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Decolorization of azo dyes by newly isolated *Citrobacter* sp. strain EBT-2 and effect of various parameters on decolourization

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ABSTRACT

Azo dyes constitute around 70% of the total dyes in the world. Almost 10%–15% of dye is released in wastewater during manufacture of the dye and its application, and is a prime source of pollution. Various physiochemical methods available for their decolorization have some or the other disadvantages like cost or time inefficiency. Hence, bacterial decolorization has been studied for cheap and efficient decolorization. In this study, *Citrobacter* sp. strain EBT-2 was isolated from a textile industry dumping site and used to optimize dye decolorization conditions for three Azodyes: methyl orange (MO), congo red (CR), and Eriochrome Black T (EBT). Decolorization was measured by UV–Vis spectroscopy analysis. The strain showed 100% decolorization for all the three dyes up to 100 mg/l concentration in 96–120 hours. It was able to decolorize till 300, 500, and 500 mg/l of dye concentration for MO, CR, and EBT, respectively. Decolorization efficiency was independent of initial dye concentration. Optimum pH for decolorization was 7, 7, and 9 for MO, CR, and EBT, respectively. The effect of agitation on decolorization was studied under static and agitated (200 rpm) condition. About 90% decolorization was observed at static condition and about 20% decolorization was observed under agitated condition in all the three dyes in 96 hours. Complete decolorization was obtained for MO and EBT at 35°C and 45°C, respectively. CR showed complete decolorization only at 35°C. The results conclude that *Citrobacter* sp. can be used for the successful dye decolorization of Azo dyes, primarily MO, CR, and EBT under optimum physiochemical conditions.

1. INTRODUCTION

Azo dyes constitute around 60%–70% of dyes synthesized globally [1,2]. These are characterized by the presence of one or more azo groups, −N=N− [3]. These further consist of phenyl and naphthyl groups modified with various functional groups [3]. Such complex modifications in their structure makes them xenobiotic in nature and highly resistive to breakdown [4]. They are resistant to light, washing, chemical and microbial attack, easy to synthesis with low energy, and low-cost consumption [4–6]. They are extensively used in textile, paper, food, leather, cosmetics, and pharmaceutical industries [7]. Almost 10%–15% of dye is released in wastewater during its manufacture and application in various industries [8]. Contaminants generated by azo dyes constitute of dye particles and their breakdown products; primarily amines that are proven to be toxic and mutagenic [9,10]. They also degrade the water quality by increasing its biological oxygen demand and chemical oxygen demand [11,12]. Many reports indicate that textile dyes and effluents have toxic effect on the germination rates and biomass of several plant species [11,13]. Azo dyes like tartrazine and carmoisine affect the functioning of vital organs like kidney and liver in rats and also induce the formation of free radicals leading to oxidative stress [14].

Various physiochemical methods like membrane filtration, adsorption on activated carbon, flocculation, electro coagulation, ozonation, froth floatation, reverse osmosis, and ion exchange are currently implied for azo dye decolorization. However, these methods have limitations like high cost, high energy input, and sludge generation [15]. Recently microorganisms have been shown to decolorize azo dyes in a cost effective and environment friendly manner [16]. Bacteria, algae, fungi, and yeast have shown cost effective and eco-friendly degradation of textile dyes [11]. Furthermore, versatility in microbial structure makes their exploitation easy for decolorization of most of the dyes [17]. Dye

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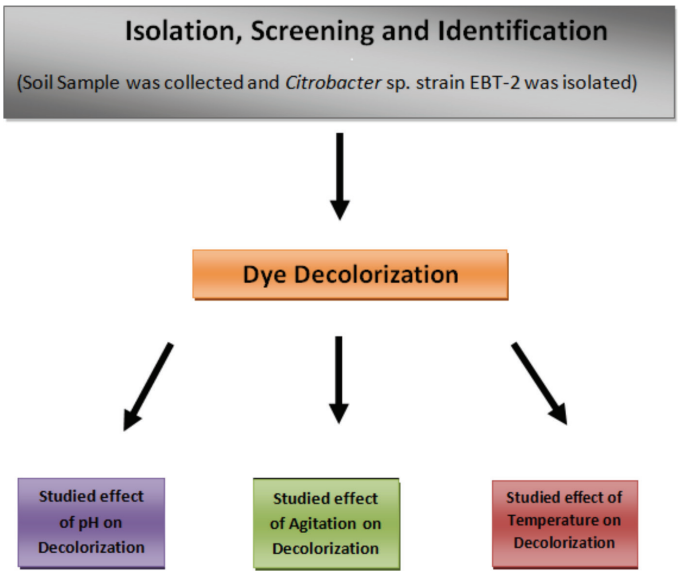
decolorization by higher order organisms like fungi is a slow process due to the slow growth of the organism [18]. Bacteria on the other hand have emerged as a very promising category for dye decolorization [19]. They proliferate rapidly under both aerobic and anaerobic condition thus can achieve dye degradation at a faster rate [20]. They primarily decolorize dyes under anaerobic conditions [21]. Few bacteria have been reported to decolorize azo dyes in aerobic conditions as well [21]. It has also been shown that under a mixture of anaerobic and aerobic conditions, complete degradation of some dyes can be achieved [22]. Often textile waste waters have high metal content and salinity; hence, it is important to utilize microorganisms tolerant to extreme environment conditions. Bacterial species like *Enterococcus faecalis* and *C. bufermentas* have been identified as highly halotolerant azo dye degrading microorganisms [23].

This study was done to isolate and identify a bacterial strain capable of decolorizing three azo dyes: methyl orange (MO), congo red (CR), and Eriochrome Black T. MO is an acid class single azo dye and was found to be mutagenic in a Salmonella/microsome assay [24]. CR is a benzidine-based anionic diazo dye and has been shown to metabolize to benzidine, a known human carcinogen [25]. Eriochrome Black T (EBT) is a single azo naphthol derived azo dye and is known to be recalcitrant to oxidative biodegradation [26]. Various physiochemical parameters like temperature and pH were also optimized for efficient decolorization of dyes.

2. MATERIALS AND METHODS

2.1. Isolation, Screening, and Identification

Soil sample was collected in a sterile glass container from dumping site of textile industry located in Bada Bagh Industrial area, New Delhi. Samples were serially diluted 10^5 times and were enriched in Luria broth (LB) amended separately with 100 mg/l of MO, CR, and Eriochrome Black Tat 37°C for 48 hours. Subsequent transfers of 1 ml of the culture were carried out in fresh media amended with dye (100 mg/l) till decolorization of the media was observed. Decolorized samples were serially diluted 10^7 times and were spread on petri plates with 100 mg/l dye concentration. Plates were incubated at 37°C for 48 hours. Morphologically distinct colonies with zone of decolorization were screened for their decolorizing ability in media supplied with dye (100 mg/l). Out of all the colonies observed on plate, EBT-2 strain showed the best decolorization zone and hence was picked up for further experiments. EBT-2 strain was identified using 16s rDNA sequencing and similarity search was conducted against database through BLASTn for 16s rDNA. MEGA 4.0 software was used to conduct evolutionary analysis by Neighbour Joining method [27]. Biochemical characteristics of EBT-2 strain were also assessed according to Bergey's Manual of Determinative Bacteriology [28].



Diagrammatic representation of Methodology

2.2. Effect of Initial Dye Concentration on Dye Decolorization

The effect of initial dye concentration on dye decolorizing potential of EBT-2 strain was measured by studying decolorization at different initial concentrations of MO, CR and Eriochrome Black T (10–500 mg/l). LB media was amended with dye concentrations ranging from 10 to 500 mg/l and inoculated with 1% EBT-2 isolate (OD 0.8–1). It was incubated at 37° for 16 hours under static condition. Samples were centrifuged at every 24-hour interval at 10,000 rpm for 10 minutes. Supernatant was collected and absorbance was measured at absorbance peak of each dye i.e., 480, 492, and 535 nm for MO, CR, and EBT respectively. Decolorization efficiency was measured for a period of 120 hours in terms of percentage decolorization [29].

% Decolorization = [(Initial absorbance – Observed absorbance)/ Initial absorbance] × 100

The experiment was done thrice, and data was represented as the mean ± Standard error of mean. Abiotic controls were always included.

2.3. Effect of pH on Dye Decolorization

Decolorization efficiency of EBT-2 strain was studied at different pH values. LB containing MO, CR and EBT at the concentration of 100 mg/l was inoculated with 1% inoculum and pH was set from 5 to 11 (with increment of 1 pH unit). pH was adjusted with 0.1 N HCL or 0.1 N NaOH. The test was conducted under static condition at 37°C for a period of 96 hours and decolorization was measured for every 24-hour interval.

2.4. Effect of Agitation on Dye Decolorization

Effect of agitation on decolorization was checked at 37°C under static and agitated state (200 rpm) for MO, CR, and EBT. Dye concentration was 100 mg/l, and the study was carried out for 96 hours with decolorization percentage measured for every24-hour interval.

2.5. Effect of Temperature on Dye Decolorization

100 mg/l dye containing LB media was incubated at 25°C, 35°C, 45°C, and 55°C at pH 7 to study the effect of temperature on dye

decolourization. The test was conducted under static condition and decolourization was measured at every 24-hour interval for 96-hour duration.

3. RESULT AND DISCUSSION

3.1. Isolation, Screening, and Identification

EBT-2 strain was identified as *Citrobacter* sp. on basis of morphological, biochemical (Table 1), and 16s rDNA sequencing (Fig. 1). It matched 86% identity with *Citrobacter* sp. and fell in the cluster of *Klebsiella* sp.

3.2. Effect of Initial Dye Concentration on Dye Decolorization

The isolate was able to decolorize MO up to 300 mg/l (Fig. 2a–c) and both CR and EBT up to 500 mg/l dye concentration (Figs. 3a–c and 4a–c). Contrary to results shown by previous works [18,30], dye decolorization did not show a gradual decreasing pattern with increase in its initial concentration. It remained independent of dye concentration and drastically fell to ~50% for 300–500 mg/l dye concentration. It has been also shown that Acid Red dye decolorization by *Acinetobacter radioresistens* is independent of its concentration [31]. A probable reason for this observation can be extracellular reduction of dye, the rate of which is independent of its concentration [32]. The decrease in decolorization efficiency beyond 100 mg/l may occur due to several reasons like the toxicity of the dye to bacteria and/or insufficient biomass concentration for the uptake of higher concentrations of dye [33].

Table 1: Phenotypic and Biochemical characters of EBT-2 strain.

Characteristics	Result
Morphology	Creamish-yellow colonies with entire margin
Gram stain	−ve, Rod
Catalase test	−ve
Mannitol salt agar test	−ve
Indole test	+ve
Methyl red test	+ve
Voges-proskeur test	-ve
Citrate test	+ve
Oxidase test	-ve
Nitrate reduction test	+ve

3.3. Effect of Agitation on Dye Decolorization

MO showed 98% decolorization in 96 hours but could only achieve 10% decolorization under agitated condition for the same time period (Fig. 5a). Similar trend was observed for CR, it showed 95% decolorization in static and 18% decolorization in shaking condition in 96 hours (Fig. 5b). Eriochrome Black T showed 90% decolorization in static and 35% decolorization in shaking condition for the same time period (Fig. 5c). Dye decolorization was reduced significantly in shaking condition as compared to static condition. This agrees with the results previously demonstrated studies [34,35]. A possible cause for this trend could be that in many bacteria, degradation of azo dyes to their corresponding amines occurs due to reduction of azo linkage with the aid of cytoplasm azoreductase enzyme. Azoreductase mediated degradation of azo dyes is inhibited by the presence of oxygen because oxygen is a preferable terminal electron acceptor over the azo groups for the oxidation of reduced electron carriers

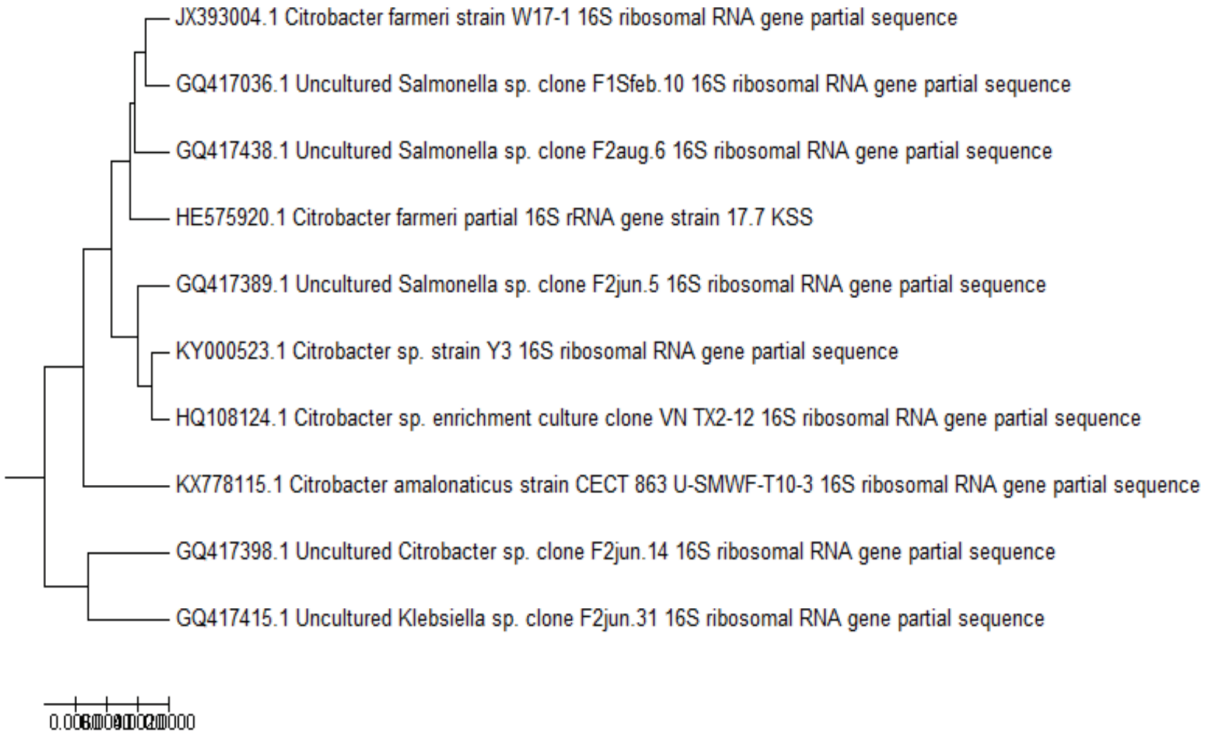
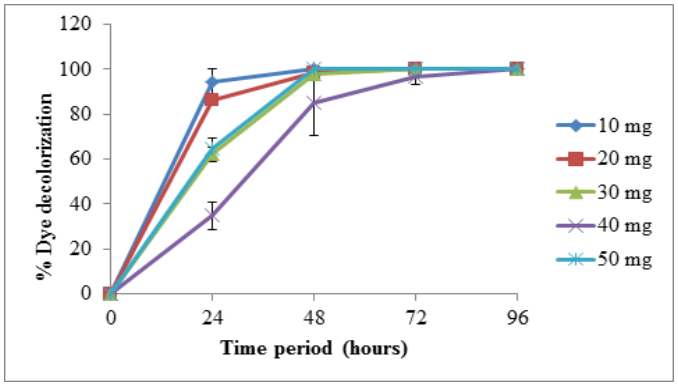


Figure 1: Phylogenetic tree of the *Citrobacter* sp. EBT-2 strain based on 16S rDNA partialsequences.



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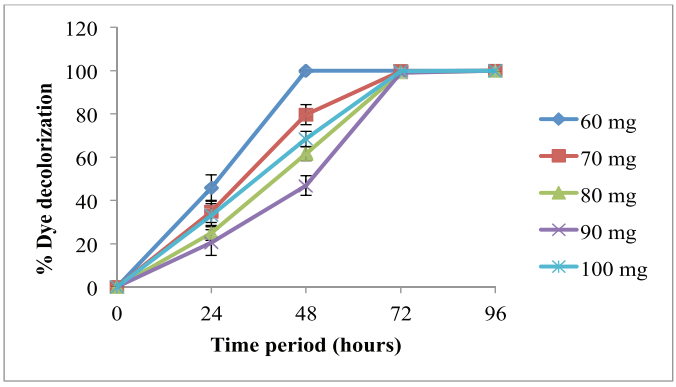

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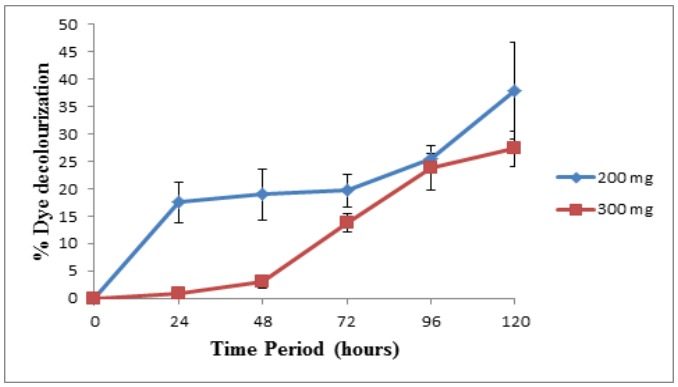
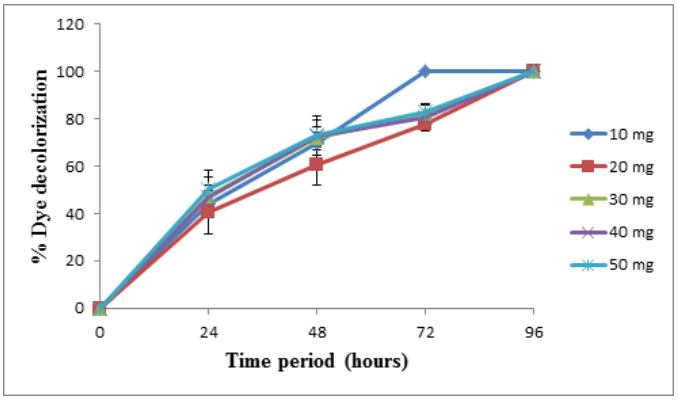


(c)

Figure 2: (a) Effect of dye concentration on dye colorization (MO: 10–50 mg/l). (b) Effect of dye concentration on dye decolorization (MO: 60–100 mg/l). (c) Effect of dye concentration on dye decolorization (MO: 200, 300 mg/l).



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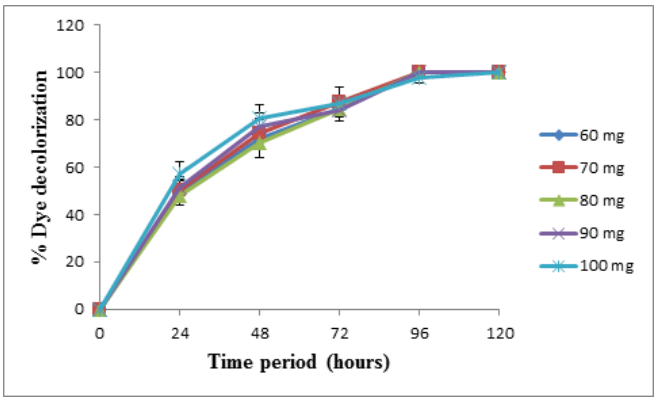

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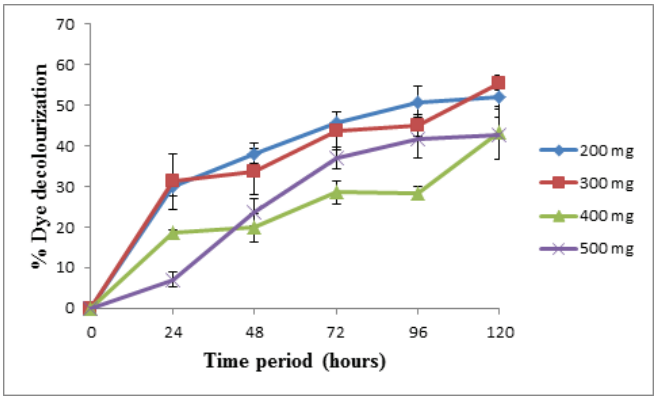


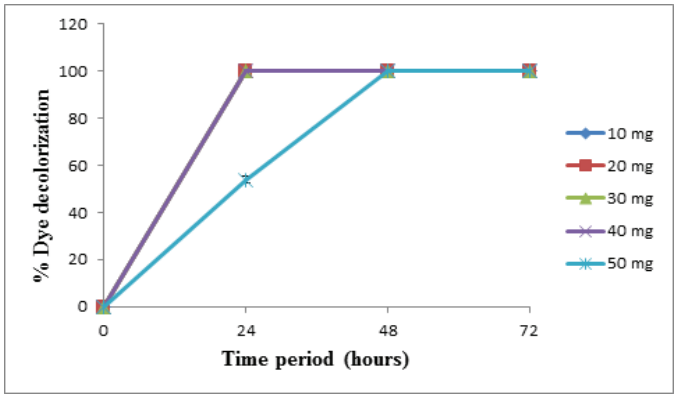
(c)

Figure 3: (a): Effect of dye concentration on dye decolorization (CR; 10–50 mg/l). (b): Effect of dye concentration on dye decolorization (CR; 60–100 mg/l). (c): Effect of dye concentration on dye decolorization (CR: 200–500 mg/l).

such as nicotinamide adenine dinucleotide - hydrogen (reduced) [22,36].

3.4. Effect of pH on Decolorization

MO and CR showed maximum decolorization at pH 7 in 96 hours (97.24% and 95.23%, respectively) (Fig. 6a,b). Maximum decolorization was observed in a range of pH 7–9 (~85%) for Eriochrome Black T (Fig. 6c). Dye decolorization decreased at lower pH (5–6) and very high pH (10–11). It was observed that in general, azo dye show better decolorization from neutral to alkaline conditions. Similar results were observed for decolorization of several azo dyes by *Micrococcus* sp. [37]. Since alkaline environment is required for binding



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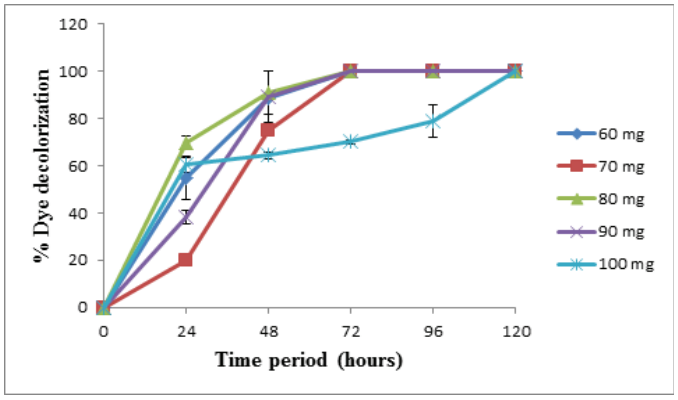

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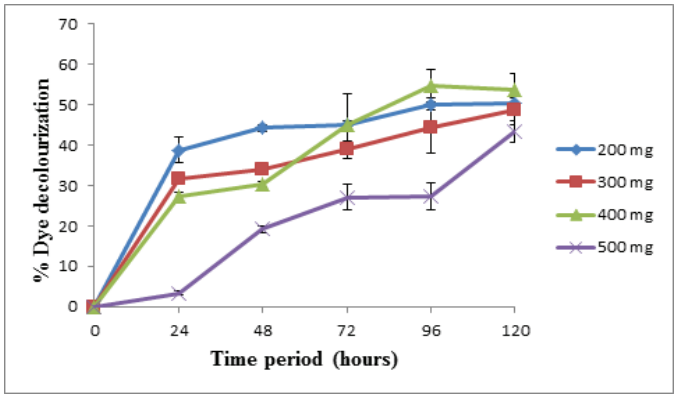

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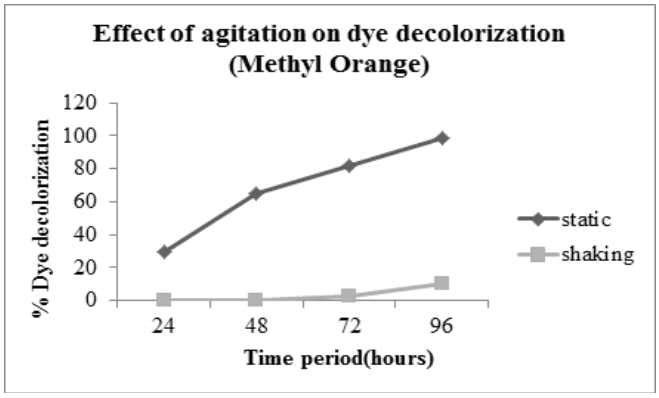
Figure 4: (a) Effect of dye concentration on dye decolorization (EBT: 10–50 mg/l). (b) Effect of dye concentration on dye decolorization (EBT: 60–100 mg/l). (c) Effect of dye concentration on dye decolorization (EBT: 200–500 mg/l).



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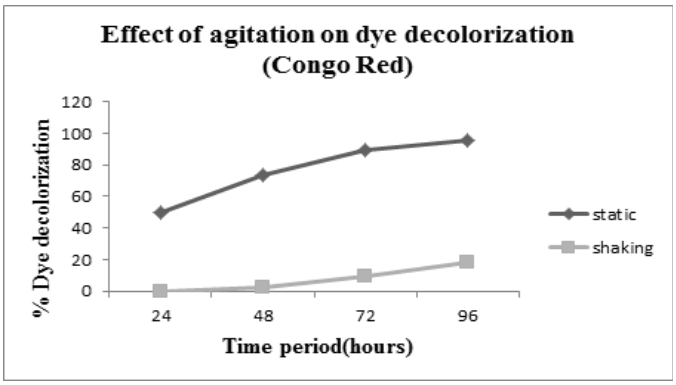

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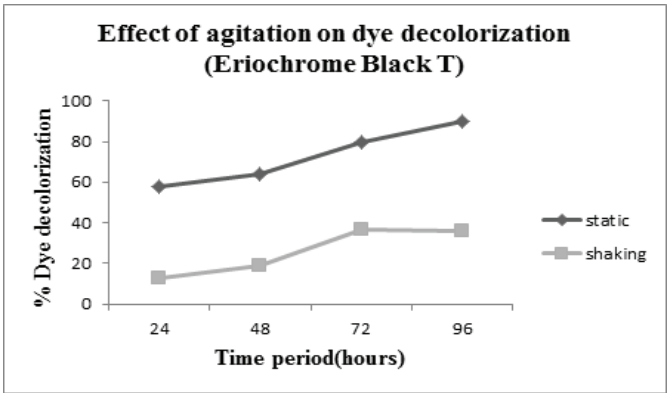


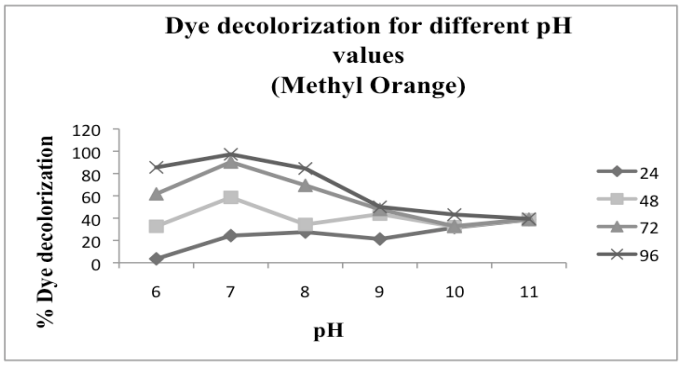
(c)

Figure 5: (a) Effect of agitation on dye decolorization (MO; 100 mg/l). (b) Effect of agitation on dye decolorization (CR; 100 mg/l). (c) Effect of agitation on dye decolorization (EBT; 100 mg/l).

of most azo dyes to fibers, dye degrading bacteria are better adapted to alkaline environment [38]. However, the dye degradation pattern for all the dyes treated with EBT-2 strain are different from each other, which prove that degradation is dependent on the chemical structure and reactivity of dyes as well.

3.5. Effect of Temperature on Dye Decolorization

EBT-2 completely decolorized MO at both 35°C and 45°C in 120 hours (Fig. 7a). It also decolorized EBT completely at both 35°C and 45°C. However, decolorization at 45°C was achieved much faster, i.e., in 72 hours as compared to 120 hours at 35°C (Fig. 7c). It decolorized CR completely only at 35°C in 120 hours (Fig. 7b). For all the three dyes, decolorization efficiency increased



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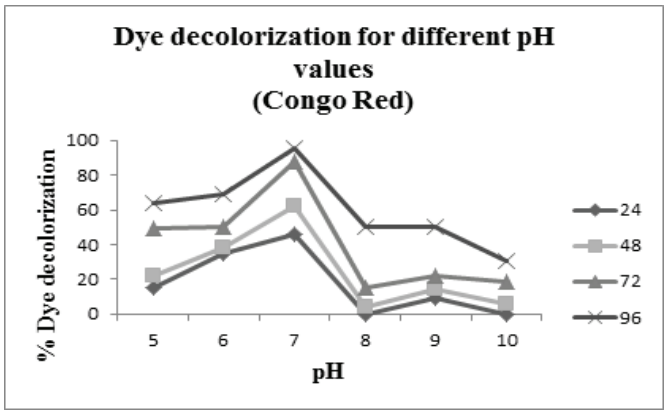

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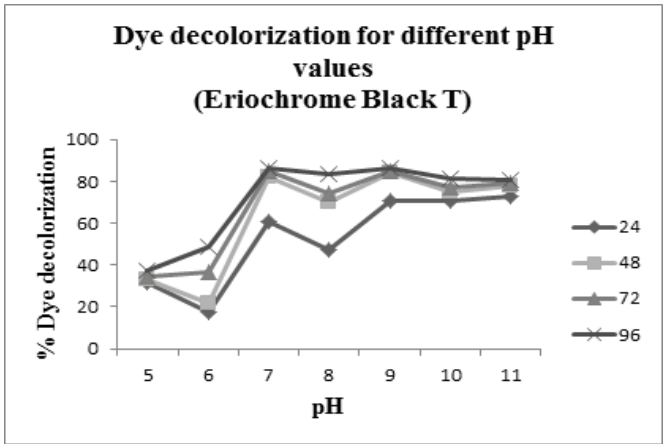

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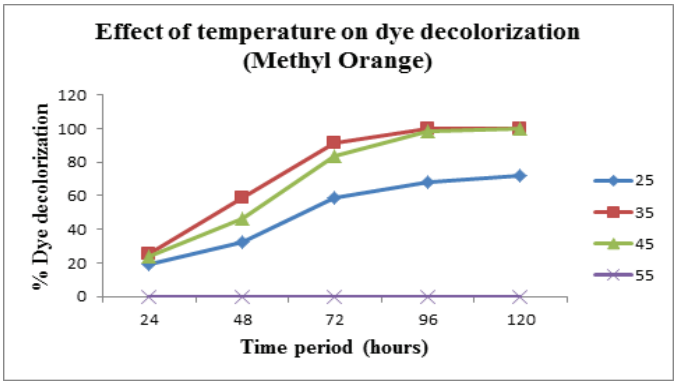
Figure 6: (a) Effect of pH on dye decolorization (MO; 100 mg/l). (b) Effect of pH on dye decolorization (CR; 100 mg/l) (c) Effect of pH on dye decolorization (EBT; 100 mg/l).

with increase in temperature from 25°C to 35–45°C. This is in accordance with results previously given for decolorization of Acid Orange by *Staphylococcus hominis* and decolorization of azo dyes by *Micrococcus* sp. [30,37,39]. Increase in temperature can increase bacterial growth hence increasing decolorization



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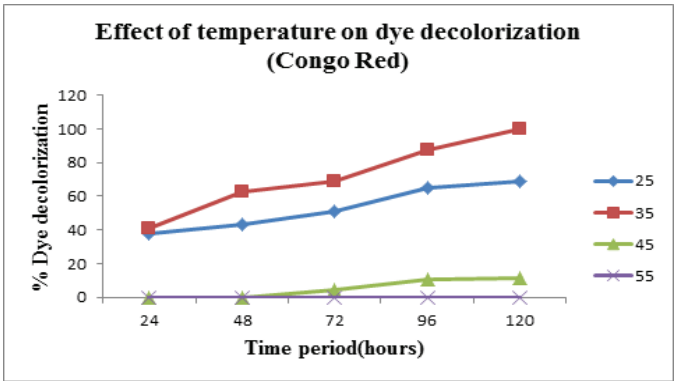

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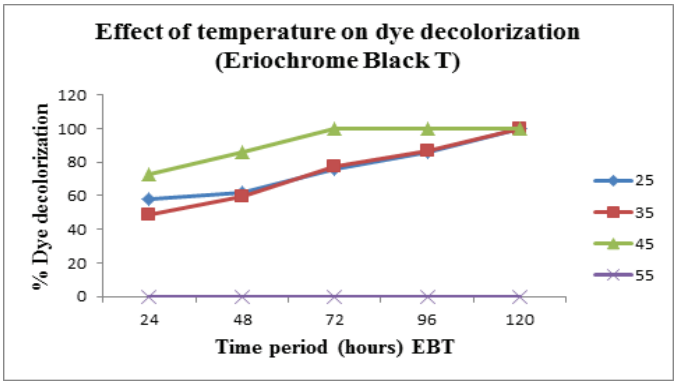

(c)

Figure 7: (a) Effect of temperature on dye decolorization (MO; 100 mg/l) (b) Effect of temperature on dye decolorization (CR; 100 mg/l) (c):Effect of temperature on dye decolorization (EBT; 100 mg/l).

efficiency [19]. No decolorization was observed at 55°C for all three dyes which can be caused due to loss of Azoreductase enzymatic activity at high temperature or decrease in cell viability [39].

4. CONCLUSION

EBT-2 strain was identified as *Citrobacter* sp. It showed complete decolorization of all the three dyes up to 100 mg/l dye

concentration in 96–120 hours. It was able to decolorize MO, CR, and EBT up to 300, 500, and 500 mg/l of dye concentration, respectively. Optimum pH for decolorization was 7, 7, and 9 for MO, CR, and EBT, respectively. Similar results were observed for decolorization of several azo dyes by *Micrococcus* sp. [37]. Effect of agitation on decolorization was studied under static and shaking (200 rpm) condition. More than 90% decolorization was observed at static condition for each dye but only 10.3%, 18.47% and 35.92% decolorization was observed at shaking condition in MO, CR, and EBT respectively in 96 hours. This agrees with the results previously demonstrated by bacterial degradation of Reactive Red 141 and Amaranth dyes [34,35]. Complete decolorization was obtained for MO and EBT at 35°C and 45°C. CR showed complete decolorization only at 35°C. This is in accordance with results previously given for decolorization of Acid Orange by *S. hominis* and decolorization of azo dyes by *Micrococcus* sp. [30,37,39]. Temperature increase can stimulate bacterial proliferation and increase decolorization efficiency [19].

The results conclude that *Citrobacter* sp. can be used for successful dye decolorization of Azo dyes; primarily MO, CR, and EBT under optimum physiochemical conditions. Further work needs to be done on complete mineralization of dyes. Scaling up the process also remains a challenge. Since metal ions like Copper, Lead, and Cadmium are present in high concentration in industrial effluents [23], it is also important to study their effects individually on azo dye bioremediation.

5. AUTHORS' CONTRIBUTION

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

6. ETHICAL APPROVAL

This study does not involve the use of animals or human subjects.

7. CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

8. FUNDING

None

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