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Kinetic study approach of remazol black-B use for the development of two-stage anoxic–oxic reactor for decolorization/biodegradation of azo dyes by activated bacterial consortium

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ABSTRACT

The laboratory-isolated strains *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides*, *Proteus mirabilis*, *Bacillus circulance*, *NAD 1* and *NAD 6* were observed to be predominant in the bacterial consortium responsible for effective decolorization of the azo dyes. The kinetic characteristics of azo dye decolorization by bacterial consortium were determined quantitatively using reactive vinyl sulfonated diazo dye, remazol black-B (RB-B) as a model substrate. Effects of substrate (RB-B) concentration as well as different substrates (azo dyes), environmental parameters (temperature and pH), glucose and other electron donor/co-substrate on the rate of decolorization were investigated to reveal the key factor that determines the performance of dye decolorization. The activation energy (E_a) and frequency factor (K_0) based on the Arrhenius equation was calculated as 11.67 kcal mol^{-1} and 1.57×10^7 mg l g MLSS^{-1} h^{-1}, respectively. The Double-reciprocal or Lineweaver–Burk plot was used to evaluate V_{max}, 15.97 h^{-1} and K_m, 85.66 mg l^{-1}. The two-stage anoxic–oxic reactor system has proved to be successful in achieving significant decolorization and degradation of azo dyes by specific developed bacterial consortium with a removal of 84% color and 80% COD for real textile effluents vis-à-vis ≥90% color and COD removal for synthetic dye solution.
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1. Introduction

Over 7×10^5 metric tones of synthetic dyes are produced worldwide every year for dyeing and printing and out of this about 5–10% are discharged with wastewater [1,2]. The amount of dye lost depends on the class of dye applied; it varies from 2% loss while using basic dyes to about 50% loss in certain reactive sulfonated dyes [3,4]. Azo dyes account for the majority of all textile dye stuffs produced and have been the most commonly used synthetic dyes in the textile, food, paper making, color paper printing, leather and cosmetic industries [5–7]. Even a small amount of dye in water (10–15 mg l^{-1}) is highly visible, affecting the aesthetic merits, water transparency and gas solubility of water bodies [8].

Dyestuffs reach the aquatic environment, primarily through dissolved or suspended form in water. The conventional treatment of wastewater from textile mills and dyestuff factories cannot remove most azo dyes effectively. The resulting dye effluents may contain some components or moieties that could be toxic, carcinogenic or mutagenic, to aquatic life [9]. With increasing use of a wide variety of dyes, pollution due to dye wastewaters is becoming increasingly alarming in terms of color removal and is of major scientific interest. Government legislation, especially in the developed countries is very stringent for concentration of dye in industrial effluents. Environmental policy in UK, since September 1997 has stated that zero synthetic chemicals should be released in to the marine environment. European community (EC) regulations are also becoming more stringent [10].

Physicochemical methods such as coagulation and flocculation [11], adsorption [12], ozonation [13], photochemical oxidation [14], membrane filtration [13] and electrochemical oxidation [15] have been used for the treatment of dye containing wastewater [16]. Limitations of these methods are the cost and generation of huge quantities of sludge leading to secondary pollution [17]. Biological degradation of azo dyes is mostly carried out by anaerobic bacteria [18]. Moreover, such decolorization/degradation is found to be environment friendly and cost effective in comparison to chemical decomposition processes [19].

Environmental biotechnology is continuously expanding within the area of bioremediation of all kinds of industrial effluents. However, several studies reported on decolorization of dyes using a pure isolated bacterial cultures [7,18,19]. These studies revealed that the isolated cultures are often specific to a type of dye used. Moreover, pure cultures cannot be easily scaled up and

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maintained in large-scale operations typical of wastewater treatment systems [20,21]. Utilization of microbial consortium offers considerable advantages over the use of pure cultures in the degradation of synthetic dyes. Prerequisite for the mineralization of many azo dyes are combinations of reductive and oxidative steps [22]. The aromatic amine intermediates, which are produced during the anaerobic phase, are toxic and carcinogenic [23], but can be further degraded during aerobic step to become less harmful products [24]. Anoxic–oxic (A-O) process is a good choice to simultaneously remove chemical oxygen demand (COD) and dye color.

This paper reports the investigation results and kinetic studies of azo dye decolorization. The present study deals with the bacterial decolorization /degradation of dye RB-B and optimization of parameters required for the bacterial consortium to decolorize the dyes efficiently. Laboratory scale treatment of RB-B and textile industrial wastewater through anoxic–oxic reactor system is also reported.

2. Materials and methods

2.1. Dyes and reagents

The dyes C.I. remazol black-B (RB-B) [2,7-napthalenedisulfonic acid, 4-amino-5-hydroxy-3,6-bis((4-((2-(sulfooxy)ethyl)sulfonyl)-azo)-tetrasodium salt] (CAS Reg. No. 17095-24-8), reactive red 11 (RR 11), reactive red 141 (RR 141), reactive orange 16 (RO 16), reactive violet 13 (RV 13), acid yellow 36 (AY 36), and direct green 6 (DG 6) were obtained from a manufacturing unit in Gujarat. The media components and chemicals were purchased from Hi-media labs, Bombay, India. All chemicals used were of analytical grade. The chemical structures of the dyes used in the study are shown in Fig. 1 except reactive violet 13, which is not available.

2.2. Dye solution

RB-B, a sulfonated diazo dye, which is soluble in water and acidic in nature, was dissolved in double distilled water to prepare stock solution (10,000 mg l^{-1}). The desired dye concentrations were obtained by successive dilutions. The other seven dyes were used only for substrate specificity and RB-B was used as a model dye for further experiments.

Decolorization test solutions were prepared by addition of dye stock solutions, growth medium and cell suspension (inoculum).

2.3. Acclimatization

The procedure for obtaining acclimatized bacterial cultures effective towards decolorization/biodegradation of RB-B was reported in previous paper [25]. The culture was gradually exposed to increasing concentrations of RB-B dye to acclimatize the microbial culture. Successive transfer of culture into fresh nutrient medium containing higher concentration of RB-B was done at 37 °C in static condition. This acclimatized microbial culture was used for all studies.

2.4. Isolation and identification of strains

The strains identified in the current study are *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides*, *Proteus mirabilis*, *NAD 1*, *NAD 6* and *Bacillus circulance*. All are Gram negative except *B. circulance*, which is Gram positive. Isolation of bacterial cultures was carried out by inoculating 0.5 ml suspended anoxic bacterial culture to 50 ml nutrient medium. This enriched culture was incubated at 37 °C when the medium became colorless and turbid; a loop of the culture was used to inoculate on agar plates. After 48-h of incubation the plates containing bacterial colonies were picked up on the basis of its ability to form clear zones. These cultures were subsequently transferred to a nutrient medium containing 200 mg l^{-1} RB-B dye. For identification, these cultures were further isolated on nutrient agar. Five colonies were picked up according to their different morphological appearance and their ability to form clear zone in petri plates. These strains were identified by the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTech), Chandigarh, India, on the basis of morphological, physiological and biochemical characteristics.

2.5. Study of physicochemical parameters

Batch scale decolorization experiments were performed in 250-ml conical flask reactors. A nutrient rich medium comprising dye RB-B, nutrient broth, inorganic salts (K_2HPO_4, KH_2PO_4 and NH_3PO_4) and glucose was inoculated with bacterial inoculum. Biomass was developed in the flask until it attained an adequate mixed liquor suspended solids (MLSS) concentration ($\approx 1600 \pm 50$ mg l^{-1}). This biomass was used as inoculum for all batch decolorization experiments. Decolorization was studied using various co-substrates/electron donors (glucose, dextrose, maltose, starch, tryptone, lactose and sucrose), at different dye concentration (25, 50, 75, 100, 200, 500, 1000 and 2000 mg l^{-1}), temperature (20, 25, 30, 35, 37, 40 and 50 °C) and pH (5, 6, 7, 8, 9 and 10). pH adjustment was done with 2N KOH or 2N HCl. Batch decolorization assay at fixed concentration (100 mg l^{-1}) was also performed for all the other chosen dyes in order to test the substrate specificity and efficiency of anoxically acclimatized bacteria. The samples were drawn at different time intervals (0, 4, 8, 12 and 24 h), centrifuged at $15{,}000 \times g$ for 10 min and analyzed for decolorization. A biotic control without cultures was always included.

2.6. Lab-scale two-stage anoxic–oxic continuous reactor

The decolorization and biodegradation experiments were carried out in continuous anoxic–oxic reactors of volume 5.2 and 5.0 l, respectively. The working volume for both the reactors was 4.2 l. The effluent of anoxic reactor, operated at different hydraulic residence times (HRT) was passed through clarification unit (230 ml) using coal bed as media. The clarified anoxic effluent was treated in aerobic reactor. The aerobic effluent was clarified in a settling unit of 1.2 l capacity. Feeding solution (influent) consisting of proposed nutrient medium with dye RB-B was fed vertically at the bottom and flows out from top of the other side of reactor. Initially, the bacterial culture was grown in the nutrient rich medium by inoculating acclimatized bacterial culture. The biomass was grown separately in the anoxic and oxic reactors till it attained an adequate mixed liquor suspended solids concentration of $\approx 1600 \pm 50$ mg l^{-1} and $\approx 2800 \pm 100$ mg l^{-1}, respectively. After the development of adequate biomass, the dye solution (100 mg l^{-1}) was fed into the reactor. Anoxic reactor was maintained at ambient temperature of 37 °C ± 0.2 and pH 7.0. The contents in the anoxic reactor were stirred at 10 rpm continuously throughout the experiment. Dye concentration (25, 50, 75 and 100 mg l^{-1}) and HRT (10, 20, 24, 30 and 35 h) was varied in the course of the study. Once the reactor was energized, the dye concentration was increased from 25 to 100 mg l^{-1} and the decolorization was monitored. At each concentration of RB-B and HRT the reactor was operated for 10 days. Before changing the dye concentration and HRT of the feed solution, reactor was operated for 2 days only with nutrient medium in the feed. Dye concentration and COD in the effluent were determined daily.

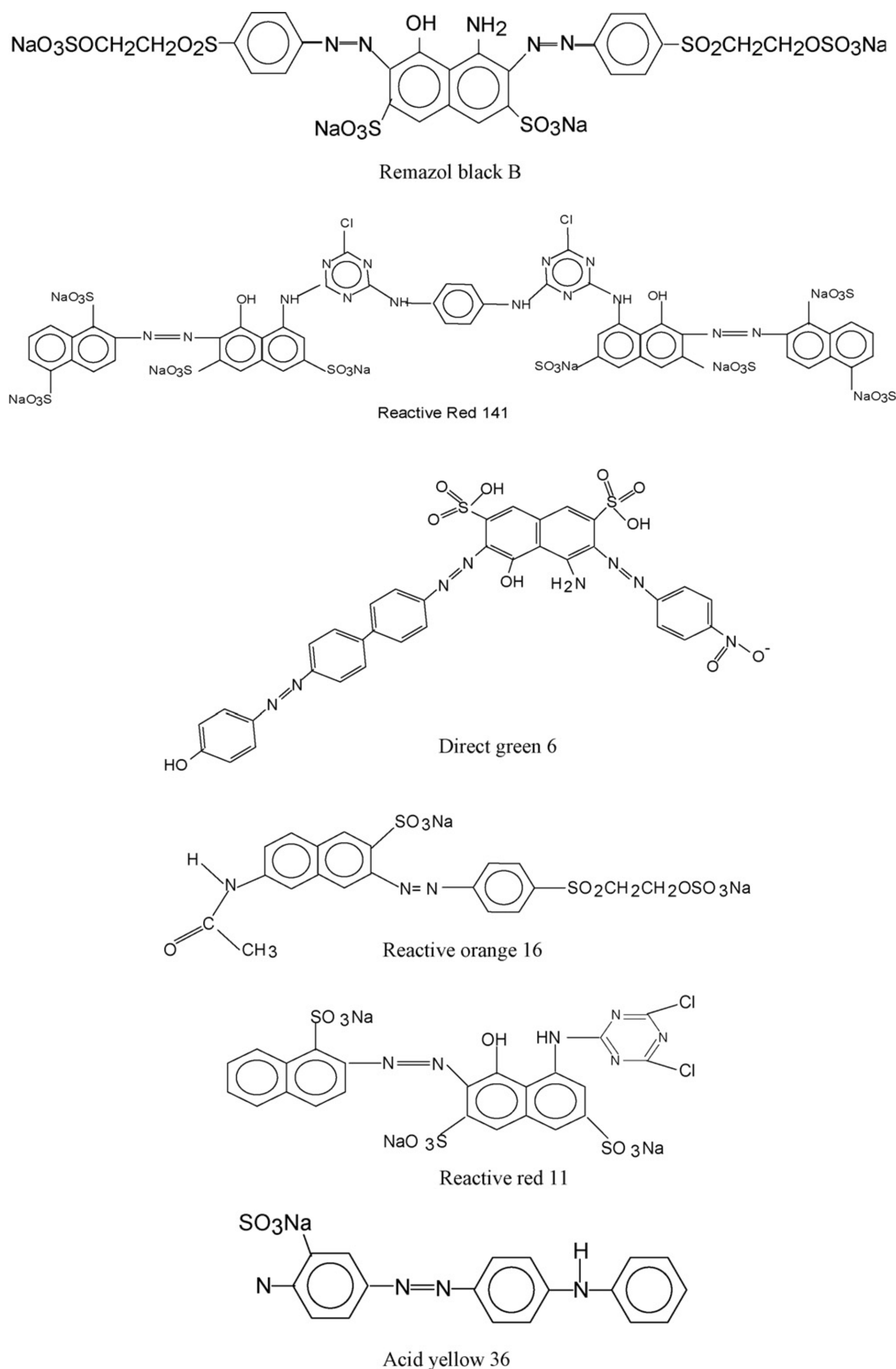

Fig. 1. Chemical structures of azo dyes used in the study.

2.7. Treatment of textile effluent

Textile industry effluent containing a mixture of dyes was procured from textile manufacturing unit locally. The wastewater was collected from the equalization tank of the effluent treatment plant. Physicochemical analyses were performed as per standard methods [26]. The textile effluent was treated in lab-scale anoxic–oxic reactor. The textile wastewater was supplemented with $2\,g\,l^{-1}$ glucose and minerals (K_2HPO_4, NH_4Cl and KH_2PO_4), which was fed in to the reactor with HRT of 24 h ($2\,ml\,min^{-1}$).

2.8. Analysis

UV–vis absorption spectra were obtained using a double-beam UV–vis spectrophotometer (Shimadzu UV-1650PC). The concentration of RB-B dye was determined by measuring the absorbance of test samples photometrically at the absorbance maximum of RB-B (λ_{max}, 597 nm) in the visible range. A calibration plot between concentration and absorbance of RB-B was used for determination of RB-B concentration extinction coefficient ($\varepsilon_{597} = 0.0304\,mg^{-1}\,l\,cm^{-1}$). The decolorization efficiency was

reported as

$$\text{percent decolorization} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 is the initial absorbance and A_t is the absorbance at incubation time, t. Percentage decolorization was calculated from the absorption values obtained against the uninoculated controls. The biomass from test samples was removed by filtration through 0.45 μm membrane filters prior to absorbance measurement. Decolorization was measured at different time intervals (4, 8, 12 and 24 h) at the wavelength in the visible range where maximum absorbance was obtained. The aromatic amines formed in the anoxic reactor were analyzed spectrophotometrically by their absorbance peaks at the wavelength in the UV range where maximum absorbance was obtained. Reaction rate constants were deduced from the slopes of $-\log[A_t/A_0]$ versus incubation time (t) and by performing linear regression. Each data point represented the mean value of at least two values obtained from duplicate tests.

COD of filtered samples (0.45 μm membrane filter paper) was determined using open reflex method and other physicochemical analysis was performed as per standard methods [26].

3. Results and discussion

3.1. Mechanism of microbial decolorization

Decolorization of dye solution by bacteria could be due to adsorption to microbial cell or biochemical reaction leading to biodegradation. In adsorption, examination of the absorption spectrum would reveal that all peaks decreased approximately in proportion to each other. If dye removal is attributed to latter phenomenon, either the major visible light absorbance peak would completely disappear or a new peak would appear.

The change of UV–visible spectra of RB-B, using the supernatant fluid of the culture, before and after decolorizing cultivation with activated bacterial culture. The absorbance peak at 597 nm completely disappeared and new peak appeared at 267 nm due to decolorization of dye. In addition, as the azo dye was reduced (phenomenon leading to decolorization), the broth returned to its original white color. These results indicate that the color removal by activated anoxic microbial consortium may be largely attributed by biodegradation.

3.2. Effect of temperature and pH

The experiments were performed at different temperatures and pH; the initial RB-B concentration was fixed (100 mg l^{-1}). Michaelis–Menten kinetics relation can describe the correlation between specific decolorization rate with dye concentration, temperature and pH. Over a range of 20–37 °C, the specific decolorization rate increased as the temperature rose (Fig. 2a). Further increase in temperature above 40 °C resulted in marginal reduction in decolorization activity of bacterial consortium. Decline in decolorization activity at higher temperature can be attributed to the loss of cell viability or the denaturation of the azo reductase enzyme [4]. The maximum specific decolorization rate of RB-B was found to be 5.81 mg g cell^{-1} h^{-1} at 37 °C. Maximum potential of *Pseudomonas* sp. to decolorize malachite green, fast green, brilliant green, congo red and methylene blue was noticed at 37 °C [27]. The results showed no thermal deactivation of the decolorization activity during normal operation temperature. Increase in the temperature from 20 to 37 °C, increase the first-order kinetic rate constant (k_1) values from 3.27×10^{-2} to 9.29×10^{-2} in batch studies. Whereas beyond that temperature the k_1 values reduced to 3.86×10^{-2} at 40 °C (Table 1a).

The effect of pH on RB-B decolorization in anoxic reactor was examined by varying pH from 5.0 to 10.0. It was observed from Fig. 2b that the specific decolorization rate increased with increasing pH from 5.0 to 7.0, which remained approximately the same for pH 7.0–8.0. This seems to indicate that neutral and slightly basic pH values would be more favorable for decolorization of RB-B. Maximum specific decolorization rate of RB-B determined as 5.58 mg g cell^{-1} h^{-1} at the pH of 7.0 (Fig. 2b). However above the pH 8.0 the specific decolorization rate dropped dramatically. The decolorization rate constant (k_1) value increase slightly from 3.22×10^{-2} to 8.92×10^{-2} with increase in pH 5.0–7.0. Further increase in pH the k_1 values reduced (Table 1b).

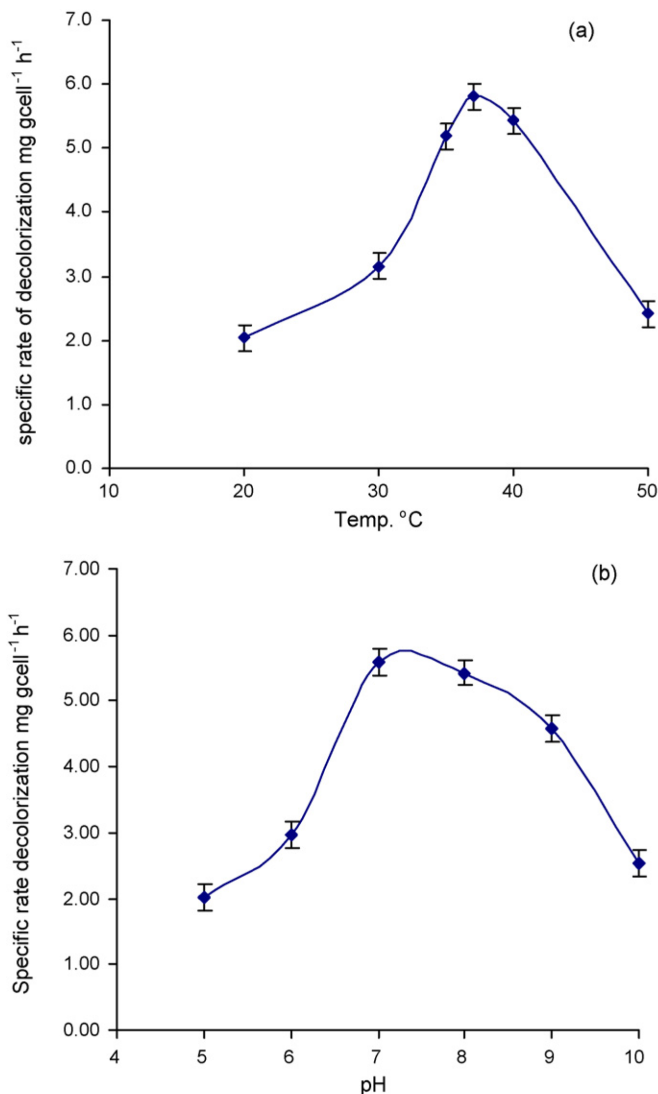

Fig. 2. Dependence of specific decolorization rate on (a) temperature; (b) pH [RB-B] = 100 mg l^{-1}.

3.3. Kinetic study of RB-B decolorization

The maximum decolorization rate of RB-B was measured by test solution containing 100 mg dye, ≈1600 ± 50 mg l^{-1} MLSS and growth medium, incubated under anoxic conditions at 37 °C for 24 h. Michaelis–Menten type rate model equation has been widely used for the kinetics of substrate conversion by enzyme and or living cells. The primary function of enzymes is to enhance rate of reaction so that they are compatible with need of microorganism. To understand how system functions we need a kinetic description of their activity.

Table 1
First-order kinetic constants obtained in anoxic batch tests decolorization of azo dyes

(a) Different temperature

Constants	20 °C	30 °C	35 °C	37 °C	40 °C	50 °C
K_1	3.27×10^{-2}	5.06×10^{-2}	8.30×10^{-2}	9.29×10^{-2}	8.69×10^{-2}	3.86×10^{-2}
R^2	0.9167	0.9694	0.9755	0.9817	0.9701	0.9366

(b) Different pH

Constants	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
K_1	3.22×10^{-2}	4.74×10^{-2}	8.92×10^{-2}	8.69×10^{-2}	7.34×10^{-2}	4.07×10^{-2}
R^2	0.9299	0.9734	0.9801	0.9715	0.9712	0.97

(c) Different concentration of RB-B

Constants	25 mg l^{-1}	50 mg l^{-1}	75 mg l^{-1}	100 mg l^{-1}
K_1	2.277×10^{-1}	1.527×10^{-1}	1.118×10^{-1}	9.06×10^{-2}
R^2	0.9015	0.969	0.9782	0.9827

(d) With or without addition of glucose at different concentration of RB-B; [Glucose] = 2 g l^{-1}

Constants	100 mg l^{-1}	200 mg l^{-1}	500 mg l^{-1}	1000 mg l^{-1}	2000 mg l^{-1}
K_1 (Without glucose)	3.66×10^{-2}	2.65×10^{-2}	1.91×10^{-2}	1.52×10^{-2}	1.24×10^{-2}
R^2	0.9592	0.956	0.9023	0.9656	0.9565
K_1 (with-glucose)	780×10^{-2}	515×10^{-2}	324×10^{-2}	191×10^{-2}	161×10^{-2}
R^2	0.9378	0.9657	0.8804	0.8832	0.8795

(e) Different electron donor/co-substrate; [E.D.] = 2 g l^{-1}

Constants	Blank	Glucose	Dextrose	Lactose	Sucrose	Mannitol	Tryptone	Starch
K_1	6.05×10^{-2}	9.48×10^{-2}	8.67×10^{-2}	8.53×10^{-2}	8.35×10^{-2}	7.43×10^{-2}	7.31×10^{-2}	7.13×10^{-2}
R^2	0.9771	0.9909	0.9855	0.9857	0.9913	0.9731	0.9553	0.9503

(f) Different azo dyes; [dye] = 100 mg l^{-1}

Constants	RB-B	RM-13	RR-11	RR-141	AO-16	DG-6	AY-36
K_1	9.09×10^{-2}	4.35×10^{-2}	4.21×10^{-2}	3.86×10^{-2}	2.39×10^{-2}	2.09×10^{-2}	1.40×10^{-2}
R^2	0.9817	0.9704	0.9001	0.9883	0.9122	0.8864	0.5579

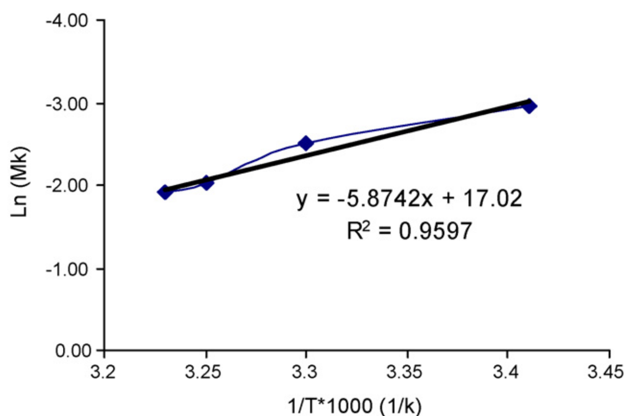

Fig. 3. Estimation of activation energy of RB-B decolorization by Arrhenius equation: [RB-B] = 100 mg l^{-1}; initial pH 7.0.

The two model parameters (V_{max} and K_m) of the non-linear rate equation. have specific rates (k_1, $k-1$ and k_2) as a function of temperature. Because of the nonlinearity, the temperature effect on the overall enzymatic rate is usually analyzed by assuming an Arrhenius equation between V_{max} and temperature but neglecting the temperature effect on K_m [28].

$$V_0 = \frac{-d[S]}{dt} = V_{max}\frac{[S]}{[S]+K_m} \quad (1)$$

A general kinetic model (Eq. (2)) of dye decolorization gives the convenience on analysis of temperature effect.

$$\frac{dA}{dt} = -kM^m A^n \quad (2)$$

where t is the time (h), M and m the cell mass concentration (MLSS, mg l^{-1}) and its reaction order (first order), respectively, A and n are the dye concentration (mg l^{-1}) and its reaction order (first order), respectively. The units of k, the specific decolorization rate, depend on the values of m and n (mg$^{(1-n)}$ l$^{(m+n-1)}$/mg MLSS^m h). This rate model (Eq. (2)) can be taken as a modified type of the Michaelis–Menten rate model (Eq. (1)) where the parameter K_m is not included. Eq. (3) gives dye concentration with time depending on the partial reaction order of dye concentration (n) and Eq. (4) depicts first order of dye concentration (n).

$$\left(\frac{A_t}{A_0}\right)^{(1-n)} = 1 - \frac{(1-n)kM^m}{A_0^{(1-n)}}t \quad (n \neq 1) \quad (3)$$

and

$$\frac{A_t}{A_0} = \exp(-kM^m t) \quad (n = 1) \quad (4)$$

Integrating the above expression with the limit from $[A] = [A_0]$ to $[A]$ and from $t = 0$ to t

$$\ln\left[\frac{A_t}{A_0}\right] = -kM^m t \quad (5)$$

$$-\log\left[\frac{A_t}{A_0}\right] = \frac{kM^m t}{2.3} \quad (6)$$

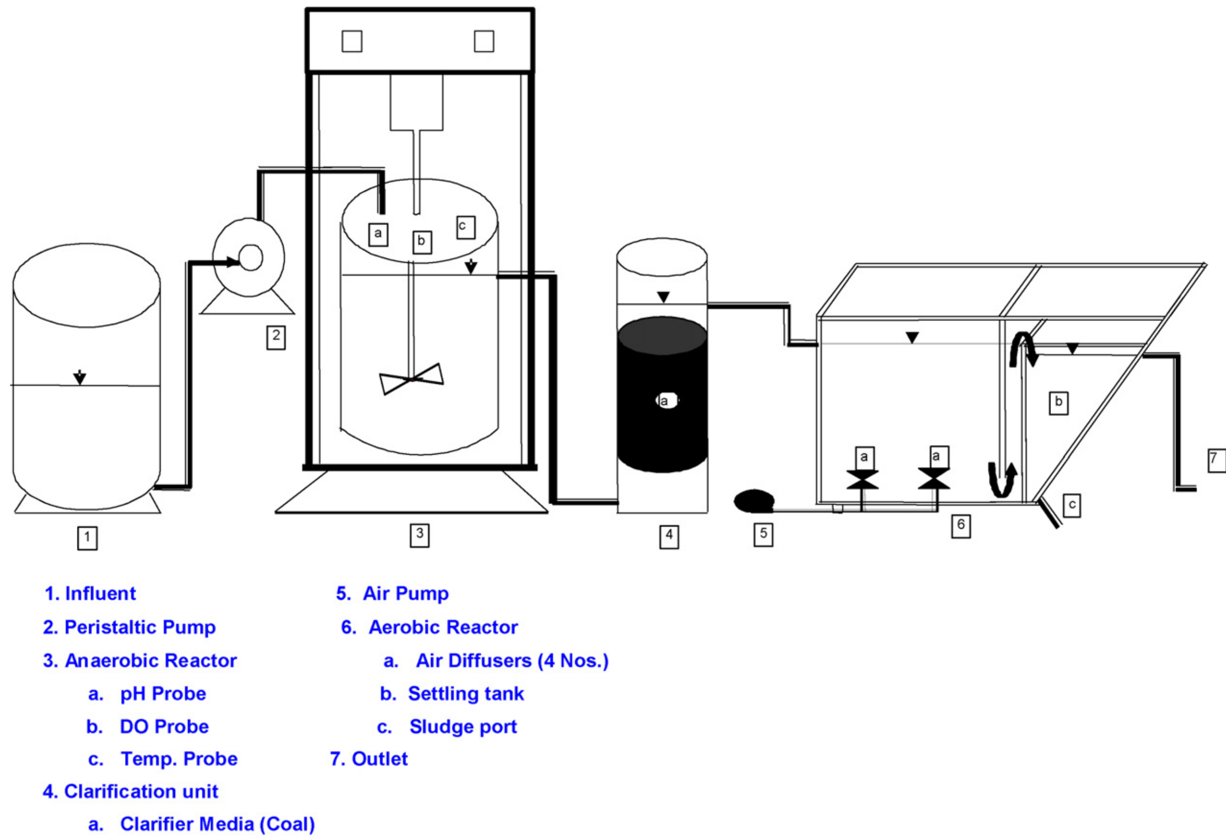

Fig. 4. Experimental set up of laboratory scale RB-B degradation by two-stage anoxic–oxic reactor system.

Since cell growth or death was not observed in the nutrient-containing test solution in 24 h, the MLSS concentration in decolorization experiment rate was a constant. For the particular case of a first-order reaction with respect to dye concentration ($n = 1$) at constant MLSS (M^m = constant), Eq. (6) can be simplified to Eq. (7)

$$\frac{A_t}{A_0} = e^{-kt} \quad (7)$$

$$-\log\frac{A_t}{A_0} = \frac{kt}{2.3} \quad (8)$$

In order to approximate the reaction by first-order kinetics with respect to the dye concentration, the values of $-\log[A_t/A_0]$ were plotted against time, using data of decolorization. A high-degree linear relationship ($R^2 > 0.90$) between the $-\log[A_t/A_0]$ and time, giving a first-order reaction ($n = 1$). The reaction rate constant is obtained graphically by plotting $-\log[A_t/A_0]$ versus t. First-order kinetics with respect to dye concentration has also been reported by several researchers [29,30], whereas some have reported zero-order kinetics [31,32]. The kinetic rate constants and correlation coefficients of various parameters relevant to the first orders are summarized in Table 1.

3.3.1. Effect of initial RB-B concentration and glucose on first-order kinetics

The specific decolorization rate mostly depends on the initial concentration of RB-B. It was observed that first-order kinetic constant decreased slightly (2.277×10^{-1} to $9.06 \times 10^{-2}\,h^{-1}$) as the concentration of RB-B increased from 0 to 100 mg l^{-1}. The correlation coefficients (R^2) were in the range of 0.90–0.98, which indicates the first-order kinetics. The results are depicted in Table 1c.

The batch studies with and without-glucose as a co-substrate was performed at higher concentration (100–2000 mg l^{-1}). The kinetic rate constants without-glucose decreased marginally, however with-glucose it varied significantly as the concentration increased from 100 to 2000 mg l^{-1}. The correlation coefficients of all these studies were determined and observed to follow the first-order kinetics. The decolorization kinetic constants with and without glucose at concentration of 100–2000 mg l^{-1} were found to be in the range of 7.80×10^{-2} to $1.61 \times 10^{-2}\,h^{-1}$ and 3.66×10^{-2} to $1.24 \times 10^{-2}\,h^{-1}$, respectively (Table 1d). Increase in dye concentration from 0 to 2000 mg l^{-1} reduced the k values from 7.80×10^{-2} to $1.61 \times 10^{-2}\,h^{-1}$ in batch study performed with RB-B and glucose. The decolorization rate constants with-glucose are significantly higher than the rate constants without-glucose.

3.3.2. Decolorization kinetics of electron donor/co-substrate and different azo dyes for optimum efficiencies

The electron donor/co-substrate was used to enhance the efficiency of decolorization. The seven electron donors were used in the studied of RB-B (100 mg l^{-1}) decolorization. The glucose was found to be better among them. The decolorization kinetic rate constant of glucose, dextrose, lactose, sucrose, mannitol, tryptone, starch and blank were found to be 9.48×10^{-2}, 8.67×10^{-2}, 8.53×10^{-2}, 8.35×10^{-2}, 7.43×10^{-2}, 7.31×10^{-2}, 7.13×10^{-2} and $6.05 \times 10^{-2}\,h^{-1}$, respectively (Table 1e). A high degree of linear relationship ($R^2 > 0.95$) between electron donor and time showed that the RB-B reduction was according to first-order kinetics.

The decolorization batch assay for substrate specificity was done by using various azo dyes. The decolorization kinetic rate constant

of reactive azo dyes viz. RB-B, RM-13, RR-11 and RR-141 were found to be 9.09×10^{-2}, 4.35×10^{-2}, 4.21×10^{-2}, $3.86 \times 10^{-2}\,h^{-1}$, respectively, which were higher than non-reactive azo dyes viz. AO-16 $2.39 \times 10^{-2}\,h^{-1}$, DG-6 $2.09 \times 10^{-2}\,h^{-1}$ and RY-36 $1.40 \times 10^{-2}\,h^{-1}$ (Table 1f). The decolorization kinetic constants of RB-B were higher among all the azo dyes tested. This might be due to bacterial consortium acclimatized in RB-B dye.

3.3.3. Activation energy (E_a) determination during anaerobic decolorization

The anoxic decolorization of RB-B by anoxically acclimatized bacterial consortium followed a first-order kinetic with respect to dye concentration. Thus, the first-order constant k_1 (h^{-1}) was determined in each temperature tested. In order to calculate E_a values $\ln(k_1)$ versus $1000/T$ was plotted, and the slope E_a/t was obtained by the linear regression. This ratio was multiplied by the universal gas constant ($R = 8314\,J\,K^{-1}\,mol^{-1}$ or $19{,}987\,cal\,K^{-1}\,mol^{-1}$) to obtain the E_a value.

A significant effect of temperature on decolorization of the diazo dye (RB-B) by anoxically activated bacterial consortium was obtained. The decolorization increased from temperature 20 to 37 °C and declined above 40 °C and it depends upon activation energy of the reaction as given by Arrhenius equation

$$k = K_0 \exp\left[-\frac{E}{RT}\right] \tag{9}$$

where K_0 is the frequency factor and has the same unit as k, E_a the activation energy ($cal\,K^{-1}\,mol^{-1}$), R the gas constant and T is the temperature (K). Eq. (9) gives the overall relationship among dye concentration, temperature and cell mass concentration.

$$\ln\left[\frac{A_t}{A_0}\right] = \ln(MK_0) - \frac{E}{RT} \tag{10}$$

where A_0 and A_t are dye concentrations ($mg\,l^{-1}$) initially and at time t (h), and M is the cell mass concentration (g MLSS). The left-hand side of Eq. (10) was measured and plotted against the reciprocal of temperature as shown in Fig. 3.

The high-degree linearity ($R^2 > 0.90$) between two variables gives reliable estimations of the activation energy (E_a) and frequency factor (K_0), which usually within 4–20 kcal mol^{-1} range, mostly about 11 kcal mol^{-1} [28]. Fig. 3 represents the Arrhenius equation, which could describe the anoxic decolorization of RB-B by bacterial consortium at different temperature, which was used to calculate E_a, and K_0 values.

$$E_a = 11.67\,\text{kcal mol}^{-1}, \qquad K_0 = 1.54 \times 10^{7}\,\text{mgl g MLSS}^{-1}\,\text{h}^{-1}$$

3.3.4. Application of steady-state kinetics for determination of K_m and V_{max}

The Michaelis constant, K_m and the maximal rate, V_{max} can be readily derived from rates of catalysis measured at a various substrate (RB-B) concentrations. The K_m values for a system depends upon the specific dye (RB-B) and on environmental conditions viz. pH, temperature, etc.

A double reciprocal or Lineweaver–Burk plot $1/[V_0]$ versus $1/[S]$, yields straight line with an intercept of $1/V_{max}$ and a slope of K_m/V_{max}. The decolorization of RB-B by acclimatized bacterial consortium was studied at different concentration ranging from 25 to 200 $mg\,l^{-1}$ in proposed nutrient medium at optimum environmental conditions. The double reciprocal plot of decolorization rate $mg\,l^{-1}$ against RB-B concentration $mg\,l^{-1}$ was used to calculate V_{max} and K_m.

$$V_{max} = 15.97\,h^{-1}, \qquad K_m = 85.66\,mg\,l^{-1}$$

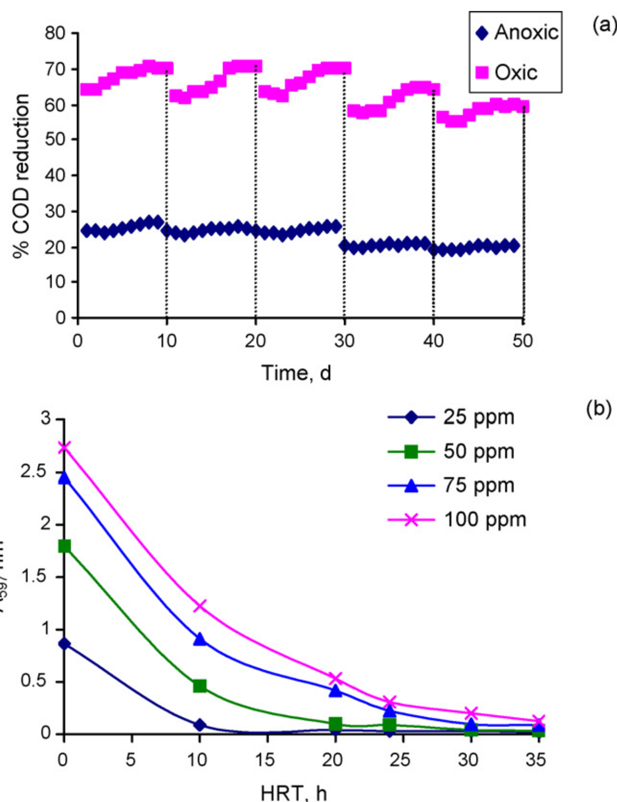

Fig. 5. (a) Profile of COD reduction in combined two-stage anoxic–oxic reactor: [RB-B] = 100 $mg\,l^{-1}$; (b) effect of initial dye concentration and change in HRT on decolorization of RB-B; initial pH 7.0; temperature = 37 °C; St = 10 rpm.

3.4. Lab-scale biodegradation in two-stage anoxic–oxic continuous reactor

The decolorization of RB-B dye in two-stage anoxic–oxic reactor was performed using a two-stage anoxic–oxic reactor (Fig. 4). The major carbon sources are from glucose and partially from nutrient broth that was added to the system, which could enhance the color reduction efficiency. Two-stage anoxic–oxic process performance regarding the COD removal was very high, i.e. 90–97%, but maximum COD removal was observed in the aerobic reactor. The COD of the reactor system effluent was determined at each HRT viz. 10, 20, 24, 30 and 35 h. It was observed that during the period of run at each HRT, COD removal in the initial 4–5 days was low and fluctuating but after this period the reactor system stabilized. The efficiency of stabilized reactor system was high and constant. The COD removal was optimum at HRT of 24 h. About 70% COD was removed during aerobic condition while 26% COD was removed under anoxic condition (Fig. 5a). The reactor was operated at different HRTs and concentration of RB-B. The 25 and 50 ppm concentration of RB-B were decolorized within 12 and 20 h, respectively, in anoxic–oxic reactor. Further increase in concentration of RB-B to 75 and 100 ppm, about >90% color was removed in 24 h. While it increases to 96% at 35 h HRT (Fig. 5b).

It was found that the intensity of absorption peaks of RB-B at 597, 398 and 330 nm decreased drastically within 24 h and these peaks disappeared completely after 35 h. Further, a new peak was observed at λ_{max} = 267 nm consistently in all the spectra of final samples. This suggests the formation of an intermediate compound during anoxic decolorization of RB-B (Fig. 6a). This was attributed to cleavage products of azo linkage in the dyes [25]. There was further increase in the decolorization efficiency up to 95% in the next 11 h.

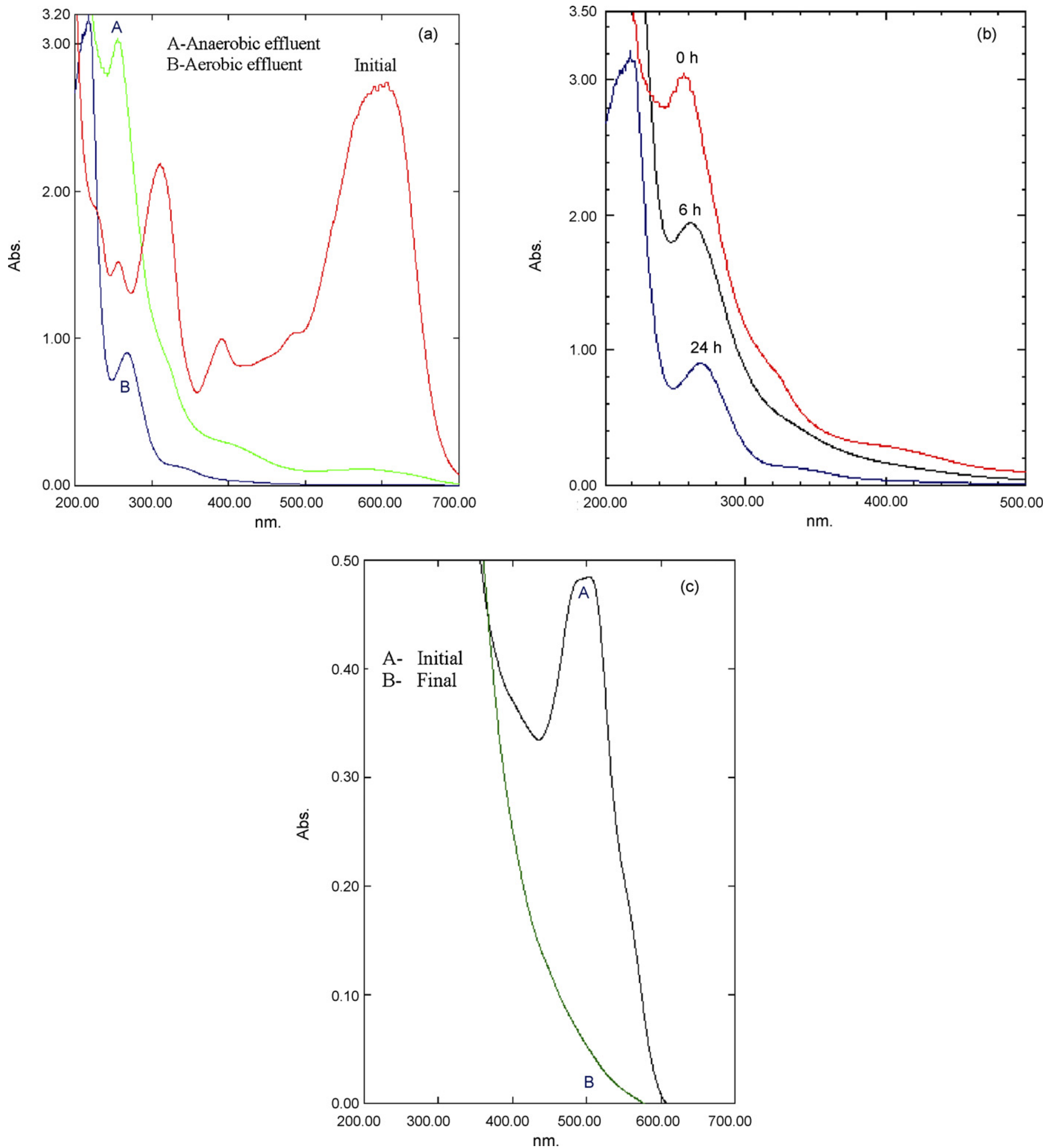

Fig. 6. (a) UV–vis spectral scan (200–700 nm) depicting decolorization/degradation of RB-B in two-stage anoxic–oxic reactor: [RB-B] = 100 mg l^{-1}; (b) spectra depicting amine reduction formed in anoxic treatment by aerobic treatment: [RB-B] = 100 mg l^{-1}; (c) spectra depicting the degradation of textile wastewater in lab-scale reactor. Temperature = 37 °C; initial pH 7.0; St = 10 rpm.

The UV–vis spectral changes represent disappearance of RB-B and formation of a metabolite during anoxic decolorization reaction. More than 90% decolorization was achieved in the anoxic reactor as found earlier in this study. The intensity of the peak at 267 nm was further reduced in the aerobic tank. An absorbance maxima was seen at 267 nm decreased as a function of time (Fig. 6b). The concentration of the metabolite was reduced by atleast 73%. Aerobic reaction promoted the oxidation of the metabolites formed in the anoxic reaction chamber. This is in accordance with the literature reports [33,34,25].

The industrial textile wastewater was efficiently treated in laboratory scale anoxic–oxic reactor. The physico-chemical analysis of influent and effluent of treated textile wastewater is given in Table 2. The maximum color removal was observed in 24 h and

Table 2
Physicochemical characteristic of the influent and treated effluent of industrial textile dye bath wastewater from two-stage anoxic–oxic reactor system

S. No.	Parameters	Influent concentration ($mg\,l^{-1}$)	Effluent concentration ($mg\,l^{-1}$)
1	TDS	3580	2868
2	pH	11.5	7.4
3	COD	1412.8	182.6
4	BOD	367.12	38.28
5	Sulfate	252.95	11.35
6	Chloride	503.88	420.64
7	TKN	162.4	67.55
8	TOC	226.4	32.89
9	Color intensity (A_{502})	0.49	0.09

N.D.: not detected; TDS: total dissolved solids; TKN: total kjeldahl nitrogen. BOD: biological oxygen demand; COD: chemical oxygen demand; TOC: total organic carbon.

hence the reactor was operated at optimum HRT of 24 h. The efficiency of treatment was enhanced by supplementing wastewater with $2\,g\,l^{-1}$ glucose and minerals. Color removal of 84% was obtained in the anoxic reactor. Formation of black particles in the reactor was observed due to formation of sulphides. The aromatic amines and black color sulfide particles formed in the anoxic reactor were significantly removed in the aerobic tank. More than 80% of COD was removed by the two stages. The works regarding the complete removal of black particles (black color) are in progress. The spectrum of influent and effluent decolorization is depicted in Fig. 6c.

4. Discussion

In order to develop an efficient bacterial decolorization process, knowledge regarding the kinetics of decolorization and the environmental factor affecting the rate of decolorization need to be well identified. However, this kind of information has been severely lacking in the literature. The kinetic properties of substrate (dye) concentration and other rate dependent environmental parameters (temperature, pH, co-substrate and different substrate specificity of other dyes) were characterized quantitatively. Two-stage anoxic–oxic decolorization has been demonstrated as an effective and stable method in laboratory facilities under well-controlled conditions [35]. A high degree of linear relationship ($R^2 > 0.99$) between the ln [dye] and time showed that the color was removed according to first-order kinetic in all batch decolorization tests. The decolorization rate constants of RB-B dye were significantly higher than the rate constants of other dye. In other words, the RB-B azo dye decolorizes much faster than the other dyes.

Monoazo dye decolorization has been reported to follow first-order kinetics with respect to dye concentration by several authors [36,30], where as other studies mention zero order [37,38] or even half order kinetics [39]. Furthermore, for the case of diazo and polyazo dyes, only the first part of decolorization profiles has been reported to follow first-order kinetics with respect to the dye concentration [30]. Post studies showed that the kinetic constants decreased as the dye concentration were increased through simultaneous color and substrate removals [40,30].

The results of kinetic studies of this work provided fundamental information for the design and operation of two-stage anoxic–oxic reactor system for the treatment of synthetic azo dyes and azo-dye-contaminated textile effluent. The effluent of a completely anoxic reactor and the effluent of a combined A-O reactor provide evidence for the removal of aromatic amines. The individual strains may attack the dye molecule at different positions or may use decomposition products produced by another strain for further decomposition [41]. Alternatively, it would be interesting to obtain an active microbial culture towards toxic or recalcitrant compounds. Maximum efficiency was obtained when the reactor was supplemented with $2\,g\,l^{-1}$ of glucose and the DO content was maintained at $<0.5\,mg\,l^{-1}$ [25]. A co-substrate was also reported to be essential for the decolorization of several other dyes [42]. The reason for accumulation of COD in the aerobic reactor at high HRT is due to non-mineralized amines or slow degradation to another cleavage product as mentioned by Tan and Field [43]. In these studies it was demonstrated that the aerobic stage eliminated the additional COD and attributed to removal of aromatic amines. The color was removed under anaerobic conditions while the complete mineralization of amines to CO_2, H_2O and NH_3 occurs under aerobic conditions [44]. The decrease or disappearance of the new peaks is in the range between 250 and 300 nm was observed in the HPLC chromatogram [34,45] and UV spectrophotometer [46,47], all indicates removal aromatic amines.

Azoreductases, a class of enzyme involved in the reductive cleavage of the azo bond (–N=N–) is used in anaerobic degradation by various organisms [48]. Azoreductase enzyme system helps bacteria to decolorize high concentration of dyes with a co substrate/electron donor under anaerobic conditions [29,49]. Taking these characteristics into consideration, the effect of increasing RB-B concentration and other physicochemical parameters on decolorization of azo dyes were determined.

5. Conclusions

Azo dyes with different chemical structures can be effectively decolorized under anoxic conditions by specialized bacterial consortium. The results of the kinetic studies provided fundamental information for the design and operation of laboratory scale two-stage anoxic–oxic reactor system for the treatment of synthetic azo dyes and azo-dye-contaminated textile effluents.

Decolorization of RB-B in an anoxic system was found to fit first-order equation with respect to dye concentration, temperature, pH, electron donor/carbon source and substrate (azo dyes). The calculated activation energy value and frequency factor were found to be $11.67\,kcal\,mg^{-1}$ and $1.54 \times 10^7\,mg\,l\,g\,MLSS^{-1}\,h^{-1}$, respectively. The K_m and V_{max} was found to be $15.97\,h^{-1}$ and $85.66\,mg\,l^{-1}$, respectively by using Lineweaver–Burk plot.

The two-stage anoxic–oxic reactor system proved to be successful in achieving significant decolorization/degradation of azo dyes by specific bacterial consortium with a removal of 84% color and 80% COD for real textile effluents vis-à-vis ≥90% color and COD removal for synthetic dye solution.

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