medium without dye and inoculum was used as blank while medium with dye but without inoculum taken as control. All the experiments were carried out in triplicates and the mean value was taken. The decolorization of dyes was calculated by the given formula (Ola et al., 2010) as; Decolorization efficiency (%) = I-F/I x 100, where I = Absorbance of media prior to incubation, F = Absorbance of decolorized media. After calculating the percentage decolorization, the cultures showing maximum decolorization of dyes up to 50h were sorted out as dye degrading bacteria**.**

Identification of dye decolorizing bacteria

The bacterial **i**solate showing maximum decolorization of reactive dyes was initially studied for phenotypic characteristic features on the nutrient agar followed by the classification based on the Gram's staining (Aneja, 2003).The isolate was outsourced to Bioaxis, Hyderabad for 16S rDNA sequencing. The sequence obtained was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov/) using nBLAST tool and corresponding sequences were downloaded (Altschul, 1990). The phylogenetic tree was constructed using MEGA4 package by the neighbor joining method (Tamura, 2007).

Physiochemical parameters optimization

The potent bacteria isolated was further studied for the physiochemical parameters in order to monitor their effect on the decolorization efficiency of dyes using different carbon sources (1%), nitrogen sources (1%), pH (4, 5, 6, 7, 8, 9 and 10), temperature (25, 30, 35 and 40ºC), various dye concentrations (150, 200, 250, 300 and 350mg/l), different concentration of inoculum (100, 150, 200, 250 and 300µl) and static /shaker condition. The dye degradation study by bacteria was carried out in 10ml nutrient broth amended with 150mg/l of individual dyes, inoculated with 100µl of overnight grown culture further incubated at 35ºC for 24h and decolorization was monitored as described earlier section.

Decolorization of dyes using optimized parameters

Using all the physiochemical parameters decolorization analysis of dyes was carried out with respect to time where after every 2h interval, aliquot of 1ml decolorized media was withdrawn and centrifuged. The supernatant was used for monitoring the percentage decolorization while the cell pellets obtained were dried at 85°C and weighed mass was then expressed in mg/ml.

Other analysis

Decolorization mode of isolate on dyes

Decolorization of dyes may take place by adsorption (Aravindhan et al., 2007) or degradation (Kumar et al., 2007). In the case of adsorption, dyes are only adsorbed onto the surface of bacterial cells, whereas new compounds come into being when dyes are degraded by bacterial enzymes during the degradation process. Dye adsorption can be also easily judged by an evidently colored cell pellet, whereas those retaining their original colors are accompanied by the occurrence of biodegradation (Chen et al., 2003). The two sets of standard nutrient broth (25ml) were inoculated (1%v/v) with the dye decolorizing bacteria further incubated for 24h. Before addition of dyes (individually150mg /l in separate flask), one set of bacterial culture was autoclaved at 121◦C for half an hour while the unautoclaved served as control. Dye decolorization assay was carried out as described in earlier section.

Scanning Electron Microscope (SEM)

Further visualization of the isolates under SEM was performed to determine the effect of dyes on the morphology of the bacterial isolate. Following 24h incubation of cultures in optimized and 150mg/l of dye amended nutrient broth, cell pellets obtained after centrifuged at 10,000rpm for 10mintues were fixed by the 2.5% (v/v) gluteraldehyde and dehydrated by successive immersion for 10 minutes in ethanol at the following concentrations: 30% (v/v), 50, 70, 80, 90 and 100%. This was then followed by critical point drying, gold palladium coating and then visualized under JEOL 840 SEM (Kassongo and Togo, 2010).

Toxicity study

Microbial toxicity and phytotoxicity test were performed in order to assess the toxicity of selected dyes and their degraded metabolites. The degraded products of each 150mg/l Reactive red 31 and Reactive yellow 81 from the 50ml media were extracted in ethyl acetate, dried and

dissolved in 5ml sterilized distilled water to make a final concentration of 500 ppm and 1000ppm. These final concentrations were then used for the microbial toxicity studies carried out in relation to *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Aspergillus niger* while respective concentrations of dyes were taken as control. The zones of growth inhibition of microbes (diameter in cm) were recorded after 24h of incubation at 35ºC. The phytotoxicity study of 150mg/l Reactive red 31, Reactive yellow 81and their degraded products (extracted from 50ml media in ethyl acetate, dried and final concentration of 100 ppm in 5 ml) was also carried out (at room temp) in relation to *Triticum aestivum* and *Phaseolus mungo* (10 seeds each) by watering separately with 5 ml sample of control Reactive red 31 and Reactive yellow 81 and their degraded product (100 ppm per day) while control set was watered with distilled water simultaneously. Germination (%), length of plumule (shoot) and radicle (root) was recorded after 10 days.

RESULT AND DISCUSSION

Isolation and identification of dye decolorizing bacteria

Total of five bacterial strains were isolated and only bacterial isolate VBHI showed decolorization of the dyes Reactive red 31 and Reactive yellow 81. After 50h of incubation, significant percentage decolorization of 41.21 and 45.93 % was recorded for the Reactive red 31and Reactive yellow 81, respectively (Fig.2).

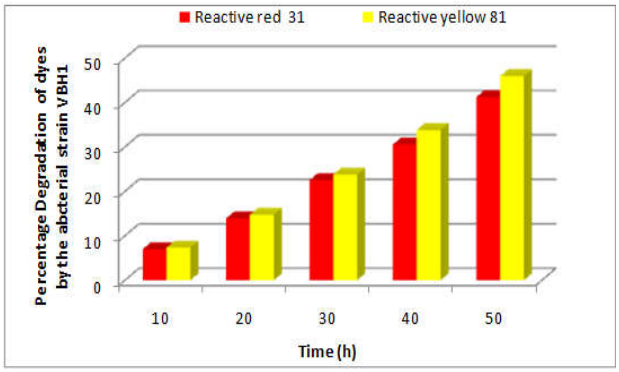


Fig.2: Screening of dye degradation bacteria for Reactive red 31 and Reactive yellow 81 with respect to time.

The colonies of VBH1 were smooth, circular, transparent dew drop like whitish blue and 1mm in diameter. The isolate VBH1 was recorded as Gram-positive cocci and its 16S rDNA sequence analysis showed highest similarity of 100% with the *Streptococcus* sp. oral clone ASCE01 (AY923132). Based on the phenotypic characteristics and phylogenetic analysis, strain VBH1 was identified as *Streptococcus* sp. The Fig.3 shows the phylogenetic relationship between the isolate VBH1 and *Streptococcus* sp. The sequence of the isolate *Streptococcus* sp.VBH1 has been deposited in GenBank with the accession number JQ72669.

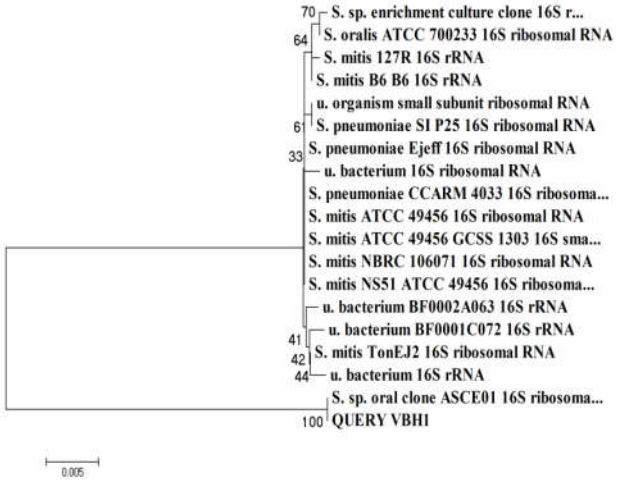


Fig.3: Phylogenetic tree of the isolate VBH1 with relation to genus *Streptococcus*.

Analysis of physiochemical parameters

Effect of carbon and nitrogen sources

The decolorization efficiency of the isolate *Streptococcus* sp.VBH1was monitored with different carbon and nitrogen sources as supplement in the nutrient broth for the decolorization of dyes. It was observed that among the different carbon sources, glucose showed maximum decolorization of 49.62 and 47.85% for the Reactive red 31 and Reactive yellow 81, respectively (Fig. 4). Two opinions have been argued for many years: one deems that dyes are not a carbon source since the anaerobic bacteria obtains energy from the glucose instead of the dyes and glucose can enhance the decolorizing performance of biological systems (Sarioglu and Bisgin, 2007); while another deems that glucose can inhibit the decolorizing activity (Chen et al., 2003). The variability may be due to the different microbial characteristics involved. Our results showed that 1% concentration of glucose as carbon source was good effective carbon supplement for the *Streptococcus* sp. VBH1 for the decolorizing process. Effects of some other carbon sources on bacterial decolorization performance have been studied in former researches where lactate, peptone, succinate, yeast extract, and formate were proved to enhance decolorization, while sucrose and dextrin resulted in lower decolorization activities (Xu et al., 2006).

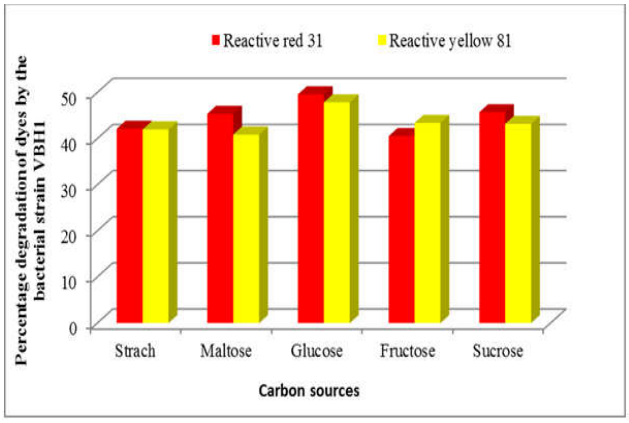


Fig.4: Effect of different carbon sources on the dye decolorization.

Whilst among the different nitrogen supplements tryptone was recorded with maximum decolorization of 49.99% and 48.12% for the Reactive red 31 and Reactive yellow 81, respectively as effective nitrogen supplement (Fig. 5). It was reported that addition of inorganic nutrients like nitrogen does not always enhance degradation of organic compounds, because there are many other factors which many decrease microbial activity [Steffensen and Alexander, 1995]. Urea when dissolved in liquid culture causes a shift of pH more towards acidic side, which decreased the color removal, growth as well as enzyme activity of strains. Presence of nitrate in culture media might slow down process of color removal (Carliell et al., 1995; Panswad and Luangdilik, 2000).

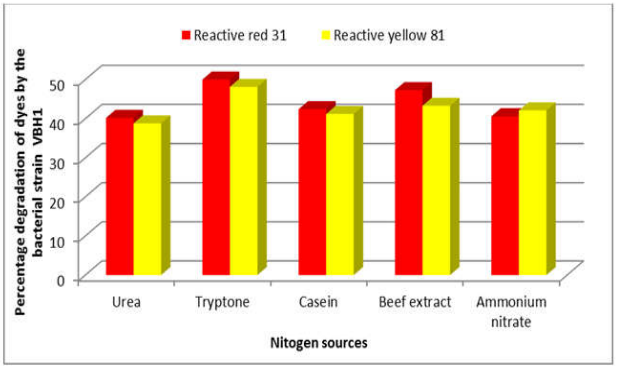


Fig.5: Effect of various nitrogen sources on the dye decolorization.

Effect of pH and temperature

It was monitored that strain *Streptococcus* sp.VBH1 showed lowest degradation of dyes at pH4, which increased as pH shifted towards pH8 and on further increment in pH, depression in the decolorization peeks were observed (Fig.6). The strain VBH1 showed elevated decolorization profiles of 51.54 and 55.45% for Reactive red 31and Reactive yellow 81, respectively at pH 8 after 24h of incubation. The results concluded that pH has major effect on the efficiency of dye decolorization and the optimal pH for color removal is often between the range of 6 and 10 (Guo et al, 2007; Kilic et al., 2007).

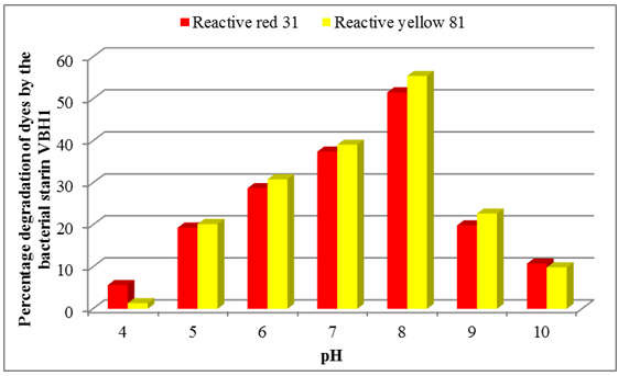


Fig.6: Decolorization of dyes on using different pH for the nutrient media.

With an increase in temperature from 25 to 35ºC, the decolorization rate increased and a further increase in temperature to 40ºC adversely affected the dye decolorization activity of the strain *Streptococcus* sp.VBH1 (Fig.7). The optimum temperature for dye decolorization was found to be 35ºC at which the isolate showed 55.67 and 59.89 % decolorization for the Reactive red 31and Reactive yellow 81, respectively. Decolorizing activity was significantly suppressed for 45ºC, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 42ºC (Panswad and Luangdilik, 2000; Cetin and Donmez, 2006).

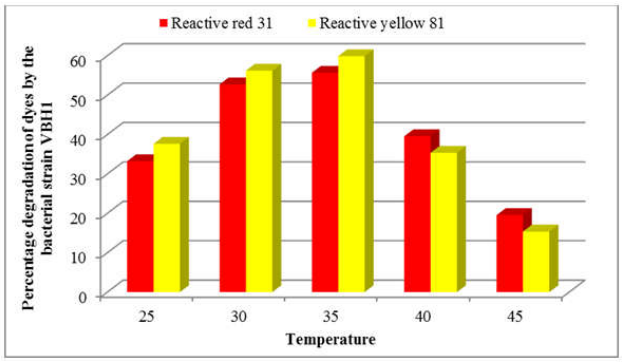


Fig.7: Decolorization of dyes with respect to varying temperature.

Effect of static and shaker condition

The strain *Streptococcus* sp.VBH1 showed significant decolorization profiles for the reactive azo dyes in both shaker (190rpm) and static condition, but it was observed that the decolorization percentage was higher on the shaker as compared to static condition. The isolate *Streptococcus* sp.VBH1 decolorized 53.30 and 57.21% of the Reactive red 31and Reactive yellow 81, respectively on the static condition whereas on the shaker (190rpm) higher decolorization of 62.23 and 63.12% for the Reactive red 31 and Reactive yellow 81, respectively after 24h incubation (Fig.8). These results concluded that decolorization activity was significantly correlated with the dissolved oxygen concentration which must create the micro-aerophillic environment in the glass vessel for the aerobic bacteria bringing about the decolorization of dyes at a faster rate (Hu, 1998).

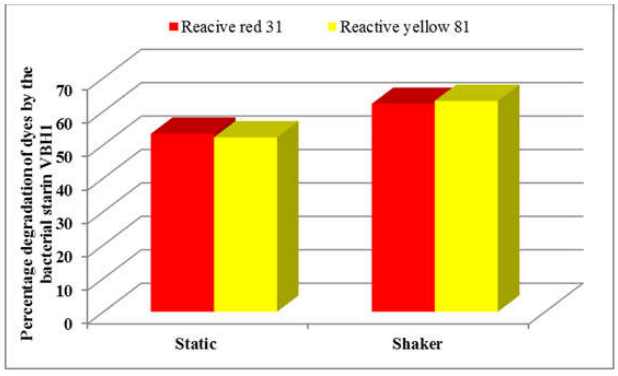


Fig.8: Illustration of dye decolorization with respect to static and shaker condition.

Effect of different inoculum and dye concentrations

The effect of different inoculum concentration and dye concentration were studied for the bacterial isolate

Streptococcus sp.VBH1. The decolorization of 62.23 and 63.12% was recorded for the 150mg/l of reactive red31 and Reactive yellow 81,respectively with 100µl inoculum concentration whereas 100 % decolorization was recorded with the use of 300µl inoculum concentration for the Reactive red 31 and Reactive yellow 81 (Fig.9). This can be well explained on the basis that for 100µl inoculum concentration, 150mg/l dye concentration acted as inhibitor for the normal bacterial growth and resulted in less decolorization whereas 300µl inoculum able to overcome the inhibiting effect of dyes resulted in significant degradation of 150mg/l dyes as substrate.

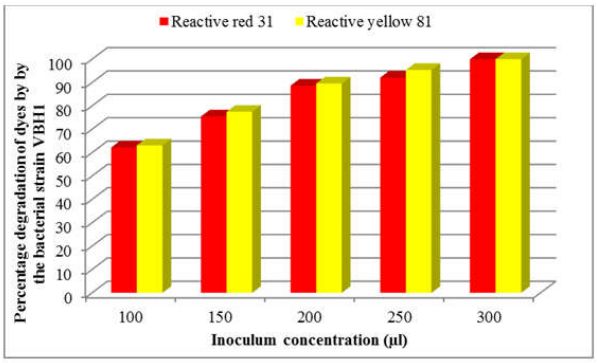


Fig.9: Monitoring of dye decolorization with respect to different inoculum concentration.

Also the study also recorded that with increase of the initial dye concentration, 150mg/l, 200mg/l, 250mg/l, 300mg/l and 350mg/l for 100µl inoculum, the decolorization efficiency of the inoculum over the same time interval decreased. At 150mg/l concentration the isolate showed 100% decolorization which decreased to 32.23and 40.12 % for the 350mg/l of Reactive red 31and Reactive yellow 81, respectively (Fig.10). It was documented that the concentration of dye substrate can influence the efficiency of dye removal through a combination of factors including the toxicity of the dye at higher concentrations, and the ability of the enzyme to recognize the substrate efficiently at the very low concentrations (Pearce et al., 2003).

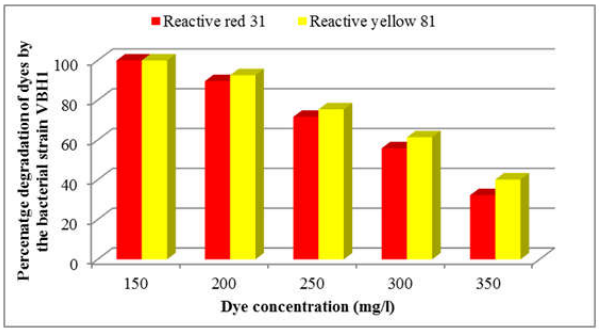


Fig.10: Decolorization efficiency with respect to different dye concentration.

Effect of total physiochemical parameters

Using all of the optimized parameters, the decolorization of 150mg/l Reactive red 31 and Reactive yellow 81 was calculated with respect to time. The figure 11 shows that the bacteria degraded 13.13% (0.16mg/ml) and 14.76% (0.17mg/ml) of Reactive red 31and Reactive yellow 81 within 2h whilst 100% (3.17 mg/ml) degradation at 20h for Reactive yellow 81 and 100% (3.28mg/ml) at 22h for Reactive red 31 (Fig.11). This proves that the combine effect of optimized parameters attributed to the decolourization of the dyes by isolated bacteria *Streptococcus* sp.VBH1.

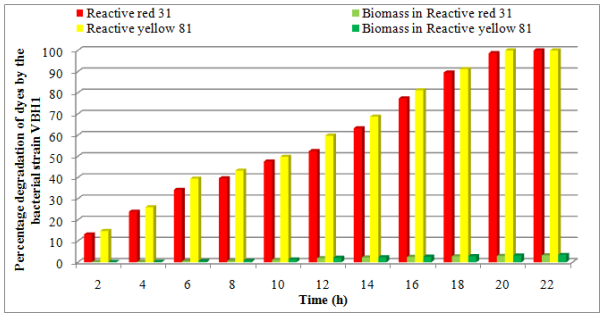


Fig.11: Monitoring of decolorization of dyes with optimized parameters with respect to time.

Analysis of decolorization mode of *Streptococcus* sp.VBH1 and SEM analysis

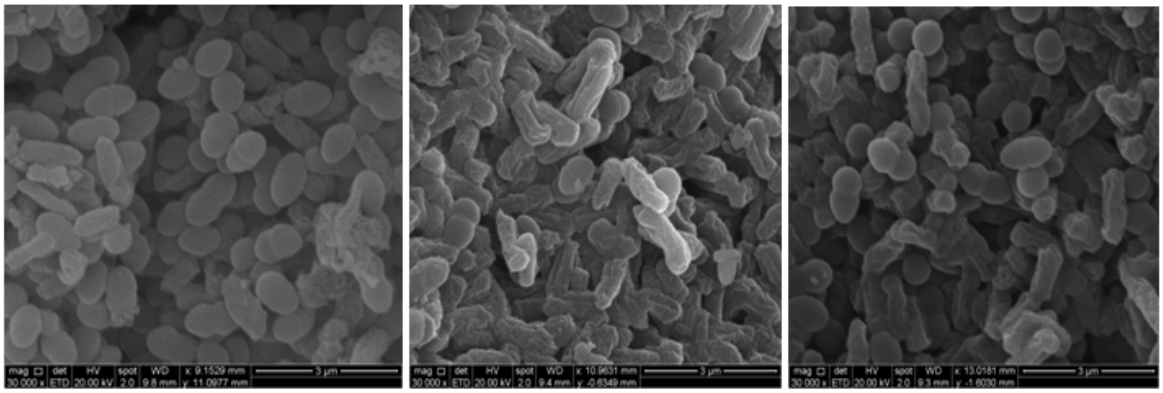
In the heat-killed bacterial cultures, only 8.76 and 9.22% decolorization of Reactive red 31and Reactive yellow 81 was recorded after 24h incubation, which might be due to the adsorption by dead bacterial cells and this was also confirmed by the presence of colored cell pellets. In the control culture 100% decolorization of reactive red and reactive yellow was obtained in 24h and the cell pellets were not pigmented. The scanning micrographs showed that there was no change in the morphology of the bacteria whereas membrane folding was observed for the bacterial cells taken from the dye treated media depicted in Fig.12 (A, B, C).

Microbial toxicity and phytotoxicity study

The inhibition zones were observed with control Reactive red 31 and Reactive yellow 81 with all bacterial strains studied for microbial toxicity whereas the dye degraded products by the isolate *Streptococcus* sp. VBH1 did not show any growth inhibition (Table 1). This inferred that degradation of respective dye by the isolates did not result in any metabolic products more toxic than the parent dye. During phytotoxicity study, germination (%) of the both *Triticum aestivum* and *Phaseolus mungo* seeds was less with parent dye as compared to their degraded products and distilled water. In comparison to the degraded products, untreated respective dyes, significantly affected the length of the plumule and radical, verified no toxicity of the degraded products (Table 2). The non-toxicity of the dye degraded products can be suggested because of hydroxylase and oxygenase produced by the bacteria (Pandey et al., 2007).

Conclusions

This study has been aimed at isolating and characterizing the potential dye degrading bacteria from the local paper industry waste water. It was inferred that aerobic bacterial isolate *Streptococcus* sp.VBH1showed 100% degradation of dyes with optimized physiochemical parameters. Since, the dye degraded products have no toxic effect on the microbes and



A B C

Fig. 12. Scanning micrographs of *Streptococcus* sp. VBH1 taken from normal nutrient media (A), Reactive red 31 amended nutrient broth (B) and Reactive yellow 81 amended nutrient broth (C)."

Table 1: Microbial toxicity study Reactive red 31, Reactive yellow 81 and its degraded product

Dyes		Diameter of zone of inhibition in growth of Bacteria (cm)		
		Staphylococcus aureus	*Aspergillus niger*	*Pseudomonas aeruginosa*
Reactive red 31	Control (500 ppm)	0.6	0.7	0.3
	DP-VBH1 (500 ppm)	NI	NI	NI
	Control (1000 ppm)	1.2	0.9	0.8
	DP-VBH1(1000 ppm)	NI	NI	NI
Reactive yellow 81	Control (500 ppm)	0.8	0.3	0.3
	DP-VBH1 (500 ppm)	NI	NI	NI
	Control (1000 ppm)	1.2	0.6	0.5
	DP-VBH1 (1000 ppm)	NI	NI	NI

* NI- No Inhibition, DP = Degraded product

Table 2: Phytotoxicity study of Reactive red 31, Reactive yellow 81 and their degraded product by the *Streptococcus* sp.VBH1

Selected plants used (10 seeds in each plate)			Parameter studied		
			Germination (%)	*Plumule (cm)	*Radical (cm)
Triticum aestivum	Reactive red 31	H_2O	90	15.68 ±1.21	8.56 ± 1.0
		Dye	70	3.5 ± 0.25	1.2 ± 0.45
		DP-VBH1	90	7.13 ± 0.57	3.97 ± 0.76
	Reactive yellow 81	H_2O	100	15.68 ±1.21	8.56 ± 1.0
		Dye	75	5.4 ± 0.39	1.7 ± 0.20
		DP-VBH1	90	6.35 ± 0.65	4.18 ± 0.54
Phaseolus mungo	Reactive red 31	H_2O	100	2.12 ± 0.29	1.82 ± 0.24
		Dye	75	1.45 ± 0.25	0.49 ± 0.04
		DP-VBH1	95	1.82 ± 0.33	1.82 ± 0.42
	Reactive yellow 81	H_2O	100	2.12 ± 0.29	1.82 ± 0.24
		Dye	80	0.88 ± 0.10	0.24 ± 0.04
		DP-VBH1	86	1.62 ± 0.23	1.41 ± 0.32

* NI-No Inhibition, DP = Degraded product, H_2O = UV treated water. *Plumule and *Radical length from all seed's was taken in average of germinated seeds

plants; the conversion mechanism of respective enzyme on the dye is likely based on the reduction and cleavage of the azo-bond resulting in the formation and accumulation of colorless non-toxic products rather than aromatic amines. This implies that maximum degradation of a range of different dyes can be meted out by employing the potential bacterial strains from the local industrial wastes using optimized physiochemical parameters as it renders the ability to bio transform the toxic dyes into non-toxic products without any additional treatment. This study further recommends the identification and purification of the enzymes and their kinetics involved in the degradation of dyes by the isolates and exploitation of potential bacterial consortium from the local industrial waste in the treatment of dye polluted waste water which would be cost-effective for the treatment and eradication of the dye pollution from the water bodies.

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