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Original Research

Decolorization of Textile Dye Reactive Blue 221 by Bacteria Isolated from Anthropogenic Dye-Contaminated Soil

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Abstract

The present study was conducted to evaluate the degradative potential of indigenous bacterial isolates for Reactive Blue 221. The two strains proficient in decolorization (>80%) were isolated from a waste disposal site by enrichment technique. Under optimized conditions, *Pseudomonas* sp. BDS 2 displayed efficient decolorization activity of 94.4% in Minimal Salt Medium supplemented with 50 mg/l of RB 221 after 96 h of incubations, whereas 88.5% of color was removed by *Alcaligenes sp.* BDS 9 by the end of 112 h. UV-Visible and FTIR spectroscopy confirmed that in the case of *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9*,* decolorization was the ultimate consequence of biodegradation and adsorption, respectively. Toxicological studies revealed the nontoxic nature of degradative metabolites.

Keywords: Decolorization, Reactive Blue 221, *Pseudomonas* sp., *Alcaligenes* sp.

Introduction

Wastewater is a concerning environmental pollutant. In the industrial sector, wastewater generated by textile industries poses a great environmental threat [1] and is among the prominent sources of polluted effluents both in terms of volume and composition as it makes use of large quantities of water and a variety of dyes in the dyeing process [2]. The number of commercially available dyes is more than 100,000, and the world's annual production of colorants is more than $7x10^5$ tons [3-5].

Some quantity of dye is lost during its application, and its discharge in surrounding water bodies can vary from 2 to 50% for basic and reactive dyes, respectively, and thus contribute to severe contamination of surface and ground waters [6]. Inappropriate methods of disposal in water bodies greatly influence the physicochemistry of water by reducing the sunlight penetration that affects the photosynthetic activity. This in turn leads to decreased levels of dissolved oxygen, thereby negatively affecting the aquatic flora and fauna. The majority of dyes and their degradation products (i.e. aromatic amines) are toxic, mutagenic, and carcinogenic [7].

Reactive dyes constitute an important class of textile dyes. Due to their intensive application in the textile industry almost 45% of annually manufactured textile dyes belong to this class. So it can be concluded that reactive dyes constitute a major proportion of textile wastewaters. Due to their extensive use, highly water-soluble nature, and limited biodegradability in an aerobic environment, these dyes are among the most problematic and challenging agents in textile effluents [8].

Over the last few decades treatment of textile wastewater has emerged as a challenge [9]. Various methods, i.e. physical, chemical, and biological, are used

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for removal of dyes from textile effluents. Due to chemical stability of synthetic dyes, conventionally practiced wastewater treatment techniques have not contributed to promising outcomes [3]. But biological methods are usually preferred over physical and chemical methods because they are specific, less energy consuming, cost effective, and eco-friendly as they bring about partial or complete biotransformation of organic pollutants to stable and nontoxic end products [10, 11]. A large number of different microorganisms such as bacteria, fungi, yeasts, algae, and actinomycetes are capable of decolorization and even complete mineralization of a wide range of dyes (especially azo dyes) [7, 10].

Bacteria are among the most frequently used microorganisms for the bioremediation of textile wastewaters as they can rapidly grow, are adaptable to extreme conditions of temperature and salinity, are easy to cultivate, and produce different types of oxidoreductases [12, 13]. Normally bacterial decolorization is relatively faster than fungal [2]. There are many reports of dye decolorizing bacteria either in pure culture or in consortium [2-6, 10, 12-14]. Dye decolorizing bacteria can be isolated from a variety of anthropogenic polluted environments, e.g. soil, water, contaminated food items, human and animal waste matter, etc. In addition, colored effluents released from the dye manufacturing and textile industries are also an important reservoir for the isolation of such types of bacteria [15]. In the present study the dye degradation potential of bacteria isolated from the waste disposal site of a textile industry was evaluated. Secondly, the physiological conditions of these potent bacterial isolates were optimized for the degradation of Reactive Blue 221. Finally, toxicity of metabolites produced from biodegradation of Reactive Blue 221 was assessed by their effect on flora and fauna.

Material and Methods

Chemicals and Dyestuffs

All chemicals and microbiological media were purchased from Oxoid (USA) and Sigma-Aldrich (USA) and were of analytical grade. The textile dye Reactive Blue 221 (RB 221) (Fig. 1) subjected to investigation was courteously provided by the local textile industry.

Isolation of Microorganisms

A soil sample was collected from the textile industry waste disposal site in Lahore, Pakistan. Bacterial strains having potential for the degradation of textile industry dye RB 221 were isolated from the soil sample through enrichment technique using RB 221 (50 mg/l) supplemented nutrient broth and MSM media. The composition of MSM used was (g/l): Na_2HPO_4 3.6, KH_2PO_4 1.0, $MgSO_4$ 1.0, $(NH_4)_2SO_4$ 1.0, $CaCl_2 \cdot 2H_2O$ 0.1, and Fe (NH_4) citrate 0.01. To this media, trace element solution (10 ml/l) of the following composition was added: $ZnSO_4 \cdot 7H_2O$ 10.0, $MnCl_2 \cdot 4H_2O$ 3.0, $CoCl_2 \cdot 6H_2O$ 1.0, $NiCl_2 \cdot 6H_2O$ 2.0, $Na_2MoO_4 \cdot 2H_2O$ 3.0, H_3BO_3 30.0, and $CuCl_2 \cdot 2H_2O$ 1.0. Final pH of the medium was adjusted by pH meter (Sartorious professional meter PP15) to 7.0 using 1N HCl and 1N NaOH solution [4].

Screening for Decolorization of RB 221 by Bacterial Isolates

In order to select the most efficient RB 221 dye-degrading bacteria, the isolates were subjected to screening in liquid medium (MSM). Therefore, an inoculum of 10 ml of absorbance (A_{600}) at 600 nm was used to inoculate 90 ml of MSM supplemented with 50 mg/l of RB 221 dye, and media was dispensed in 250 ml flask Erlenmeyer flasks. The flasks were incubated under shaking (150 rpm) as well as static conditions at 37ºC for eight days.

The aliquot (3 ml) of culture media was aseptically withdrawn after every 24 h and centrifuged at 14,000 rpm for 15 min. The supernatant obtained after centrifuging was read at 615 nm i.e. λ_{max} of RB 221 using a UV-Visible spectrophotometer (Agilent 8453). Biotic and abiotic controls were carried out side by side. The decolorization activity (%) of each isolate was calculated using the following formula [16].

$$\text{Decolorization (\%)} = \frac{(I - F)}{I} \times 100$$

...where I = initial absorbance of coloured sample and F = Final absorbance of the decolorized sample.

Taxonomic Characterization of Bacterial Isolates

The most proficient RB 221 decolorizing isolates were selected and identified using established microbiological methods, including colony morphology, Gram's staining, and standard conventional biochemical tests according to Bergey's Manual of Systematic Bacteriology [17], followed by the use of an API Kit identification system (API 20E and 20NE, Biomerieux).

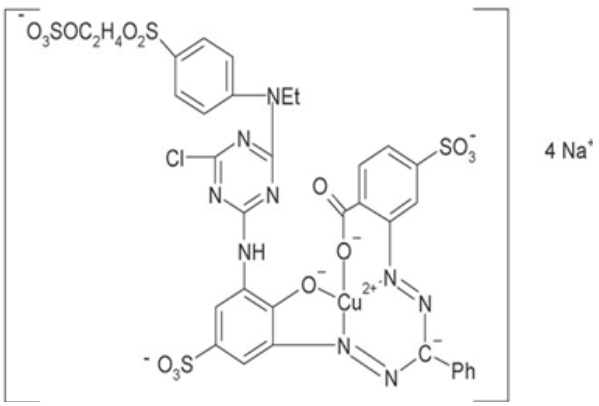

Fig. 1. Chemical structure of C.I. Reactive Blue 221 [14].

Effect of Different Environmental Parameters on Dye Decolorization

Decolorization potential of both isolates for RB 221 was investigated at different pH (5-9); temperature (30-45ºC); inoculum size i.e. 2-10% (v/v); carbon sources (glucose, lactose, sucrose, starch, and mannitol); nitrogen sources, including organic and inorganic (yeast extract, peptone, potassium nitrate, ammonium sulphate, sodium nitrite); concentrations of glucose, i.e. 0-4 g/l, yeast extract i.e. 0-2 g/l; different initial RB 221 dye concentration, i.e. 50-300 mg/l, NaCl i.e. 0-100 g/l; and metal compounds (10 mg/l), including $CuCl_2$, $CoCl_2$, $CdCl_2$, $AgNO_3$, and $HgCl_2$. The classical method of process optimization was adopted by altering one parameter at a time in each experiment while keeping the previously optimized once as constant. Decolorization efficiency at the respective optimized conditions for each of the selected isolates was determined. In addition, the relationship between the growth and decolorization processes was also monitored. For this, optical density (OD) at 600 nm was measured using a UV-Vis spectrophotometer (Agilent 8453).

Biodegradation Analysis

UV-vis absorption spectrum of the control and treated dye samples was studied in the range of 200-800 nm using a UV-Visible spectrophotometer (Agilent 8453). Dye concentration was monitored at 615 nm i.e. λ_{max} of RB 221 using standard dye solution.

FTIR analysis of control and decolorized RB 221 samples (Perkin Elmer, spectrum 65 FTIR spectrometer, equipped with ATR) was done and transmission spectrum was recorded. This analysis was performed in the mid-IR region of 650-4000 cm^{-1} with 16 scan speed.

Toxicity Study

By using *Raphanus sativus* (radish) seeds, phytotoxicity of the treated samples was determined [18] and cytotoxicity of samples was tested by brine shrimp (*Artemia salina*) lethality assay [19].

Results

Isolation and Taxonomic Characterization of Dye Decolorizing Bacteria

Twenty-seven morphologically distinct colonies isolated from sludge sample were screened by incubating under shaking and static conditions. The isolates under static condition showed better decolorization as compared to shaking. Two bacterial strains with the potential of up to 80% decolorization were selected and subjected to identification and optimization. On the basis of their cultural and biochemical properties and gram-staining reaction, isolates were identified and designated as *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9 (Table 1).

Effect of Different Environmental Parameters on Dye Decolorization

Decolorization process depends on the presence and availability of co-substrate that acts as an electron donor for dye reduction. In the presence of glucose as a co-substrate, *Pseudomonas* sp. BDS 2 gave 61.3% decolorization, while *Alcaligenes* sp. BDS 9 showed up to 65% color removal among various carbon sources (0.1 g/l) tested. With all other carbon sources the rate of decolorization was not significant (Fig. 2a). Results of effective glucose concentration evaluation depicted that in the control (0 mg/l glucose) the extent of color removal was greatly inhibited and an insignificant decolorization percent, i.e. 33.1 and 47.7%, was observed in the case of *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9, respectively. With an increase in glucose concentration a progressive increase in color removal was observed. At 1 g/l glucose concentration, maximum decolorization of 90.8 and 93.9% was attained in the case of *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9, respectively (Fig. 2b).

Nitrogen sources are considered essential medium supplements for the reduction of dyes by microorganisms, so different organic and inorganic nitrogen sources were evaluated for the decolorization process. *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9 showed best

Table 1. Results of Gram's staining and biochemical tests.

Strains	Microscopic Examination				Biochemical Test												
					Fermentation			SIM									
	Color	Shape	Arrangement	Gram's Reaction	Sucrose	Dextrose	Lactose	H_2S Test	Indole Test	Motility Test	TSI Test	MR Test	VP Test	Citrate Test	Catalase Test	Oxidase Test	Identified strains
BDS 2	Pink	Rods	Scattered short rods	–	–	–	–	–	–	±	K/ NC	–	–	+	+	+	*Pseudomonas* sp.
BDS 9	Pink	Rods	Coccobacilli	–	–	–	–	–	–	+	–	–	–	±	+	+	*Alcaligenes* sp.

Key: (+) = Positive; (−) = Negative; (±) = Variable reaction; A = Acid production; K = Alkaline reaction; H_2S = Hydrogen sulphide; K/NC = Red/ No change; K/A = Red/yellow.

decolorization in the presence of yeast extract as respective 93.9 and 95.3% color removal was observed. With all other nitrogen sources, decolorization activity is less pronounced (Fig. 2c). With a progressive increase in concentration of yeast extract from 0-1 g/l, a linear increase in decolorization rate was observed and 1 g/l was found as optimum concentration, as 89-91% decolorization by the selected isolates was detected at this concentration (Fig. 2d).

The pH of culture medium is another important factor and plays a critical role for the optimal physiological performance of microbial cells and has a marked effect on cell growth. For decolorization process an optimal pH was selected from the range of 5-9. At pH 7 best decolorization results (70.6%) were observed by *Alcaligenes* sp. BDS 9, while at pH 8 the decolorization percentage was reduced to 62.8%. With *Pseudomonas* sp. BDS 2, 63.3 and 62.7% decolorization was achieved at pH 7 and 8, respectively. At pH 5, 6, and 9, further reduction in percentage decolorization of both isolates was observed (Fig. 3a).

Incubation temperature is a critical process parameter and it varies among different microorganisms, and slight changes in temperature may affect growth and ultimately rate of decolorization. In the case of *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9, highest color removal was achieved at 35°C (94.4%) and 37°C (84.4%), respectively. For both the isolates, a decline in the decolorization percentage was observed at higher temperature ranges (Fig. 3b).

Dyes, being toxic compounds, have an inhibitory effect on the decolorization process at higher concentrations

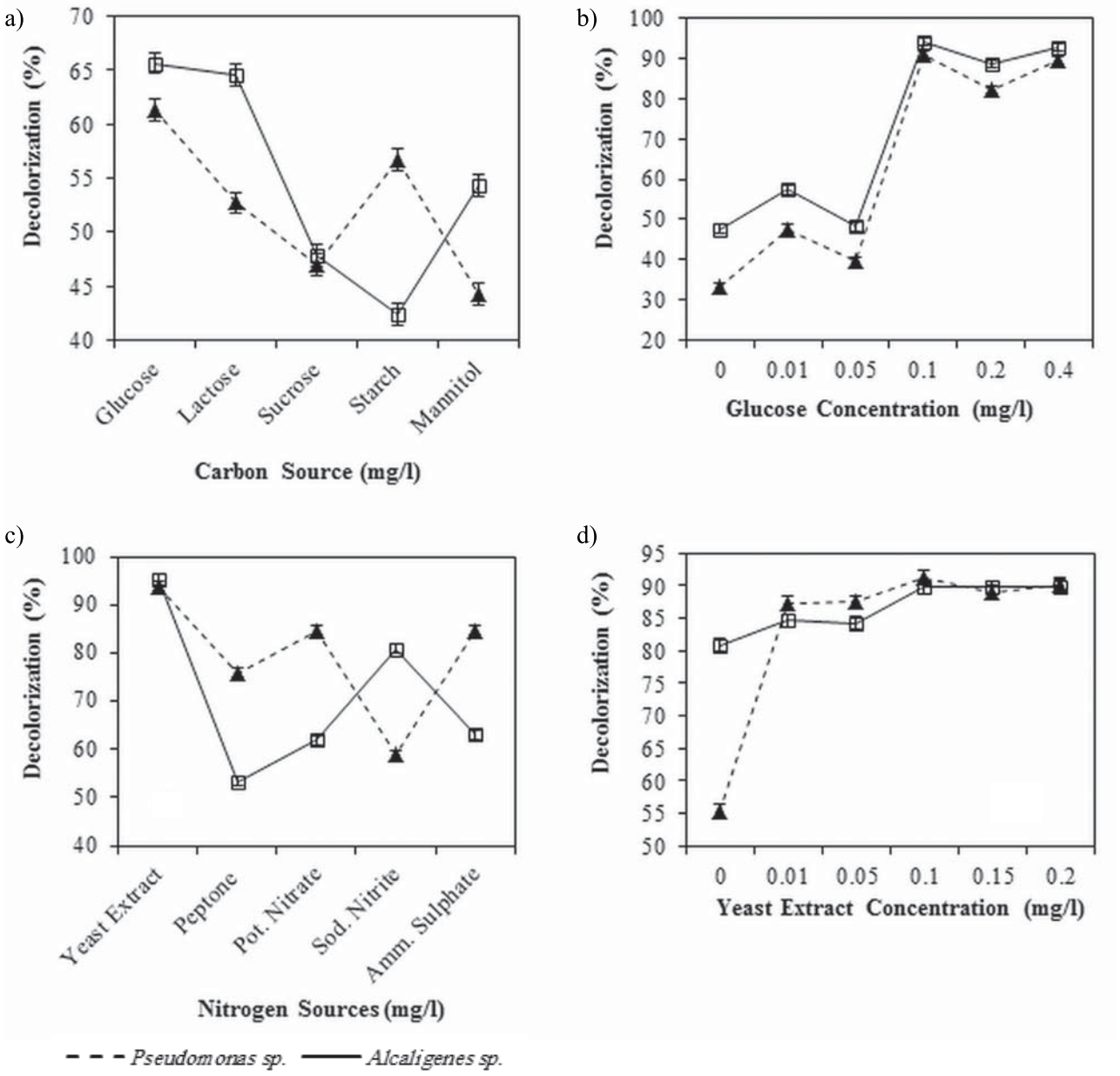

Fig. 2. Optimization of nutritional requirements: (a) carbon source, (b) glucose concentration, (c) nitrogen source, (d) yeast extract concentration.

so the influence of different dye concentrations was evaluated by taking 100, 200, 300, 400, and 500 mg/l of RB 221. At an initial concentration of 50 mg/l, 94.1 and 93.6% decolorization was displayed by the *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9, respectively. At 100 mg/l dye concentration, more than 90% decolorization was attained. A progressively decreasing trend in decolorization rate was observed on further increase in dye concentration (Fig. 3c).

Textile effluent generally contains chloride salts of sodium and potassium, which are frequently employed for salting out of dyes and are therefore discharged into the effluent. Hence, decolorizing efficiency of both isolates over a range of NaCl concentrations (0 to 100 mg/l) was assessed under static conditions at 37ºC. Results indicated that in the control medium (0 mg/l NaCl), 91.2 and 93.6% decolorization was observed in the case of *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9, respectively. By increasing concentration from 0-20 mg/l, no severe inhibitory effect was noticed as decolorization was still more than 85%. However, *Alcaligenes* sp. BDS 9 displayed good decolorization (83.5%) results, even at 40 g/l NaCl concentration (Fig. 3d).

Generally decolorization rate increases with increases in inoculum size. In the present study the effect of inoculum on decolorization was checked and it was found that at an inoculum size of 2% only 18-22% decolorization was achieved with both strains. Upon further increase in inoculum sizes to 4, 6, and 8%, progressive increases in the decolorization ability (31-33%, 37-45 and 50-54%) were observed, and maximum decolorization (63-64%) occurred at an inoculum size of 10% (Fig. 3e).

Textile effluents frequently contain different metal compounds along with different salts, which can interfere with the microbial mediated decolorization process. To check their effect the medium was supplemented with metal compounds. Results showed that *Pseudomonas* sp. BDS 2 displayed a variable response as it exhibit decolorization percentage with $CuCl_2$ (97.50%), $CdCl_2$ (95.5%), $CoCl_2$ (66.8%), $AgNO_3$ (9.4%), and $HgCl_2$ (13.4%). While *Alcaligenes* sp. BDS 9 was found to be affected by all the metal compounds with a decolorization percentage of $CoCl_2$ (72.7%), $CuCl_2$ (64.8%), $CdCl_2$ (26.4%), $AgCl_2$ (22.8%), and $HgCl_2$ (1.8%) (Fig. 3f).

At optimized cultural conditions a decolorization percentage of 94.4% was achieved with *Pseudomonas* sp. BDS 2. A gradual increase in rate of decolorization resulted, with maximum activity after 96 h, later on no significant increase was observed. Comparison of growth pattern with that of decolorization showed a relative trend over a period of 64 h. After this the growth leveled off but decolorization rate continued to increase for 96 h

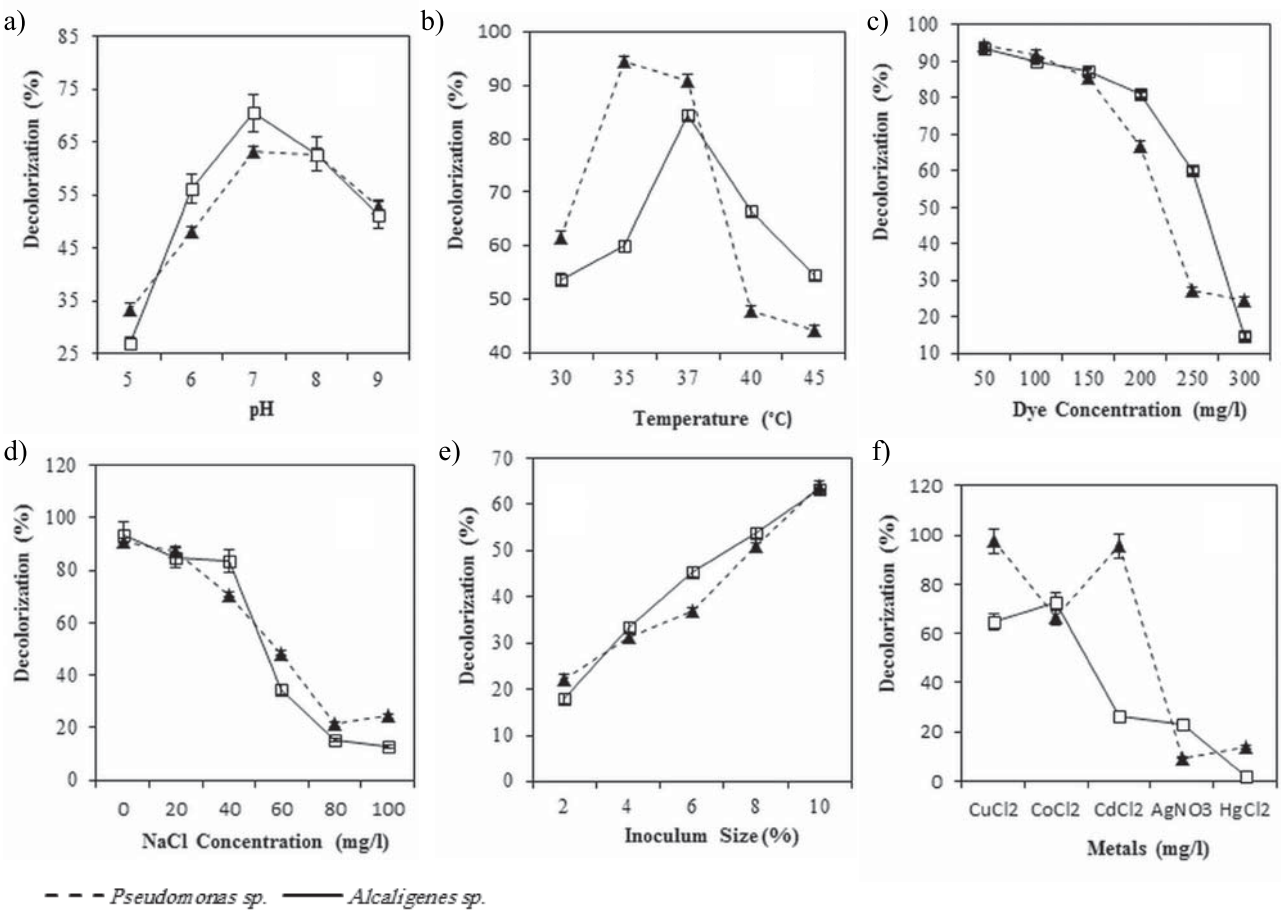

Fig. 3. Optimization of (a) pH, (b) temperature, (c) dye concentration, (d) NaCl concentration, (e) inoculum size, (f) metals.

(Fig. 4a). The highest decolorization percentage obtained with *Alcaligenes* sp. BDS 9 at its optimal conditions was 88.5%. The rate of decolorization progressively increased and reached its climax after 112 h of incubation. On extended incubation no further increase in the decolorization percentage was observed (Fig. 4b).

Biodegradation Analysis

In the UV-Vis spectrum (200-800 nm) of the control dye sample, a single peak in the visible region at 615 nm was observed, corresponding to the λ_{max} of RB 221. In addition, two intense peaks in the UV region – one near 250 nm and other near 300 nm – were observed. The decolorized samples obtained after treatment with *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9 had a similar type of spectrum. In these spectra the peak at 615 nm was decreased without showing any shift in λ_{max} until complete decolorization. Additionally, one peak observed in the UV region near 300 nm became less broad compared to the control sample (Figs. 5a, b).

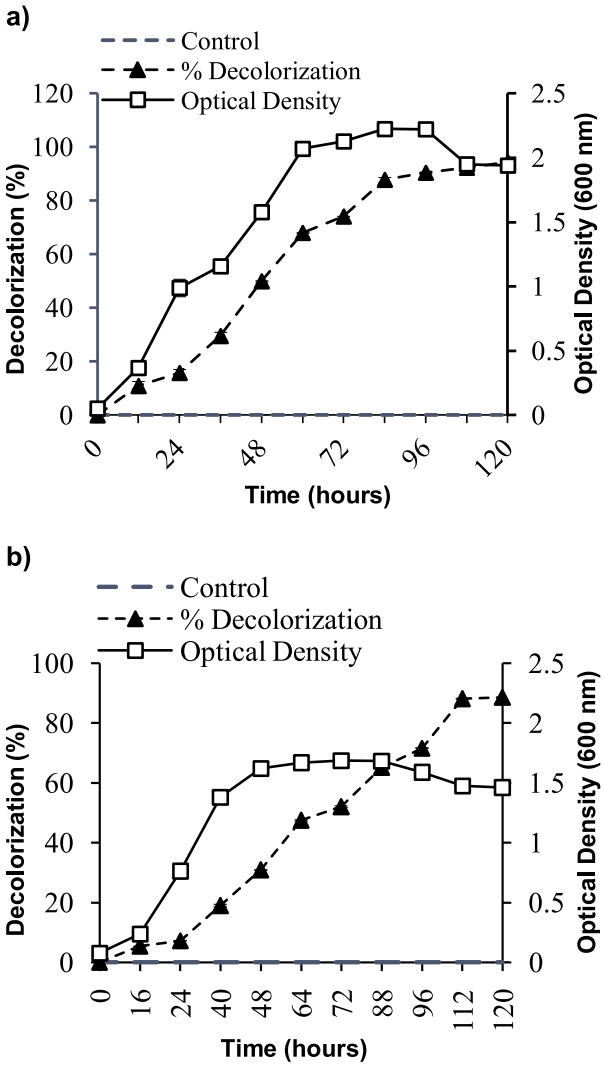

Fig. 4. Relationship between decolorization and growth kinetics of (a) *Pseudomonas* sp. BDS 2, (b) *Alcaligenes* sp. BDS 9.

FTIR spectrum obtained for control dye sample RB 221 showed peaks at 3264.74 cm^{-1}, 2931.26 cm^{-1}, 2529.81 cm^{-1}, 1842.97 cm^{-1}, 1635.18 cm^{-1}, 1511.66 cm^{-1}, 1314.11 cm^{-1}, 1154.54 cm^{-1}, 1407.11 cm^{-1}, 1038.28 cm^{-1}, 1027.47 cm^{-1}, 972.04 cm^{-1}, and 735.68 cm^{-1} (Fig. 6a). In the case of the *Pseudomonas* sp. BDS 2-treated sample, FTIR analysis showed some new peaks at 3012.99 cm^{-1}, 2156.09 cm^{-1}, 1448.34 cm^{-1}, 1335.57 cm^{-1}, and 1313.10 cm^{-1} (Fig. 6b). In addition to these, two new peaks at 2047.43 cm^{-1} and 1087.49 cm^{-1} were observed for *Alcaligenes* sp. BDS9 treated sample (Fig. 6c).

Toxicity Study

The use of untreated and treated dyeing effluents has a direct impact on the environment. Thus, it was of concern to assess the phytotoxicity and cytotoxicity of the dye before and after degradation. The results of relative sensitivity test of *Raphanus sativus* seeds to RB 221 and its degradation metabolites showed 55% germination with control dye sample. Whereas germination of 90 and 75% was observed with the treated sample of *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9, respectively. The mean root and shoot lengths obtained with most of the decolorized samples were higher than those obtained with control dye (Table 2).

Cytotoxicity assay revealed a decrease in toxicity of RB 221 after treatments. As with the control dye sample the percentage mortality of brine shrimp larvae was 40%. In the case of *Pseudomona*s sp. BDS 2 treated samples the percentage mortality was 10%, whereas no cell death was observed with *Alcaligenes* sp. BDS 9-treated samples (Table 3).

Discussion

The potential RB 221 decolorizing bacteria, i.e. *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9, were isolated from dye-contaminated sludge samples collected from the waste disposal site of a textile industry; such anthropogenically polluted sites provide a major likelihood for the presence of such microflora [20]. A total of 27 dye-decolorizing bacteria were isolated from the sludge sample through enrichment techniques. Similarly, seven bacterial strains were isolated from dye containing effluent [15] and 200 isolates from the textile industry wastewater sample [21]. Moreover, *Pseudomonas* sp. [2, 14, 22-26] and *Alcaligenes* sp. [27, 28] have been well documented for their dye decolorizing potential by several studies.

Results indicated that both the isolates showed up to 80% decolorization under static conditions, while agitated conditions showed poor results. Previously, various reports mentioned that no appreciable color removal was observed under shaking condition [4, 5, 29]. The low rate of decolorization under shaking condition could be due to inhibition of azo bond reduction by the presence of oxygen in the reaction mixture as it may compete with the azo bond

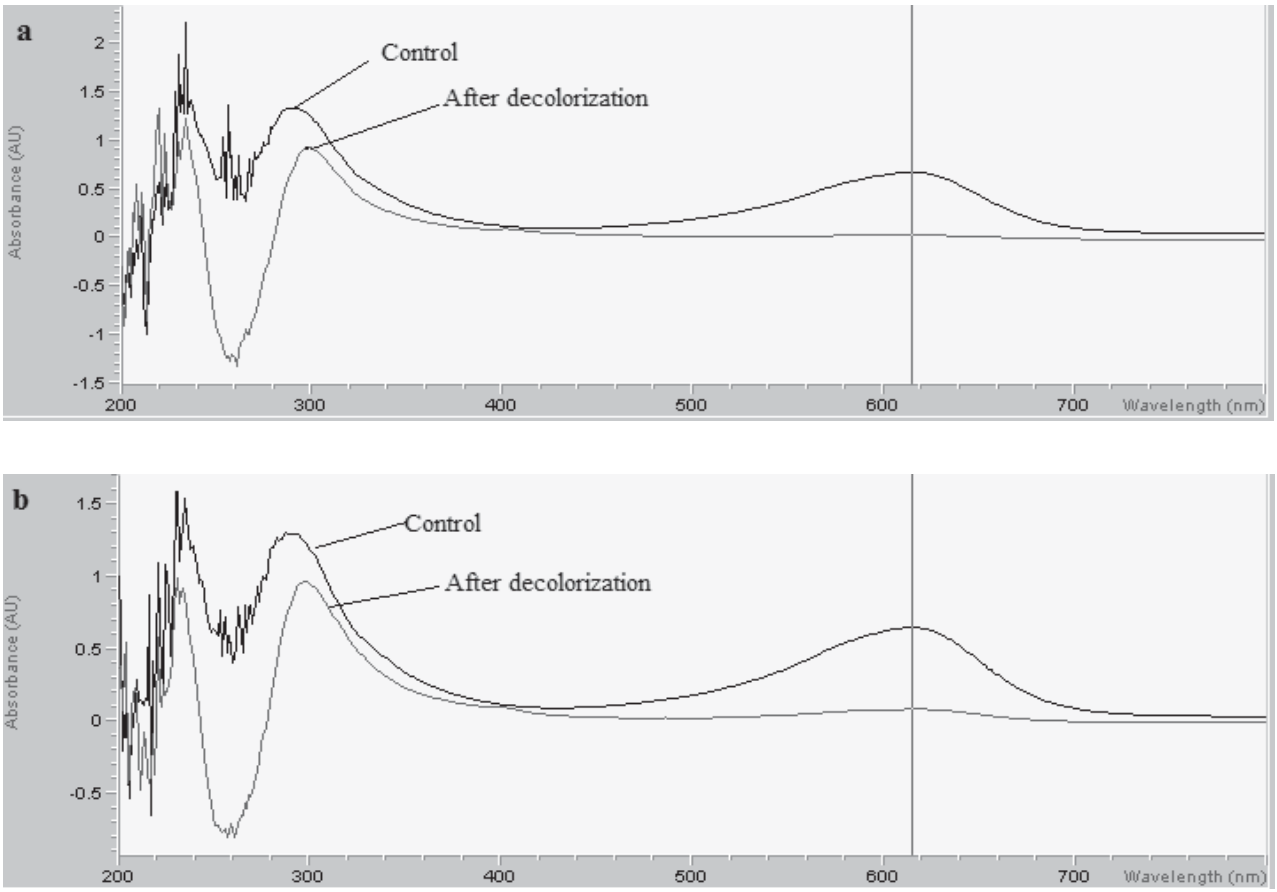

Fig. 5. UV-Visible spectra of RB 221 decolorization by (a) *Pseudomonas* sp. BDS 2, (b) *Alcaligenes* sp. BDS 9.

for NADH [30, 22]. Conversely, under static (anaerobic as well as microaerophilic) conditions reduction of azo bond by anaerobic, facultative anaerobic, and microaerophilic bacteria is rather easy due to low oxygen content in such environments [31].

The optimum pH for decolorization of RB 221 was 7. Comparable results were obtained while studying the decolorization of Scarlet R, as the highest percentage of color removal was observed within the pH range of 7-8 [32]. The *Pseudomonas* sp. GM3 showed its highest activity in narrow pH range of 7-8 and its activity dropped by about 50% when pH value deviated from the slightly alkaline level by 0.5 units [23]. The dye treatment is a pH-sensitive process and bacteria normally display best decolorization at neutral or basic pH [33].

Microbial mediated decolorization of azo dyes is highly affected by temperature as it influences their growth and enzyme activity [34]. Among different temperatures, 37ºC was found as optimum for this process. At higher temperature values, i.e. 40 and 45ºC, a decline in the decolorization percentage was observed. However, our findings are complimentary to another report, where optimum temperature for decolorization of Remazol black B by *Kluyveromyces maxianus* IMB3, was found to be 37ºC [35]. Similarly, at temperature values above the optimum range, a significant decline in the removal of Orange MR (300 ppm) by *Micrococcus* sp. was observed [36]. Loss of cell viability or denaturation of azoreductase enzyme can be the two possible reasons for a decrease in decolorization percentages at higher temperatures [30].

Among different carbon sources, maximum decolorization results were obtained with glucose. Likewise, for decolorization of Orange II by *Geobacillus stearothermophilus*, glucose was found as the best carbon source [37]. There exists a great diversification in the use of carbon sources by different microbes and thus it highly affects their decolorization rate. Glucose is usually found as the most suitable carbon source for the bacterial treatment of dyes as it is the simplest and thus most preferable carbon source [34, 38]. Actually, carbon sources perform a dual function by acting as a carbon source as well as electron donor for azo bond cleavage [5].

Nitrogen source is another crucial parameter [6]. Among different nitrogen sources evaluated during the study, yeast extract was found as being optimal for decolorization of RB 221. Similarly, yeast extract was the best nitrogen source for the decolorization of Reactive Red 198 by *Aeromonas hydrophila* [39].

Besides type, concentration of the carbon source is also crucial for the decolorization of azo dyes [40]. The present study demonstrated a direct correlation between glucose concentration and color removal percentage, and best decolorization performance was observed at 1 g/l glucose. However, further increases in glucose concentration

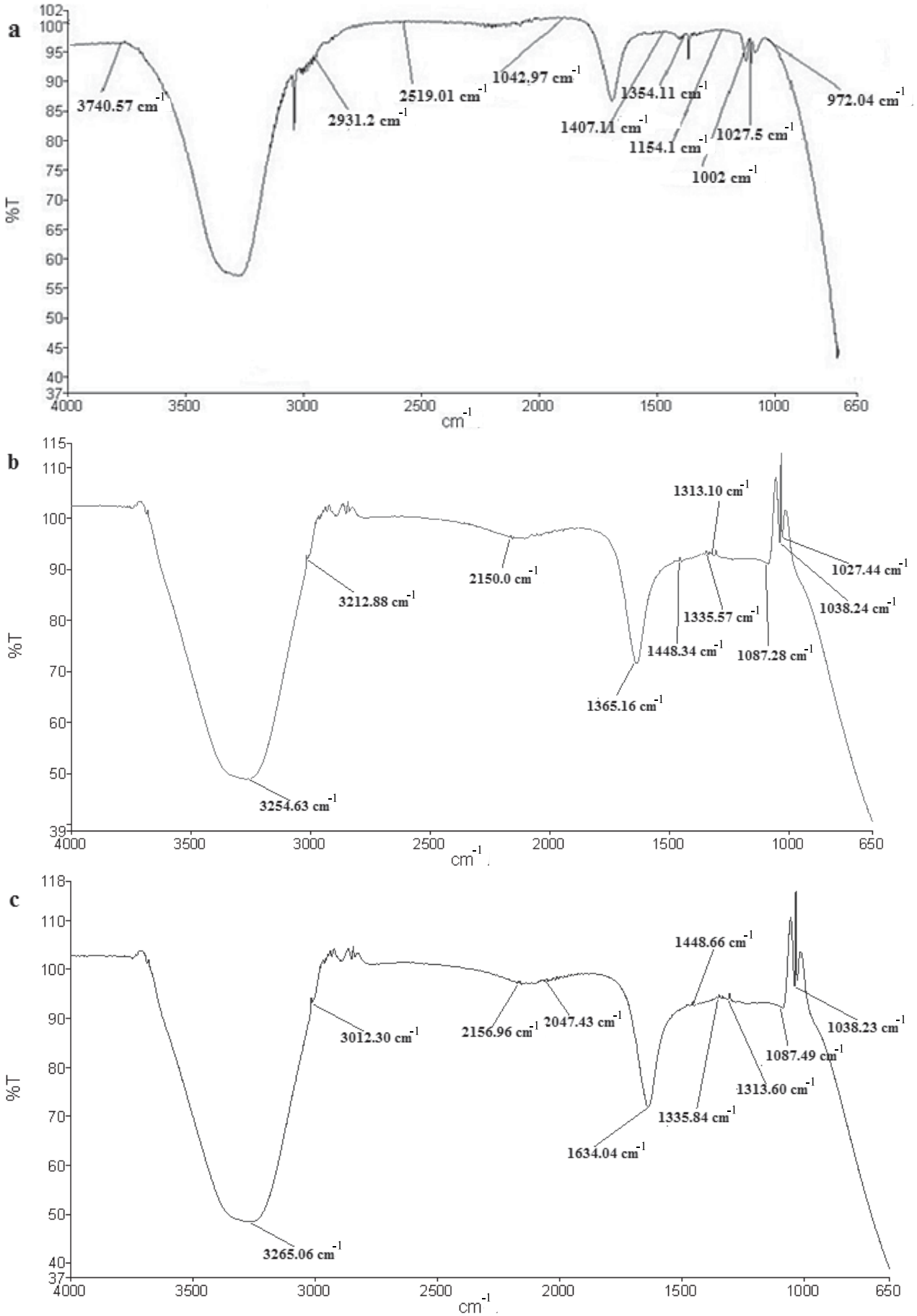

Fig. 6. FTIR spectrum of (a) control dye sample, (b) *Pseudomonas* sp. BDS 2-treated sample (c) *Alcaligenes* sp. BDS 9-treated sample.

Table 2. Phytotoxicity of treated samples.

Samples	Seeds sown (n)	Seeds germinated (n)	% of seed Germination	Mean root length (cm)	Mean shoot length (cm)
Positive Control	20	11	55	1.22±0.41	1.18±0.69
Negative control	20	19	95	1.88±0.52	1.21±0.55
Pseudomonas sp. BDS 2.	20	18	90	1.49±0.39	1.08±0.31
Alcaligenes sp. BDS 9	20	15	75	1.31±0.25	0.63±0.58

caused slight negative impact on decolorization. Decreases in the decolorization extent of *Kocuria rosea* MTCC 1532 for Malachite Green was demonstrated at higher glucose concentrations [41]. This negative impact of higher glucose concentrations can be linked to the metabolic regulatory phenomenona known as glucose/catabolic repression. During this repression, the possibility for inhibition of cyclic-AMP dependent genes (including those that might be involved in encoding azoreductase enzyme for azo dye decolorization) is increased due to higher glucose content in the medium [24]. Additionally, this negative effect of glucose at higher concentrations might have been related to reduction in pH due to the accumulation of organic acids in the reaction mixture [39].

Moreover, combination of the variables (including glucose and yeast extract) lead to >90% decolorization of the azo dye Direct Black 22 [42]. In accordance with other studies [5, 43, 44], yeast extract confers complete decolorization of RV5 within 18 h, providing better decolorization efficiency compare to other nitrogen sources [38]. Organic nitrogen sources are usually deemed as essential for the regeneration of electron donors, i.e. NADH and yeast extract being an organic nitrogen source enhancing the dye decolorization ability of many types of bacteria [4, 5, 44]. Yeast extract performs a dual role by acting as a source of carbon as well as nitrogen in decolorization of various dyes by mixed bacterial consortium PDW [45]. In the present study, 1 g/l yeast extract concentration was found as optimum. Various researchers have reported 1 g/l yeast extract as the optimum concentration for decolorization of different dyes [35, 43].

Actual concentrations of reactive dyes in dye house effluent have been reported to range from 60-250 mg/l [46]. At various initial RB221 concentrations, both isolates exhibited variable patterns of dye decolorization. Maximum decolorization was observed with 50 mg/l concentration and a decline in the color removal extent was observed as the dye concentration progressively increased from 50-300 mg/l (Fig. 3c). The highest dye concentration tolerated by both isolates was 200 mg/l, at which >65 and >80% color removal was recorded in the case of *Pseudomonas* sp. BDS 2 and *Alcaligenes* sp. BDS 9, whereas the decolorizing capability of *Alcaligenes* sp. BDS 9 was retained even at 250 mg/l, as >60% color was removed at this concentration. Similarly, in a previous study, maximum dye concentration tolerated by *P. aeruginosa* CR-25 was 350 mg/l, at which 52% of Remazol Black was decolorized [25]. Another investigator reported a decline in the decolorization percentage of Reactive Red 195 by *Enterobacter* sp., as the dye concentration was increased from 50 to 100 ppm [47]. When dye concentration was increased from 10 to 30 mM, color removal by *Kurthia* sp. was reduced drastically [16]. The decrease in color removal percentage at higher dye concentrations can be linked to their toxic effects on the decolorizing bacterial strains [5, 46]. In addition, a disturbance in the dye-to-cell ratio may also occur, which in turn can block the active sites of azoreductase enzymes [16, 32].

Table 3. Cytotoxicity of treated samples.

Samples	No. of nauplii added	Nauplii alive (n)	Nauplii dead (n)	% Mortality
Positive Control	10	6	4	40
Negative control	10	10	0	0
Pseudomonas sp. BDS 2	10	9	1	10
Alcaligenes sp. BDS 9	10	10	0	0

Activities of azo dye degrading bacteria are affected by the high concentrations of salt that are released in effluents from textile processing and dyestuff industries which in turn act as a limiting factor for the development of efficient biological treatment methods [48, 12]. Therefore, tolerance to salts in dye-degrading bacteria is an important aspect as it can facilitate biotreatment methods for dye-containing wastewaters [12]. Both isolates displayed appreciable salt tolerance along with efficient decolorization results up to 40 g/l NaCl. Later on, an inverse relationship between salt concentration and decolorization rate was observed. Due to its salt tolerance ability, it could be a good candidate for remediation of salt containing real textile effluents. These results are in accordance with a previous report in which *P. aeruginosa* CR-25 showed growth and decolorization activity up to 60 g/l NaCl, and with increasing salt concentrations (5-60 g/l) a decrease in the decolorization percentage was detected [25]. The decline in decolorization performance at higher salt concentrations can be associated with inhibition of microbial growth and activity at these concentrations due to plasmolysis [49].

An increase in color removal was positively correlated with increasing inoculum size. 10% (v/v) inoculum

size was found to be the best for the process. Similar findings were reported while studying the decolorization of Orange 3R by *P. aeruginosa* [50]. In the presence of sufficient dye and media components an increase in the inoculum size resulted in a proportionate increase in the decolorization percentage [25]. About 98% decolorization at 10% inoculum size of mixed culture for decolorization of reactive azo dye was also previously reported [40].

In addition to dye residues, salts and a variety of metals are found in textile industrial effluents that can also halt microbial activities [51]. Among different metal compounds tested, appreciable color removal was observed by *Pseudomonas* sp. BDS 2 in the presence of $CuCl_2$, $CdCl_2$, and $CoCl_2$, whereas decolorization potential of *Alcaligenes* sp. BDS 9 was retained in the presence of $CuCl_2$ and $CoCl_2$. Among all the tested metal compounds severe inhibitory effect was observed with $HgCl_2$ and $AgNO_3$. Our findings are complimentary to another report in which the decolorization of Reactive Brilliant Blue by *Rhodocyclus gelatinosus* XL-1 was severely inhibited by $ZnSO_4$, $CuSO_4$, $AgNO_3$, and $HgCl_2$ at a concentration of 1mM/l [52]. The decrease in the dye decolorization percentage in the presence of heavy metals compounds might be the result of enzyme inactivation involved in the decolorization process [53].

In the present research, *Pseudomonas* sp. BDS 2 exhibited up to 90% decolorization of RB 221 within 96 h under optimized conditions. *Alcaligenes* sp. BDS 9 was slightly slower in decolorization as it took 112 h to achieve up to 88% decolorization. With *Pseudomonas* sp., 83% decolorization of Reactive Blue 13 was achieved within 70 h [26], whereas *P. aeruginosa* took 144 hrs for decolorization of Orange 3R [50]. The variations in microbial decolorization performance can be ascribed to the differences in structure of dyes [54].

A UV-Vis scan (200-800 nm) of the decolorized sample showed a decrease in the peak intensity at 615 nm without any shift in λ_{max} until the disappearance of color. The changes occurring in spectra indicated that the molecular structure of Reactive Blue 221 changed evidently after decolorization. In addition, in *Pseudomonas* sp. the BDS 2-treated sample peak at 300 nm got less broad compared to the control dye sample that may have occurred due to some breakage of molecules having absorbance in that region [38]. These findings provide a clue that decolorization was achieved by biodegradation rather than adsorption [39]. Additionally, the cell pellets retained their original color, which also supports the biodegradative process of color removal [39]. Bacterial decolorization of dyes can occur either by adsorption or biodegradation [44]. In the case of *Alcaligenes* sp., BDS 9-treated sample peaks decreased in proportion to that of control sample but did not disappear completely, which indicated that the decolorization in these cases might have occurred by adsorption mechanism [39]. Slightly colored cell pellets also showed adsorption as a mechanism of decolorization.

The FTIR spectrum (650-4000 cm^{-1}) of the control dye sample showed different peaks, e.g. a peak was observed around 1400 cm^{-1} i.e. at 1407.11 cm^{-1} that corresponds to –N=N– band vibrations [55]. The peak around 1154.54 cm^{-1} indicated C–OH stretching vibrations [29]. Two peaks were observed at 1027.47cm^{-1} and 1038.28 cm^{-1}, which correspond to S=O stretching vibrations, and that observed at 1635.18cm^{-1} corresponds to C=N stretching. Then the peak at 1842.97 cm^{-1} corresponds to C=O stretching and the two peaks observed around 2931.26 cm^{-1} and 2529.81 cm^{-1} showed the C–H stretch of aliphatic groups. The peak near 972.04 cm^{-1} indicated the presence of substituted aromatic structures and C–Cl stretching was observed around 735.68 cm^{-1}. Finally, O–H stretching was indicated by the intense peak at 3264.74 cm^{-1} [56]. In the decolorized sample changes many original dye peaks were observed, some of the most important of which were: disappearance of peak at 1407.11 cm^{-1}, indicating cleavage of –N=N– bond [38]. Vanishing of peaks at 1842.97 cm^{-1} and 1154.54 cm^{-1} suggest changes in C=O and C–OH stretching, respectively, while changes in the intensity of peaks at 2931.26 cm^{-1} showed fluctuations in C–H stretching and distortion of the peak near 972.04 cm^{-1} suggested aromatic ring deformation [56, 57].

Toxicity assessment of degradation metabolites for determination of their environmental safety is very important, as the ultimate aim is reduction of environmental pollutants as well as their toxicity [6, 29]. Results of the phytotoxicity assay depicted moderate detoxification following bacterial treatment. Good germination rate as well as significant growth in the plumule and radical in the metabolites extracted after decolorization, as compared to dye sample, was observed. Percentage germination, and root and shoot lengths with RB Reactive Blue 13 [57] and Malachite green [41] were less as compared to their degradation metabolites. The considerable reduction in percentage mortality of *Artemia salina* larvae with treated sample is attributed to detoxification of RB 221. Likewise, the cytotoxicity assay performed with *Artemia salina* nauplii demonstrated a decrease in the toxicity of Methyl Red following treatment with *Sphingomonas paucimobilis* [58].

Conclusions

In the present polluted scenario, restoration and conservation of the environment is a huge challenge. The acquired knowledge of their hazards and strict legislation has led to the implementation of several strategies for environmental cleanup. The present study results show that both indigenous bacterial isolates have the potential to remediate dye RB 221 effectively into nontoxic metabolites with minimal nutritional requirements. The results suggest that both isolates also have potential for future application toward treatment of real dye-bearing wastewaters by using the appropriate bioreactor. Furthermore, it is suggested that dye-contaminated sites can potentially be reclaimed using the cost-effective bioremediation process with native bacterial species isolated from the dye disposal sites.

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