

**DECOLORIZATION OF SELECTIVE TEXTILE DYES USING WATERBORNE PATHOGENIC BACTERIAL STRAINS****Ranjana Das*, Arunima De, Sayan Poddar, Chiranjib Bhattacharjee**

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DOI: 10.5281/zenodo.225190**KEYWORDS:** Bacterial culture, textile dye, decolorization, methylene blue, rhodamine B, reactive green 19.**ABSTRACT**

Dyes are serious pollutants, causing environmental and health problems to human being and aquatic animals. Textile wastewater with dye contaminants presents a severe environmental peril because of persistent nature and allied toxicity along with bioaccumulation propensity. Therefore, treatment of dye effluents has become utmost important criterion prior discharged into the environment. In present study three different textile dyes namely, methylene blue, rhodamine B and reactive green 19, were selected for decolorization study using three types of water borne pathogens explicitly *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Effect of major operational process parameters like, initial dye concentration, temperature and pH were investigated for decolorization of textile dyes and present study confirms the ability of *Pseudomonas aeruginosa* and *Bacillus subtilis* as potential decolorizing bacterial with efficiency of 97.28% for methylene blue and 98.73% for Reactive Green 19. Significantly high decolorization level and simplistic process conditions illustrate the potential for these bacterial strains to be used in the biological treatment of dyeing mill effluents.

INTRODUCTION

Sustained industrial growth has resulted severe spoil of ecosystem on which quality of life depends. In case of receiving water body, pollution is primarily caused by the discharge of improperly treated industrial wastewater from several industries like, paper, plastic, textile, food, cosmetic and pharmaceuticals due to extensive use of dye stuffs for coloring their products [1](Saratale et al. 2011). The textile wastewater is rated as the most polluting among all in the industrial sectors [2, 3](Awomeso et al. 2010; Vilaseca et al. 2010). Mainstream industrial dye components being synthetic nature and having aromatic ring structure are mostly persistent and resistant to biodegradation [4](Lin et al. 2010). Improper dye effluent disposal in aqueous ecosystem leads to the reduction in sunlight penetration which in turn decreases 'photosynthesis activity', dissolved oxygen level, water quality aspect and represent acute toxic effects on aquatic flora and fauna, causing a severe wide-reaching environmental problem. Textile industry is classified as one of the biggest threats to the environment as different processes in the textile industries produce huge amount waste and waste water measuring approximately 200L per Kg of textile processed [5](Beydilli et al. 1998). In India, textile mills were reported to produce on average, 60 × 10⁴ m of fabric and discharge approximately 1.5 million liters of effluent per day with high BOD/COD ratios, indicating the presence of non-biodegradable substances [6](Koch et al. 2002). Another statistical report on dye utilization illustrate that about 10-15% of the total colorant produced were lost during synthesis and dyeing processes (Ghaly et al. 2014)[7] supporting Koch et al. (2002)[6]. It was reported that the global import and export market for acid dye, dispersed dyes, direct dye and derivative were 680,000 tonnes, 570,000 metric tones, 181,998 tones in 2011 respectively (Ghaly et al. 2014)[7]. In India, production of dyes and dyestuffs were estimated about 42,000MTs during 2009-10 among which about 70% dye stuffs were consumed by textile industries (Shah et al. 2013)[8] and the global dyes and pigments market is predicted to grow at a compounded annual growth rate (CAGR) of 3.6% from 2013 to 2018 and to reach 11 million metric tons by 2018. The export of dyes from India is projected to touch about US\$ 2.6 billion in 2020. The disposal of these wastes into receiving waters cause damage to the environment because of the presence of synthetic dyestuffs along with high alkalinity and traces of chromium, which adversely affect the aquatic life and also interfere with the biological treatment processes leading to distraction of the entire ecological and symbiotic stability of receiving water streams (Puvanewari et al. 2006) [9]. The high concentration of nitrogen in the textile industrial effluents can cause the 'eutrophication' thereby increasing the biochemical oxygen demand of the receiving water and in turn reduce the reoxygenation process and hence hamper the growth of photoautotrophic organisms (Nese et al. 2007)[10]. Dyes in water reported to cause diseases like skin ulcer, haemorrhage, nausea, severe skin irritation and dermatitis (Nese et al. 2007) [10].



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Human exposure to textile dyes has resulted in lung and skin irritations, headaches, congenital malformations and nausea (Rikta et al. 2015)[11].

In view of the adverse effects associated with textile effluent, it's become obligatory to discharge the effluent only after proper treatment. Conventionally, both chemical and physical methods and sometimes integration of both the techniques such as coagulation, ozonation, precipitation, adsorption by activated charcoal, ultrafiltration, nanofiltration, electrochemical oxidation, electrocoagulation (Alinsafi et al. 2005; Kobya et al. 2003)[12,13] were used in the treatment of the textile industrial effluents. Since, both the techniques having several shortcomings (Andleeb et al. 2010; Babu et al. 2007; Lorimer et al. 2001)[14, 15, 16] like, very expensive operational assembly, high capital investment, low efficiency, less process flexibility for wide variety of dyes and large 'foot prints', which inevitably results secondary level of land pollution. Hence, economic and safe removal of the polluting dyes is still an important issue. Currently, a large group of researchers have been focused on the biological degradation of the industrial effluents (Andleeb et al. 2010; Melgoza et al. 2004; Sapci and Ustun, 2003) [14, 17, 18]. Biodecolorisation through microorganisms has been identified as a cost effective and environment friendly alternative for disposal of textile effluent. In recent years a number of studies have been focused on some microorganisms capable of degrading and absorbing dyes from wastewater in textile industry. A wide variety of microorganisms are reported to be capable of decolonization of dyes (McMullan et al. 2001; Shah et al. 2013) [19, 9]. It mainly involves utilization of exclusively bacteria, fungi or combinations in combination with physicochemical methods (Beydilli et al. 1998; McMullan et al. 2001)[5, 19] for pollution control. The principle underlying biodecoloration probably involve absorption of chromophores to biomass or reduction of the chromophores in low redox potential environments. The attractive features of biological treatment recline in its sustainable nature, regenerative activity, insignificant or no secondary hazards and low cost (Shah et al. 2013) [9]. In biological degradation processes of dye components, key limitation arises from the toxicity of dye stuffs for bacterial strains as well as on the selective nature of the bacterial stains (Kim et al. 2002; Koch et al. 2002)[20, 6]. Hence a group of microbial species (*Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) have been attempted for decolorization of the dyes under investigation. The current study has evaluated the potential of bacterial strains for their decolorization efficiency of the textile dyes (rhodamine B, reactive green 19, methylene blue) under *in vitro* conditions and optimization of the factors influencing the process. Rhodamine B (Rh B) is reported as harmful to aquatic life being highly persistent with degradability test result 'zero percent' (OECD guideline 302) and bioaccumulative potential of 0.1mg l⁻¹ for 24day as studied on cyprinus carpio(carp). Ecotoxicity studies also reported significant toxicity for fish and water flea (*Daphnia magna*) (Nagaraja et al. 2012; Gupta and Suhas 2009) [21, 22]. Reactive green 19 (RG 19) was reported as hazardous to human life with acute eye irritation effect. It is harmful if inhaled may cause respiratory tract irritation (Rastegar et al. 2012) [23]. Methylene blue (MB) is also reported as harmful if swallowed, irritating to eyes, respiratory system and skin (Shahryari et al. 2010)[24]. Diverse approaches have been made for decolonization of these dye components (Zuorro, and Lavecchia, 2014; Rastegar et al. 2012; Sari et al. 2015; Ramamurthy et al. 2013; Wilhelm and Stephan, 2007; Baldev et al. 2013)[25, 23, 26, 27, 28, 29] but limited literatures are found decolorization of the above mentioned dyes using bacterial species. Hence novelty of this study subsists in the combination of the dye components with the bio-decolorizing species.

MATERIALS AND METHOD

Chemicals

The synthetic dyes (Methylene Blue, M9140; Rhodamine B, R6626; Reactive green19, R9378) were purchased from Sigma Aldrich, USA. Physical properties of the selected dye samples are presented in Table 1(Insert Table 1 here). All other chemicals unless mentioned were purchased from E. Merck India. All experimental runs were carried out with ultrapure water from Arium® Pro VF (Sartorius Stedim Biotech) of 18.2MΩcm resistivity.

Microorganism and culture medium

Pure bacterial cultures [*Bacillus Subtilis* (NCIM 2655) *Staphylococcus aureus* (NCIM 2127) and *Pseudomonas aeruginosa* (NCIM 2036)] were purchased from NCIM, Pune India. All bacterial stains were cultured in sterile nutrient broth [beef extracts (1.0 g), NaCl (0.5 g), peptone (1.0 g), in 100 mL water] and incubated at 37°C for 36 h. The pH of the medium was adjusted to 7.0 before autoclaving at 15lbs for 20 minutes.



Decolorization experiment

All experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL nutrient broth, and desired concentrations of dyes (50mg l⁻¹-500mg l⁻¹). The flasks were sealed with cotton plugs. After the sterilizing the dye solution, 1mL microbial cultures of grown cells (36h, with respective OD_{max} 1.0) were added to the flasks and were incubated at 37±1°C under the shaking conditions (orbital shaker, 140 rpm) for 72h (set 1). A replicate of the above set were maintained in static condition (set 2) under similar incubation environment. All experiments were performed in triplicates to maintain the accuracy. After 72h, aliquots (5mL) of the culture media were withdrawn, centrifuged at 10,000 rpm for 10 minutes at room temperature to separate the bacterial cell mass. The supernatants were used for spectral analysis. Absorbance of the supernatants withdrawn at different time intervals were measured at the respective absorbance maximum for all dye samples (methylene blue, λ_{max} 660 nm; RG 19 λ_{max} 630nm; Rhodamine B λ_{max} 556nm) using UV-Vis spectrophotometer (Cary® 50Bio, Varian). The percentage of decolorization with time was estimated using Eq. 1 and average decolorization rate was estimated using Eq. 2 (Saratale et al. 2009)[30].

$$DP_t = \frac{Abs_i - Abs_t}{Abs_i} \times 100 \quad (\text{Eq. 1})$$

$$ADR = \frac{C \times DP_t \times 1000}{100 \times t} \quad (\text{Eq. 2})$$

Where, DP_t is decolorization percent; Abs_i and Abs_t were initial absorbance and absorbance at the time of observation (t). C is the initial dye concentration.

To determine the effect of process parameters like pH, concentration, temperature on extent of decolorization, the fully grown culture was inoculated in conical flasks containing 100 ml nutrient broth at varying medium pH 4-10 within concentration range of 25-500mg l⁻¹ and temperature range of 20-50°C.

Estimation of Chemical oxygen demand (COD)

To understand the degree of biodegradation of selected dye samples, reduction in the chemical oxygen demand (COD) (34) after 72 h of incubation with selected bacterial stains were determined.

Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA) and reading were considered significant when p<0.05.

RESULT AND DISCUSSIONS

Estimation of decolorization efficiency

The bacterial cells characterize an inexpensive and potential means for the removal of various dyes from textile dye effluents (Saratale et al. 2009; Telke et al. 2008; Jadhav et al. 2008)[30, 31, 32]. The ability of bacterial species to decolorize a variety of textile dyes like, reactive green 19, rhodamine B and methylene blue (50 mg l⁻¹ each) were tested in the nutrient broth at 37°C under anoxic condition in both static and shaking mode. The results are presented in Table 2 showing that the bacterial species have selective decolorization ability against various industrial dyes (Insert Table 2 here). No abiotic losses were observed for dye samples after incubation period suggesting that decolorization was due to biological degradation rather than adsorption on bacterial cells. For methylene blue decolorization was almost complete (96.95%) in 72h with average decolorization rate of 670.62 μgh⁻¹ with *P. aeruginosa*. In case of rhodamine B and reactive green 19 maximum decolorization observed was 47.47% and 97.28% in 72h with average decolorization rate of 329.66 μgh⁻¹ and 675.58 μgh⁻¹ respectively in static mode for similar bacterial strains.

Effect of physical conditions on decolorization efficiency of dye components

The operational conditions affect the efficiency of the microbial degradation process such as dye concentration, pH, temperature, agitation and oxygen. Since the incubation maintained in anoxic condition so, effect of all other parameters except oxygen was discussed in this section with detail explanations.



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Azo and reactive dyes are electron deficient in nature and this property makes them less susceptible to oxidative catabolism (Knackmuss, 1996) [33]. They are also hydrophilic in nature and hence pass through the conventional aerobic process untreated. However, under strict anaerobic conditions, decolorization of dye can be unreasonably achieved (Jing-yi, et al. 2007) [34].

In case of RG 19, decolorization percent observed was 97.28% and 94.79% under static and shaking condition, respectively (Figure 1a) using *B. subtilis* and decolorization efficiency was found in the order *B. subtilis* > *P. aeruginosa* > *S. aureus*. Similar results were also observed by other research groups using various bacterial strains (Wilhelm and Stephan, 2007) [28]. The growth of *B. subtilis* (represented by maximal cell weight concentration) was better under static (9.1g L^{-1}) as compared to shaking condition (8.2g L^{-1}) (Figure 2). Shaking condition disfavors the decolorization possibly due to the competition of oxygen and dyes for the reduced electron carriers under aerobic condition (Kodam and Kolekar, 2015) [35]. Since, the microbial degradation of azo dyes involves the reductive cleavage of azo bonds (-N=N-) either by direct chemical reaction with bulk biogenic reducing agents or biological reactions using azo-reductase, hence the presence of oxygen typically inhibits the azo bond reduction activity (Wilhelm and Stephan, 2007) [28], thus anaerobic condition was necessarily maintained during bacterial decolorization. Decolorization by *B. subtilis* was studied at various temperature ($25\text{--}50^\circ\text{C}$), and over the pH range of 3–10, the highest decolorization for RG 19 was observed at 38°C and at pH 7.1. Further increase in the temperature and pH resulted in an insignificant reduction in the decolorization activity. For the runs with dye concentrations in the range of $25\text{--}500\text{ mg L}^{-1}$, the decolorization of RG 19 was significantly high upto 50 mg L^{-1} however, at higher dye concentrations ($100\text{--}200\text{ mg L}^{-1}$), less decolorization efficiency was observed, about 45% and 24% respectively, beyond that range negligible decolorization was observed (Figure 3a). The decline in the decolorization percent may happen due to the increased toxicity of the dye to bacteria cells or decreased cell to dye ratio resulting insufficient biomass concentration for the uptake of higher concentrations of dye (Baldev et al. 2013)[29]. The reduced decolorization efficiency for *S. aureus* and *P. aeruginosa* was may be due to presence of sulfonic acid group (SO_3H) in RG 19 which greatly inhibits the growth of microorganisms (Wilhelm and Stephan, 2007) [28]. To evaluate the actual bio-decolorization level of RG 19 with *B. subtilis*, initial and final COD was also measured and was found decreased from 2134 mg L^{-1} to 640 mg L^{-1} with significant drop of COD load (70%) after 72h incubation, however no change in COD level in control set (nutrient media with RG 19) was observed under similar incubation condition. The results suggest that the *B. subtilis* can utilize RG19 as the carbon source, causing mineralization of the dye compound. Thus, from environmental point of view, *B. subtilis* could be a good alternative to physicochemical methods for RG 19 dye decolorization as it serve the purpose of decolorization as well as reduction in COD level significantly.

In case of MB, redox decolorization involves reduction of MB into colorless form which may re-oxidized to colored form again when exposed to atmosphere with time (Jing-yi et al. 2007, Ong et al. 2005)[34, 36]. However, in the indirectly biological reduction (microbial decolorization) of MB, bacterial species acts as redox mediator, and cause an increase of the biodegradation rate with drastic change in peak positions at 610nm (for dimer) and 660nm indicating decolorization. The decolorization performance was observed 96.57% and 98.74% under shaking and static respectively (Figure 1b) using *P. aeruginosa* and decolorization efficiency was found in the order *B. subtilis* < *S. aureus* < *P. aeruginosa*. The growth of *P. aeruginosa* (as maximal cell weight concentration) was observed slightly better under static mode (8.5g L^{-1}) as compared to shaking condition (7.8g L^{-1}). Since, the microbial decolorization of MB involves the reductive changes in chemical structure hence the presence of oxygen inhibits decolorization potency (Jing-yi et al. 2007; Ong et al. 2005)[34,36], thus anaerobic static condition was maintained during bacterial decolorization of MB. Decolorization by *P. aeruginosa* was studied in temperature range of $25\text{--}50^\circ\text{C}$ and pH range of 5–9. Maximum decolorization for MB was observed at 37°C and at pH 7.0. Further increase in the temperature and pH results diminished decolorization efficiency (Figure 4). Effect of initial dye concentrations was also observed in the range of $25\text{--}500\text{ mg L}^{-1}$ and results shows significant toxicity for MB at higher concentration level (500mg L^{-1}) but comparatively less than RG 19. The decline in the decolorization percent may be due to decreased cell to dye ratio value as observed in case of RG 19 (Baldev et al. 2013) [29]. The reduced decolorization efficiency for *S. aureus* and *B. subtilis* was may be due to presence of positive charge in active part of MB which may results less interaction with both these gram positive bacterial species. To evaluate the actual biodegradation level of MB with *P. aeruginosa*, initial and final COD was also measured and was found decreased about from 1534 mg L^{-1} to 276 mg L^{-1} with significant drop of COD load (82%) after 72h incubation, conversely no change in COD value was observed in control set (nutrient media with MB) under similar incubation



condition. The results suggest that the *P. aeruginosa* can be utilized from environmental point of view, as a potential alternative to conventional decolorization techniques.

Rhodamine B (RhB) is widely used as textile color, food colorant, and as water soluble fluorescent tracer (Richardson et al. 2004)[37]. Its harmful consequences to human and animals, the carcinogenicity, reproductive toxicity, developmental toxicity, chronic toxicity and neurotoxicity toward humans and animals have been well documented (Suwannawong et al. 2010) [38]. Hence, it was considered worthwhile to develop novel techniques to degrade RhB from aqueous medium using inexpensive resources. Though dyes removal from wastewater can be performed by various methods such as co-precipitation, adsorption and chemical oxidation but, involve significant snags like chemical uses, waste sludge formation, complex operational control as well as high cost (Nigam et al. 2000)[39]. From this perspective, application of biotechnology in waste water treatment has been increasing due to its ecofriendly benefits and mild condition prevailing. An interesting approach for dye decolorization is bacterial treatment of dye effluent. This approach being environmentally friendly is a promising technique for future green industries. Though enzymatic decolorization of a xanthenes dye, Rhodamine B has been reported by Lan et al. (2006)[40] but there has no report extant describing RhB decolorization by bacterial strains. This research aims to investigate the decolorization of Rhodamine B by different water borne pathogenic bacterial strains in absence of any redox mediator. The underlying principle of decolorization of rhodamine involves redox discoloration which is enhanced in presence of redox mediator (Suwannawong et al. 2010) [38]. In present case, decolorization experiment was performed without any redox mediation to study solely the efficiency of bacterial strains.

The decolorization performance was observed 49.05% and 47.47% under static and shaking condition, respectively (Figure 1c) using *S. aureus* and decolorization efficiency was found in the order *B. subtilis* \approx *P. aeruginosa* $<$ *S. aureus*. Decolorization by *S. aureus* was studied in temperature range of 25–50°C and pH range of 4–10. Maximum decolorization for RhB was observed at 37°C and at pH 7.0. Effect of initial dye concentrations was also observed in the range of 25-500 mg l⁻¹ and results shows significant toxicity for RhB comparatively higher than MB but lower that RG 19 (Figure 3c). Measurement of COD being an effective technique to measure the organic strength of waste water was used as a measure of the oxygen equivalent of the organic content in a sample that is susceptible to oxidation to CO₂ and water by a strong oxidant. The COD of the dye solution before and after the treatment was estimated. COD of the 50mg l⁻¹ dye solution was estimated 140mg l⁻¹ which after decolorization reduced to 50 mg l⁻¹ with percent reduction of 64.28% (Nagaraja et al. 2012)[21].

CONCLUSION

Economic and environmentally safe decolorization of textile dye is a tricky procedure for textile industries as well as wastewater treatment plants. Additionally, utilization of pathogenic bacterial strains in decolorization of the dye stuffs is an economic and environmentally friendly way with less or no probability of producing toxic byproducts. Bacterial species are adaptive in nature and can degrade contaminants by natural integrated mechanisms. The results of these findings suggest great potentials for such bacterial strains to be used for removing color from textile wastewaters. *B. subtilis* and *P. aeruginosa* were found most promising in reducing the color of methylene blue and RG 19. For RhB dye *S. aureus* showed significant decolorizing activity through a degradation mechanism. The ability of the strain to tolerate and decolorize dyes at a high concentration illustrates potential of the strains but need further trials with real dye effluents. High color removal efficiency by these strains and significant reduction in COD, indicate the biodegradation of complex organic dyes and show the applicability of these strains for wide variety of individual dye and the mixture of dyes. This bio-treatment offers an easy, cheap and effective option for color removal of textile dyes. The potential of these bacterial strains can be exploited for the removal of residual dyes from the wastewater streams for environmental cleanup and renovation of ecosystem. Hence, economical and ecofriendly techniques using bacteria can be a possible pathway for fine-tuning of wastewater treatment.

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Table 1: Physical properties of selected textile dye compounds

Dyes	Molecular weight g.mole ⁻¹	Classification	Use	Color Index number (C.I.)	Solubility in water (g.L ⁻¹)
Rhodamine B (Basic violate10)	479.01	Cationic, Xanthene dye	For dyeing synthetic fibers	45170	50
Reactive Green 19	1418.92	Reactive dye, Azo type	For dyeing protein fibers	205075	150
Methylene Blue (basic blue)	373.90	Cationic, Phenothiazine dye	For dyeing cellulosic fibers	52015	0.050

Table 2: Average decolorization rate (μgh^{-1}) for dyes for different bacterial cultures at 72h

Name of dye	λ_{max} (nm)	Bacterial strain	Time (h)	Decolorization percent (DP)	Average decolorization rate (μgh^{-1})
<i>Rhodamine B</i>	556	<i>B.subtilis</i>	72	2.66	18.47
		<i>S.aureus</i>	72	49.05	329.66
		<i>P.aruginosa</i>	72	3.02	16.69
<i>Methylene blue</i>	660	<i>B.subtilis</i>	72	34.69	228.70
		<i>S.aureus</i>	72	71.46	228.70
		<i>P.aruginosa</i>	72	98.73	670.62
RG19	630	<i>B.subtilis</i>	72	97.28	675.58
		<i>S.aureus</i>	72	31.04	211.29
		<i>P.aruginosa</i>	72	17.26	211.29

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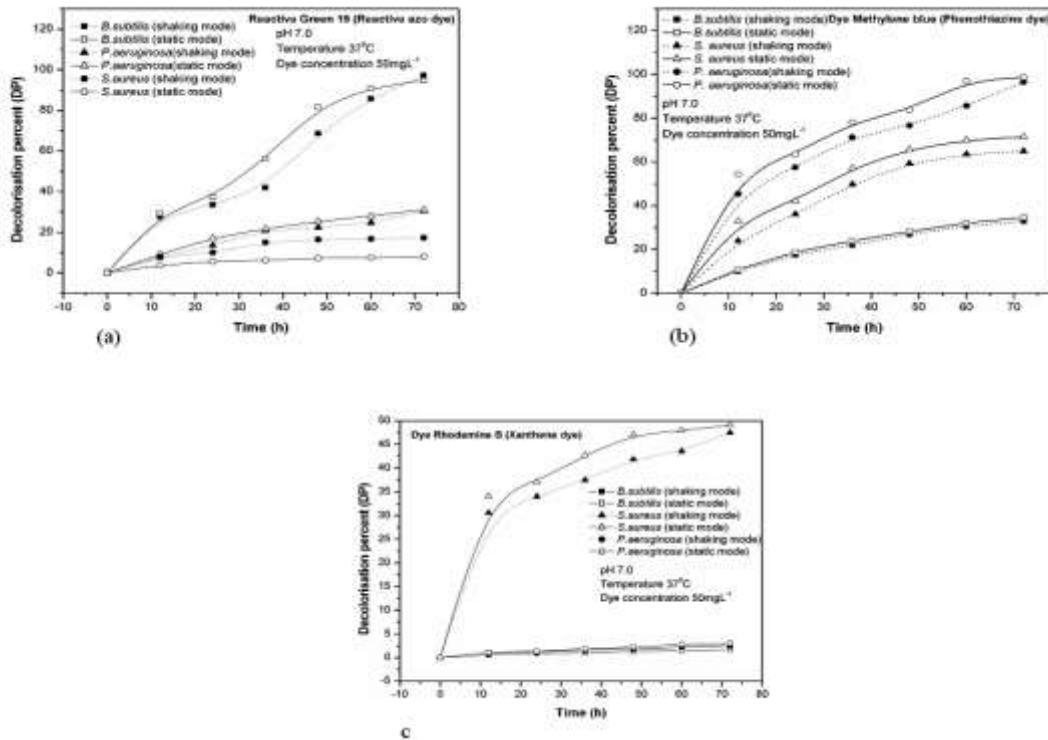


Figure 1: Extent of decolorization for selected dye compounds as function of time for various bacterial pathogens (a) Reactive Green 19 (b) Methylene blue (c) Rhodamine B



Figure 2: Experimental observation of the decolorization of RG 19 using B. subtilis after 72h of incubation period

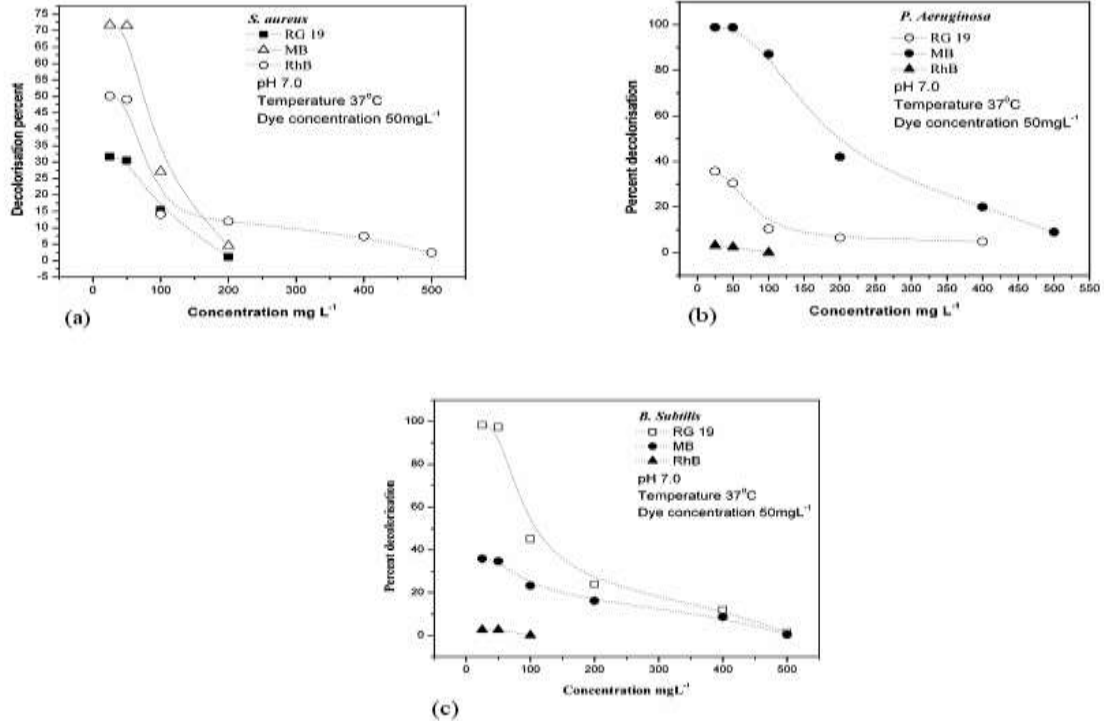


Figure 3 Effect of initial concentrations on decolorization percent of the selected dye compounds under similar reaction condition for various bacterial pathogens (a) *S. aureus* (b) *P. aeruginosa* (c) *B. subtilis*

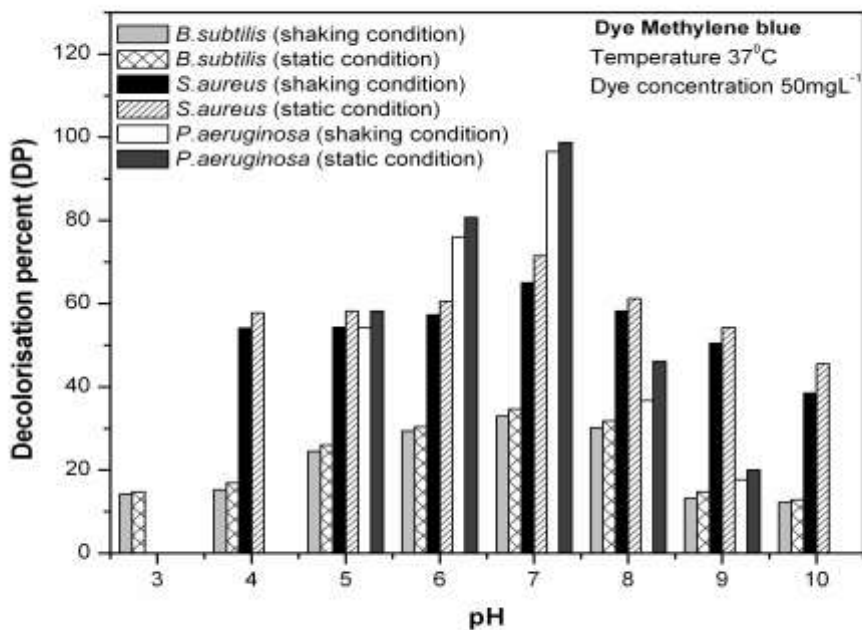


Figure 4: Effect of pH on the decolorization performance of methylene blue using various bacterial pathogens



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