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ORIGINAL ARTICLE

Decolorization of mixture of azo dyes by a bacterial strain isolated from dye contaminated sites

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ABSTRACT

The current study aims at microbial decolorization of a dye mixture by a bacterial strain JKAK isolated from soil. The effect of pH and temperature on decoloriztion of the dye mixture by JKAK was studied at a pH range 5.5-10.5 and temperature 30°C -50°C where decolorization was optimum at pH of 8.5 and temperature 40°C. JKAK could decolorize 200 mg/L of individual dyes Ponceau 4R, Amaranth RI, Fast Red E, Tartrazine, Orange G, Methyl Orange, Bismarck Brown and Amido Black within 7, 8, 10, 11, 14, 16, 18 and 25 h of incubation respectively. While, the mixture of these dyes taken in 200, 400, 600, 800 and 1000 mg/L concentrations were decolorized by JKAK in 40, 40, 40, 40 and 48 h of incubation respecitvely. Decolorization of 200, 400 and 600 mg/L of the dye mixture by JKAK and other previously isolated strains AK1, AK2 and VKY1 was also studied. JKAK was able to remove 88.04%, 74.03% and 57.20% of the dye mixtue, AK2 could remove 76.20%, 62.82% and 56.60% of the dye, AK1 was able to decolorize 70.56% and 48.00% dye, while VKY1 could remove 68.45%, 52.08% and 44.00% of 200, 400 and 600 mg/L dye mixture respectively after 40 h of incubation.

KEY WORDS: *Azo dyes, Effluent, Bacteria, Decolorization, JKAK*

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INTRODUCTION

Soil and water form a basic, crucial and essential part in the lives of all living organisms. Water plays a vital role in hydration, cleaning and provide a habitat to many living organisms. However, the fast-growing worldwide population has led to a rapid industrial development. This in turn, threatens the availability of these vital resources resulting in serious health, social and economic concerns. Dye-stuff related industries release around 10-15% of the industrial dyes, contained in the untreated effluents to the environment [1]. This in turn results in major water and land pollutions..

Azo dyes make up the most widely used group of dyes utilized in textile, food, pharmaceutical, printing as well as cosmetic industries [2]. They are made up of various functional and structural groups and possess either single or multiple double bonds. The bonds are the main color contributors and breakage of these bonds renders the dye colorless [3]. Most dyes are toxic, recalcitrant and resistant to biodegradation causing acute toxicity to the receiving land or water bodies. About 17- 20% of freshwater pollution is caused by toxic textile effluents which poses hazardous effects on the aquatic lives. Dyes are designed to resist most forms of degradation and hence persist in the environment exerting their mutagenic, carcinogenic and toxic effects on the organisms. Therefore, treatment of dye-containing wastewater is essential before discharge on to the land or water bodies.

Treatment involves degradation of harmful chemicals in the dyed wastewater to prevent toxicity to the environment. The difference in dye structures is an important aspect in biodegradation in that it greatly determines the rate and efficacy of decolorization. Dyes with low molecular weights or less number of azo bonds are easily decolorized compared to those of high molecular weights or multiple azo bonds [4].

The fuctional group also plays a major role in decolorization, for example, dyes containing amino and hydroxyl groups are decolorized easily compared to those containing Nitro, Sulpho and Methyl groups [5]. Dye related industries utilizes dyes of varied molecular and structural formulas and thus, the disposed effluents are characterized by varied chemical composition, pH, organic and inorganic solids, dissolved oxygen among others [6]. In this case, the means of effluent treatment should not be specicific to a particular dye but rather able to decolorize a mixture of various dyes. There are a number of conventional methods employed in the treatment of dye-contaminated water. However, majority of these methods are costly, ineffective and impractical for use on large scale. Moreover, most of these methods may lead to formation of more toxic intermediates. Hence, scientists are constantly researching on a safe, efficient and cost effective method of effluent treatment. Bioremediation involves the use of biological methods such as bioadsorption, biotransformation and biodegradation among others which are all ecofriendly, less expensive and efficient. In the case of microbial treatment of dyed effluents, the selected microbes should be able to remove a wide range of dyes both individually and as a mixture to prove the potential efficacy in industrial application.

In the current study, a newly isolated bacterial strain *Pseudomonas aeruginosa* JKAK, capable of decolorizing up to eight selected textile dyes was used in studies of decolorization of dye mixture. Other strains already available in our laboratory (*Bacillus* sp. AK1, *Lysinibacillus* sp. AK2 and *Kerstersia* sp. VKY1) [7] were also subjected to decolorization of the dye mixture at different concentrations to provide a comparison with the newly isolated JKAK.

MATERIAL AND METHODS

Dyes and chemicals

The eight selected dyes Ponceau 4R, Amaranth RI, Fast Red E, Tartrazine, Orange G, Methyl Orange, Bismarck Brown and Amido Black 10B (Table 1) were purchased from Hi-Media Laboratories (Mumbai, India) alongside the media components. Where as, NADH was procured from Sigma, Steinem, Germany. All chemicals used in this study are of scientific and analytical grade.

Microorganisms and media

The bacterial strain *Pseudomonas aeruginosa* JKAK was isolated from soil around textile areas of Tirupur, Tamil Nadu, South-India. The strains *Bacillus* sp. AK1, *Lysinibacillus* sp. AK2 and *Kerstersia* sp. VKY1 were already available in our laboratory. The mineral salt medium was prepared with K_2HPO_4; 6.3 g/L, KH_2PO_4; 1.8 g/L, NaCl; 5 g/L, $MgSO_4.7H_2O$; 0.1 g/L, $MnSO_4$; 0.1 g/L, $CaCl_2.2H_2O$; 0.1 g/L, $FeSO_4.7H_2O$; 0.1 g/L, $Na_2MoO_4.7H_2O$; 0.006 g/L, 0.5 g/Lyeast extract and pH was adjusted to pH 8.5.

Isolation of bacterial strain capable of dye decolorization

Sample collection was carried out following a method reported before with slight modifications [8]. The dye contaminated surface soil were collected 0-15 cm deep using stainless steel spade, dried and sieved. The soil sample was homogenized and stored in polythene bags for subsequent studies.

The sample was dissolved in water, filtered and the filtrate was used as a microbial source which was streaked onto nutrient agar plate containing the dye. The colonies that formed clear zone on the agar plate were selected and grown in 50 ml MS media containing 0.5% yeast extract and 200 mg/L of the dye and incubated at 40 °C temperature under static conditions (Figure 1). Decolorization was observed in three flasks, however, the colony showing the highest and rapid decolorizing ability on individual and dye mixture was selected for further subsequent studies. Microbial purification was performed using streak plate and spread plate techniques followed by serial dilutions.

Identification of the dye decolorizing bacteria

This strain was identified through morphological, biochemical and 16S rDNA sequencing. 16S rDNA sequencing carried out by the National Collection of Industrial Microorganisms (NCIM) Pune, India identified the isolate as *Pseudomonas aeruginosa*, which we gave the strain name, JKAK. 16S rRNA gene based phylogenetic placement of *Pseudomonas aeruginosa* strain, JKAK was also determined.

Decolorization study

The eight dyes used in preparation of the dye mixture were subjected to individual decolorization to ensure all selected dyes could be completely decolorized by JKAK. The experiments were performed in 250 mL Erlenmeyer flasks containing 0.5% yeast extract, 50 ml MS and 1 ml inoculum. The mixture was prepared by mixing eight dyes at a concentration of 25 mg/L each to make a final concentration of 200 mg/L. This study was performed to determine the decolorization efficiency of the dye mixture by the JKAK.

Measurement of dye decolorization

To determine the percentage decolorization at a given time, samples were withdrawn after 3 h intervals and optical density was determined at the respective nanometeres using UV-Vis spectrophotometer

(Specord 50, Germany). All experiments were carried out in triplicates and decolorization efficiency was expressed as percentage decolorization using the formula:

$$\% \textit{ Decolorization} = \frac{(i-f)}{i} \times 100$$

Where, *i*= initial absorbance and *f* = absorbance of decolorized medium

Effect of various parameters on decolorization of the dye mixture

Decolorization was studied at different variables such as initial dye concentration, temperature and pH. The effect of concentration of the dye mixture on decolorization was analysed at 200, 600 and 800 mg/L of the dye at pH 8.5 and incubated at 40 °C. Studies on the effect of pH on decolorization was also carried out at pH range of 6.5-10.5 and the effect of temperature was between 30 °C-50 °C. All experiments were carried out under static conditions.

Treatment of real textile effluent

Textile effluents were collected from sites around textile areas in Tirupur, Tamil Nadu India. Naturally, textile effluents discharged consists of different types of dyes with diverse molecular and structural formulas. The decolorization of real textile effluent by JKAK was studied. In this case, the control flask contained 100 ml of the effluent sample while the test sample contained 100 ml of the effluent sample and 1 mL of JKAK inoculum. The two flasks were supplied with 0.5% yeast extract and incubated at 40 °C under static conditions.

Phytotoxicity analysis

This was carried out to determine the toxicity of the dye mixture before and after decolorization. This study was coducted using Green gram (*Vigna radiata*) seeds, a plant commonly grown across most parts of the world. The seeds were soaked in water overnight and planted the following day in glass cups containing loam soil. The experiments were performed in three sets named Control set, Test set 1 and Test set 2 each containing five healthy, viable and certified seeds. Control set, Test set 1 and Test set 2 were watered with distilled water, decolorized dye and dye solution (2000 ppm) respectively.

RESULTS AND DICUSSION

Isolation of the dye decolorizing bacterium

This site was chosen due to its exposure to textile dyes. The microbes thriving in these soil expresses considerable tolerance to textile dyes in the environment. This tolerance might result from direct assimilation of the dye through the cell membrane, biological adsorption or through enzymatic degradation of the dye [9]. The collected soil was dissolved in water, filtered and the filtrate was used as a microbial source.

Decolorization of individual dyes by JKAK

JKAK could decolorize completely 200 mg/L of individual dyes Ponceau 4R, Amaranth RI, Fast Red E, Tartrazine, Orange G, Methyl Orange, Bismarck Brown and Amido Black within 7, 8, 10, 11, 14, 16, 18 and 25 h of incubation respectively. According to earlier report [10], dyes with simple structures and low molecular weights are decolorized faster than those with complex structures and high moleculaer weights. Moreover, the functional group attached affects the rate of dye decolorization [11]. The number of azo bonds also plays an important role in dye decolorization as triazo and diazo dyes are decolorized much slower than mono azo dyes [12]. Further, substituents on the para position of the azo bond are prone to decolorizaton than those on meta and ortho positions [13]. The variations in the structure different dyes in the mixture directly affects the rate and percentage of decolorization of the dye mixture [14]. In the current study, these variations showed minimal effects on decolorization efficiency of the bacteria on the dye mixture.

Decolorization of the mixture by JKAK, AK2, AK1, VKY1 at different concentrations

Textile effluent consists of varied and mixed dye composition. Therefore, bacterial decolorization of a mixture of eight dyes was carried out. To determine the effect of dye concentration on decolorization, the dye-mixture of final concentration between 200-600 mg/L was subjected to decolorization by JKAK, AK1, AK2 and VKY1. The process was monitored over 40 h period of incubation. JKAK was able to remove a maximum of 88.04, 74.03 and 57.20 mg/L of the dye mixture at a concentration of 200, 400 and 600 mg/L respectively after 40 h. AK1 decolorized a maximum of 70.56%, 64.11% and 48.00% of 200, 400 and 600 mg/L of the dye mixture respectively after 40 h. On the other hand, AK2 decolorized a maximum of 76.20%, 62.82% and 56.60% of 600 mg/L of the dye mixture respectively after 40 h. The fourth strain, VKY1 could decolorize 68.45%, 52.08% and 44.00% of the 200, 400, 600 mg/L dye respectively after 40 h of incubation (Table 2). The current study showed higher decolorization percentage compared to reports recorded earlier [15] where only 32-46% decolorization was recorded at a concentration of 20 mg/L. At 10 mg /L, 48 - 57% of

the dye was removed after 120 h of incubation. At a concentration of 30 mg/L of the dye mixture, only 10–16.3% of the dye was removed.

Dye concentration affects the decolorization of dyes significantly as shown in this study. Decolorization of the dye mixture decreased significantly with the increase of the dye concentration (200-600 mg/L). Decolorization process was also prolonged as the dye concentration was increased. This effect can be attributed to the toxicity of the excess dyes on microbial cells and inhibition of decolorization by blocking the active sites of the concerned enzyme. This agrees with results reported earlier [16] where 90% of only 206.3 mg/L Remazol Blue was decolorized after 30 h of incubation. Similar results have also been reported [17] where the decolorization of Reactive Black 5 by *Enterobacter* sp. EC3 decreased with an increase in dye concentration.

Effect of pH on decolorization of the dye mixture by JKAK

pH demonstrates significant effect on bacterial decolorization of dyes as most decolorization studies reports high decolorization efficiency at neutral to low basic pH [18]. In the current study, the optimum decolorization rate and percentage was realized at a slightly alkaline pH 8.5-9.5 where JKAK strain decolorized a maximum of 91.36% and 88.24% of the dye after 40 h of incubation. The isolated strain JKAK was slightly tolerant to pH changes as shown by the presence of decolorization at weak acidic pH 6.5 and strongly alkaline pH 10.5 where the strain removed 37.02% and 56.49% of the dye after 40 h of incubation (Figure 2). Though these percentages are of course far less compared to decolorization efficiency at optimum pH 8.5, its still clear that the enzyme is not completely inhibited by these pH levels. These findings are in accordance with the previous reports [19] on decolorization of RB-5, DR-81 and DO-3 by *Shewanella putrefaciens*, AS96 which showed high toletrance to pH between 6-10. The optimum pH for the decolorization of RB-5, DR-81 and AR88 was 7 and 9 while the optimum pH for decolorization of DO-3 was 7-8. Increased decolorization at slightly basic media might result from elevated bacterial growth and enzyme activity at alkaline pH [20]. The studies carried out on decolorization of a mixture of two dyes (Methyl orange and Naphthol Green) by *S. oneidensis* MR-1 shows a sharp decrease in decolorization at a pH below 6.5 [21]. Dye decolorization through adsorption is reported to increase the pH inside the cell which results in a significant decrease in decolorization process which eventually hinders the bacterial growth and inhibits the dye decolorization process [22]. Generally, an increase or decrease from the optimal pH results in a decrease in dye decolorization.

Effect of temperature on decolorization of the dye mixture by JKAK

Temperature directly affects the rate chemical reactions in biological systems. Optimal temperature is therefore essential for growth, metabolism and enzymatic activities of bacterial strains, resulting in optimized dye decolorization. In the present study, an increased rate of decolorization was realized with the rising temperature from 30 °C-40 °C beyond which there was a drastic decline. At 30 °C, 35 °C, 37 °C and 40 °C, JKAK strain was able to remove 48.62%, 70.44%, 87.33% and 94.20% of the dye mixture after respectively after 40 h of incubation (Table 3). In this case, the optimum tempearuture was 40 °C with decolorization percentage of 94.20%. Any change in temperature on either side of the 30 °C and 40 °C resulted in a drastic decline in decolorization. A decline was observed at 50 °C with decolorization efficiency of 56.11% after 40 h of incubation.

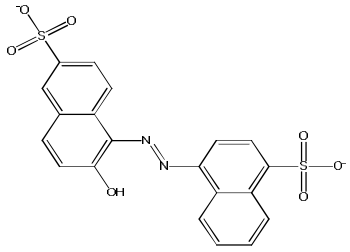
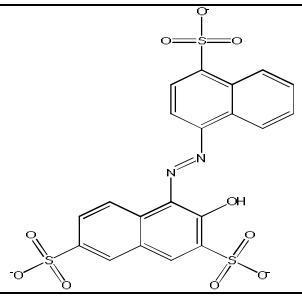
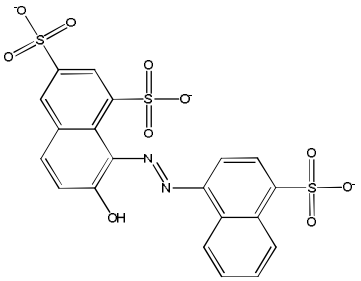
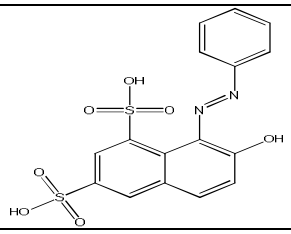
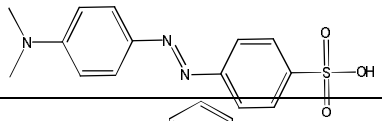
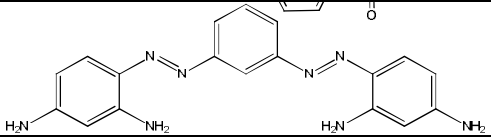
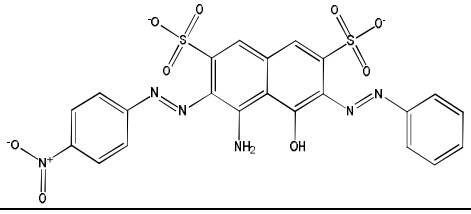
The results shows that JKAK can tolerate moderately high temperatures as depicted by 56.11% decolorization at 50 °C. This proves JKAK as more thermo-tolerant and could decolorize a detectable percentage of the dye over a wide range of temperatures (30 °C-50 °C). These results proves JKAK as more efficient compared where *Pseudomonas aeruginosa*, NBAR12 which showed 3.8 times reduction in decolorization of the individual dye, Reactive Blue 172 at 50 °C in the previous report [23]. Decolorization by *Pseudomonas aeruginosa*, JKAK was also better compared to the results obtained by [24] where *Alcaligenes faecalis* was able to remove only 14.75 to 25.0 mg/L of Reactive Orange 13 per hour at 25°C-37°C. The increased decolorization rate with an increase in temperature to the optimum level can be due to increased bacterial growth as well as enzymatic activities of the strain at a favorable temperature [25]. On the other hand, inhibited decolorization at low temperatures might be attributed to inhibition of major cell processes such as growth and enzymatic activities. Further, the drastic decrease of decolorization percentage at 50 °C might result from the loss of cell viability and enzyme inactivation caused by excess heat.

Treatment of real dye effluent

The textile industries utilize a wide range of dyes proving the mixed dye content of the effluent. This study was performed to determine the action of JKAK on real textile effluents. In the study, 100 mL of the sample was inoculated and incubated at 40 °C under static condition. After 9 h of incubation, there was no change observed on the control flask which contained the effluent and 0.5% yeast extract. On the other hand, sedimentation and coagulation was evident on the test flask containing the effluent sample, 0.5%

yeast extract and JKAK inoculum. After 9 h of incubation, floccules were observed while the upper layer gets clearer and fade. The floccules formed settled at the bottom of the flask to form a thick sediment. As suggested in earlier reports [27], this might prove JKAK to possess both decolorization and flocculation abilities.

Table 1. Details of the various dyes studied

Dye Name	Molecular formular	Molecular structure	Uses
Fast Red E	$C_{20}H_{12}N_2Na_2O_7S_2$		Used in drugs, cocktails, tinned fruits, biscuits, chocolates, bakeries, snacks industries and textile industries.
Amaranth RI	$C_{20}H_{11}N_2Na_3O_{10}S_3$		Dying natural and synthetic fibres, leather, paper, phenol-formaldehyde resins, food color, medicine color, chemical indicator.
Ponceau 4R	$C_{20}H_{11}N_2Na_3O_{10}S_3$		Coloring of alcoholic and non alcoholic beverages, coloring of food stuff.
Orange G	$C_{16}H_{10}N_2Na_2O_7S_2$		Maker in agrose gel electophorosis, biological stain, Microbiology stain, Textile (wool and silk), paper, leather.
Methyl Orange	$C_{14}H_{14}N_3NaO_3S$		Titration indicator, textile industry, printing, paper, food, pharmaceutical industries.
Bismarck Brown	$C_{21}H_{24}N_8.2HCl$		Biological stain (stains acid mucin to yellow and mast cell granules to brown) textile, leather
Amido Black 10B	$C_{22}H_{14}N_6Na_2O_9S_2$		Stain for total protein on transferred membrane blots, criminal investigation (detect blood with latent fingerprints)

Phytotoxicity

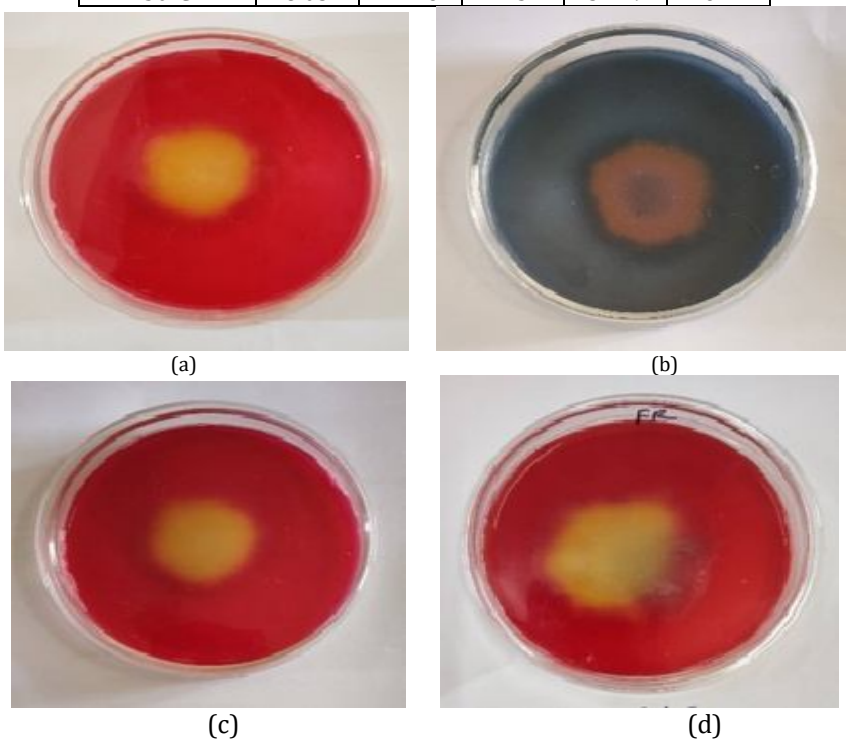
The saplings from the three sets were uprooted after 72 h to compare the developed shoot and root lengths. The control set had a shoot length of 18 cm and root length of 5.5cm whereas the test set 1 had a shoot length of 8.2 cm and root length of 2.5 cm. The test set 2 on the other hand showed a shoot length of 15.6 cm and root length of 4 cm. These results clearly indicates that the growth and health of the sapling was affected by the dye as the sapling watered with distilled water were longer followed by sapling watered with decolorized dye and the shortest of them was the sapling watered with the dye solution (Figure 3).

Table 2. Decolorization of the dye mixture by JKAK, AK1, AK2, VKY1 at different dye concentrations

Strain	Conc. (mg/L)	Percent decolorization				
		8 h	16 h	24 h	32 h	40 h
JKAK	200	13.51±0.7	28.07±0.9	48.24±0.7	65.78±0.8	88.04±0.3
	400	9.40±0.4	23.00±0.6	36.84±0.9	51.75±0.5	74.03±0.8
	600	5.6±0.2	13.60±0.1	28.80±0.2	38.80±0.7	57.20±0.6
AK2	200	16.20±0.9	27.19±0.22	43.42±0.10	66.66±0.23	76.20±0.64
	400	12.82±0.6	26.49±0.14	40.59±0.12	53.84±0.63	62.82±0.15
	600	9.60±0.13	20.00±0.21	32.88±0.31	45.20±0.7	56.60±0.4
AK1	200	14.56±0.7	35.52±0.2	55.00±0.63	70.61±0.41	70.56±0.42
	400	11.11±0.2	21.79±0.10	31.62±0.25	42.73±0.22	64.11±0.
	600	8.00±0.31	13.21±0.35	21.61±0.17	33.60±0.36	48.00±0.21
VKY1	200	11.45±0.12	27.19±0.13	42.90±0.11	60.96±0.21	68.45±0.3
	400	8.08±0.2	21.36±0.8	33.33±0.1	46.15±0.8	52.08±0.32
	600	4.00±0.15	13.60±0.62	24.00±0.56	34.00±0.41	44.00±0.16

Table 3. Decolorization of the dye mixture by JKAK at different temperature

Temperature	Percentage decolorization at different times				
	8 h	16 h	24 h	32 h	40 h
30°C	6.12	10.26	19.54	26.17	34.62
35°C	8.27	14.08	22.04	32.05	43.44
37°C	16.41	25.02	40.07	58.16	77.33
40°C	19.62	34.15	55.42	74.21	93.20
50°C	8.03	14.26	24.51	31.19	46.11



(a) (b) (c) (d)

Figure 1. Decolorization zones on agar plate obtained using JKAK for dyes (a) Amaranth RI (b) Amido Black 10B (c) Ponceau 4R (d) Fast Red E

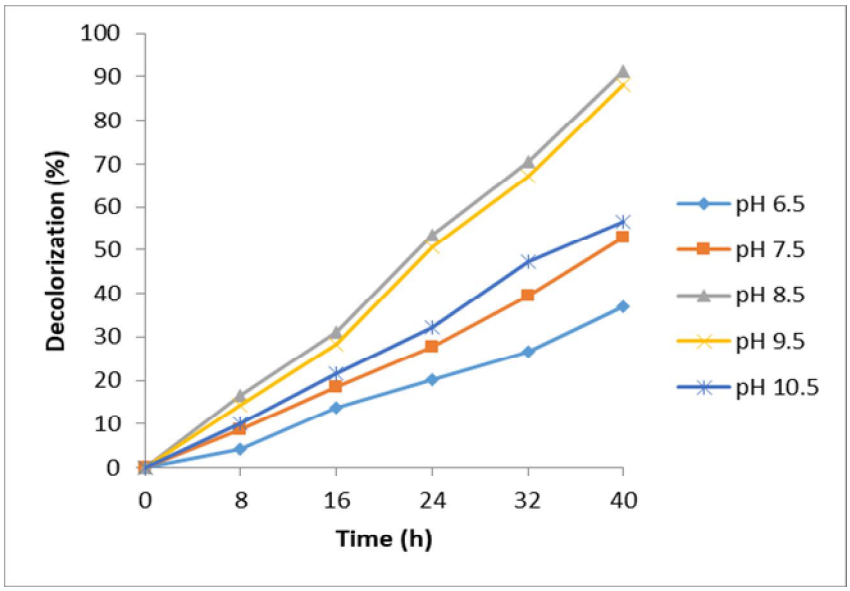


Figure 2. Decolorization of dye mixture at different pH by JKAK

Figure 3. Phytotoxicity studies on seeds of Green gram watered with (a) Distilled water (b) Decolorized solution (c) Dye solution

CONCLUSION

Decolorization efficacy of *Pseudomonas aeruginosa*, JKAK, on a mixture of eight dyes was studied for the first time. This strain shows high potentiality in decolorization of dye, the mixtures and further studies on practical application of treatment of dye-effluents before discharge. Bacterial decolorization involves either enzymatic breakdown of the azo bond or direct adsorption of the dye into the microbial system. JKAK was able to decolorize mixture of dyes under varied pH, temperature and dye concentrations. Decolorization of real dye effluent was also demonstrated. Hence, JKAK can be employed effectively in the treatment of dye effluent.

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