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Kinetics of biological decolorisation of anthraquinone based Reactive Blue 19 using an isolated strain of *Enterobacter* sp.F NCIM 5545

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HIGHLIGHTS

- Bacterial decolorisation of Reactive Blue 19 by an *Enterobacter* sp.strain F.
- The maximum decolorisation efficiency of RB 19 achieved was 90% in 24 h.
- UV–vis and FTIR analysis confirmed the decolorisation was due to biodegradation.

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ABSTRACT

In the present study, an attempt was made to evaluate the bacterial decolorisation of Reactive Blue 19 by an *Enterobacter* sp.F which was isolated from a mixed culture from anaerobic digester for biogas production. Phenotypic characterization and phylogenetic analysis based on DNA sequencing comparisons indicate that *Enterobacter* sp.F was 99.7% similar to *Enterobacter cloacae* ATCC13047. The kinetics of Reactive Blue 19 dye decolorisation by bacterium had been estimated. Effects of substrate concentration, oxygen, temperature, pH, glucose and glucose to microbe weight ratio on the rate of decolorisation were investigated to understand key factor that determines the performance of dye decolorisation. The maximum decolorisation efficiency of Reactive Blue 19 was 90% over period of 24 h for optimized parameter.

To the best of our knowledge, this research study is the report where *Enterobacter* sp.F has been reported with about 90% decolorizing ability against anthraquinone based Reactive Blue 19 dye.

1. Introduction

The textile industry consumes large quantities of water and hence produces large volumes of wastewater from different steps in the dyeing and finishing processes. Wastewater from printing and dyeing units is often rich in color, containing residues of dyes. Textile wastewater pollutants generally contain dyes that increase its BOD, COD, solid contents, color and toxicity. This water must be treated before discharge in environment. For the treatment, removal color (i.e., breakage of chromophore) is necessary. The most important chromophores are azo (—N=N—), carbonyl (—C=O), nitro (—N=O) and quinoid groups. Azo and anthraquinone are the most important chromophoric groups from which textile dyes are synthesized (He et al., 2008). It is believed that anthraquinone-based dyes are more resistant to biodegradation due to their fused aromatic structures compared to azo-based ones (He et al., 2008; Fanchiang and Tseng, 2009). Extensive studies have been carried out in the decolorization of azo dyes but there are very few studies reported for an anthraquinone dye.

For decolorisation or degradation, treatment methods generally involve various oxidation methods (cavitation (Gogate and Mishra, 2010; Pandit et al., 2011, 2013), photocatalytic oxidation, ozone, H_2O_2, Fenton's chemistry (Abdullah et al., 2007)), physical methods (flocculation, adsorption (Kumar and Ahmad, 2011)) and biological methods (fungi (Mohammad et al., 2009), algae (Khataee et al., 2011) and bacteria (Ponraj et al., 2011)). Physical/chemical methods are dealing with dye wastewater treatment but these methods have some drawbacks, such as inability to completely remove the recalcitrant dyes and generation of significant amount of sludge that may cause secondary pollution problems; substantially increases the cost of these treatment methods (Pearce et al., 2003). Compared to physical and chemical methods, biological processes are time consuming but result into complete mineralization of dye (Rai et al., 2005). Within biological processes, compared to algae and fungus, bacterial decolorisation requires less hydraulic retention time (Alalewi and Jiang, 2012).

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Reactive Blue-19 (RB-19), also known as Remazol brilliant blue is an anthraquinone based vinyl sulphone dye, which is very resistant to chemical oxidation due to its anthraquinone structure being stabilized by resonance (Fanchiang and Tseng, 2009). RB-19 has relatively low fixation ability (75–80%) due to the competition between the formation of vinyl sulphone and the hydrolysis reactions, i.e., formation of 2-hydroxysulphone, which does not attach to the fiber as a result that dye remains stable into the wastewater for a long period of time, i.e., half life of RB-19 is 46 years at pH 7 and at a temperature of 25 °C that renders transport resistance to the oxygen transfer to aquatic life (Memon and Memon, 2012). Therefore, the strategy to remove the color and to decrease its impact on the wastewater of textile environment is of significant importance.

Thus, the bacterial decolorisation of anthraquinone dye (C.I. Reactive Blue 19) by a newly isolated strain of *Enterobacter* sp.F have been reported in the present work. Kinetics of biological decolorisation of anthraquinone based Reactive Blue 19 have also been evaluated.

2. Methods

2.1. Dyes and chemicals

Glucose, nutrient broth and agar were obtained from Hi-Media Pvt. Ltd. (India). The textile dyes: Reactive Blue 19 was kindly donated by Colorband Dyestuff (P) Ltd., INDIA.

2.2. Organism and culture condition

The organism used in this study was isolated mixed culture from anaerobic digester for biogas production from Institute of Chemical Technology, Mumbai, India. It was then maintained on nutrient agar slants at 4 °C. The pure culture was grown in 250 ml Erlenmeyer flask containing 100 ml nutrient broth containing beef extract 1 g/L yeast extract 2 g/L, peptone 5 g/L and NaCl 5 g/L at 30 °C for 24 h. The 24 h culture was kept at 4 °C and used as the seed inoculum.

2.3. Synthetic wastewater preparation

Reactive Blue 19 dye with the purity of 95%, was dissolved in M9 medium (Sambrook and Russell, 2001) (Na_2HPO_4 31.7 g/L, KH_2PO_4 3 g/L, NH_4Cl 0.5 g/L, NaCl 0.5 g/L, $MgSO_4$ 0.12 g/L, $CaCl_2$ 4 mg/L, glucose 8 g/L, vitamin B 0.15 mg/L, pH 7.0) at a required concentration to create the synthetic dye loaded wastewater.

2.4. Isolation and identification of decolorizing bacteria

Aliquots (1 ml) of activated sludge collected from an anaerobic digester used for biogas production at Institute of Chemical Technology, Mumbai, India, were inoculated into 100 mL of synthetic wastewater with Reactive Blue 19 concentration of 50 mg/L, followed by incubation at 30 °C for 5 days under anaerobic condition before being transferred to fresh synthetic wastewater. The process was repeated several times until the synthetic wastewater showed significant decolorisation. Then, the samples were serially diluted 16-fold and 100 μL aliquots of each dilution were spread onto M9 agar plates with 50 mg/L Reactive Blue 19. After 12 h of incubation at 37 °C, morphologically different colonies were streaked on M9 agar for purification before being inoculated into the synthetic wastewater to check their decolorizing ability. The bacterial strain with the strongest decolorizing ability, designated as F, was isolated and preserved at −20 °C in M9 medium with 25% glycerol. Then, the isolated colony was sent for DNA sequencing in NCIM Resource Centre, CSIR-NCL, Pune. The sequence was input into National Center for Biotechnology Information (NCBI) to identify it by Basic Local Alignment Search Tool (BLAST) search.

2.5. Decolorisation experiments

An isolated colony was collected from the slant and inoculated in 250 ml Erlenmeyer flask containing 100 ml nutrient broth under optimal growth conditions (pH 7; 30 °C, and aeration and agitation) and run to its exponential phase (24 h). A microbial culture with an optical density of 1.0 (at 600 nm) was inoculated in anaerobic bottle or flask containing 200 ml M9 media. The 12 h grown cells were incubated with required concentration of RB19 dye under aerobic and anaerobic condition. To study the surface adsorption characteristics, decolorisation, using heat killed bacteria were carried out. Bacteria were inactivated at 100 °C for 25 min. To maintain the anaerobic condition in 500 ml anaerobic bottle, oxygen was removed with the help of nitrogen flushing and glass bottle containing synthetic media and culture was sealed with rubber corks. To understand the role of inactivated bacteria and aerobic decolorisation, flask containing M9 media and 50 ppm dye was kept for five days under shaking condition (150 rpm) and at 30 °C and pH of 7. Effects of various parameters including oxygen, temperature (25, 30, 37 and 42 °C) and initial pH (5, 7 and 10) initial glucose concentration (0, 1, 2, 4, 8 or 12 g/L), glucose to microbe weight ratio (100, 90, 80, 70, 60, 50 and 40), periodic and repeated additions of dye aliquots on continuous decolorisation were investigated.

2.6. Fed batch decolorisation

The fed batch decolorization of Reactive Blue 19 dye was also studied under anaerobic condition. In this study, 50 mg/L dye was added into the 12 h grown culture (200 ml M9 media) of bacterial isolate. After the decolorization further 50 mg/L dye was added to the decolorized broth without the supplement of additional nutrient. Dye was added periodically until the culture showed the decolorization ability. All decolorization experiments were performed in triplicate.

2.7. Measurement of biomass

Dong et al. (2003) had reported the method for determination of biomass by measuring optical density (OD) at a wavelength 600 nm:

$$OD_{600} = OD_{sample} - OD_{supernatant}$$

The relationship between the dry cell mass (DCM) of the *Enterobacter* sp.F and OD 600 was found to be:

$$\text{Biomass concentration (g DCM/l)} = 1.18OD_{600}$$

2.8. Measurement of decolorization efficiency

Samples (5 ml) were collected every 4 h and centrifuged at 15,000×*g* for 15 min. Decolorisation efficiency was determined by measuring the absorbance of the culture supernatant at 592 nm using a Unicam UV9100-visible spectrophotometer. Decolorisation efficiency was calculated using the following Eq. (1):

$$\text{Decolorisation efficiency } (\%) = \frac{OD_1 - OD_t}{OD_1} \times 100 \quad (1)$$

where OD_1 refers to the initial absorbance, OD_t to the absorbance after incubation; and t to the incubation time.

2.9. UV–vis spectral analysis and FTIR

Decolorisation was monitored by UV–vis spectroscopic analysis, whereas biodegradation was monitored using FTIR Spectroscopy. Decolorisation of Reactive Blue 19 dye was followed by monitoring changes in its absorption spectrum (200–700 nm) using a Hitachi UV–Vis spectrophotometer (Hitachi U-2800) and comparing the results, to those of the respective controls. The metabolites produced during the biodegradation of the dye (after decolorisation of medium) were centrifuged at 15,000×*g* for 30 min. After near complete degradation of adsorbed dye or after specified treatment period, the metabolites were extracted from supernatant by adding equal volume of ethyl acetate. The samples were used for UV–Vis spectral analysis. FTIR analysis was carried out using SHIMADZU IR Prestige 21 with connection of attenuated total reflection (ATR) (Specac Goldengate) and changes in % transmission at different wavelengths were observed. The Fourier Transform Infrared Spectroscopy (FTIR) analysis of extracted metabolites was done, and compared with control dye in the mid IR region of 400–4000 cm^{-1} with 16 scan speed.

3. Statistical analysis

All the experiment and analysis were conducted in triplicate and results presented here are the mean of triplicate ± standard deviations (SD). For the comparison of two levels of a single factor, Statistical analysis was done using student "*t*" test by Graph pad software. For the comparison of three or more than three levels of a single factor, data were analyzed by one-way analysis of variance (ANOVA) by using Tukey–Kramer multiple comparisons test (GraphPad Prism version 6.0 for Windows, http://www.graphpad.com/demos/download.cfm?demo- Products=IsDemoWin). Results in each experiment were interpreted depending on their probabilities. The *p*-values of less than 0.05 were considered to be statistically significant while those less than 0.001 were considered to be highly significant.

4. Results and discussions

4.1. Isolation and identification of decolorizing bacteria

A bacterial strain, which had high decolorization ability against Reactive Blue 19, was isolated. Significant decolorization efficiency (90%) was achieved after incubation for 108 h. The colony of bacterial isolate F was filamentous and yellow–white. The strain F was observed to be a Gram-negative.

To analyze the phylogenetic position, the 16 S rDNA sequence of the strain F was determined. Fig. 1 showed the phylogenetic relationship between the strain F and other related microorganisms found in the GenBank database. The homology assay result indicated that the strain F was in the phylogenetic branch of the *Enterobacter*. The strain F exhibited a maximum identities (99.7%) to *Enterobacter cloacae ATCC 13047*. The strain F has been deposited

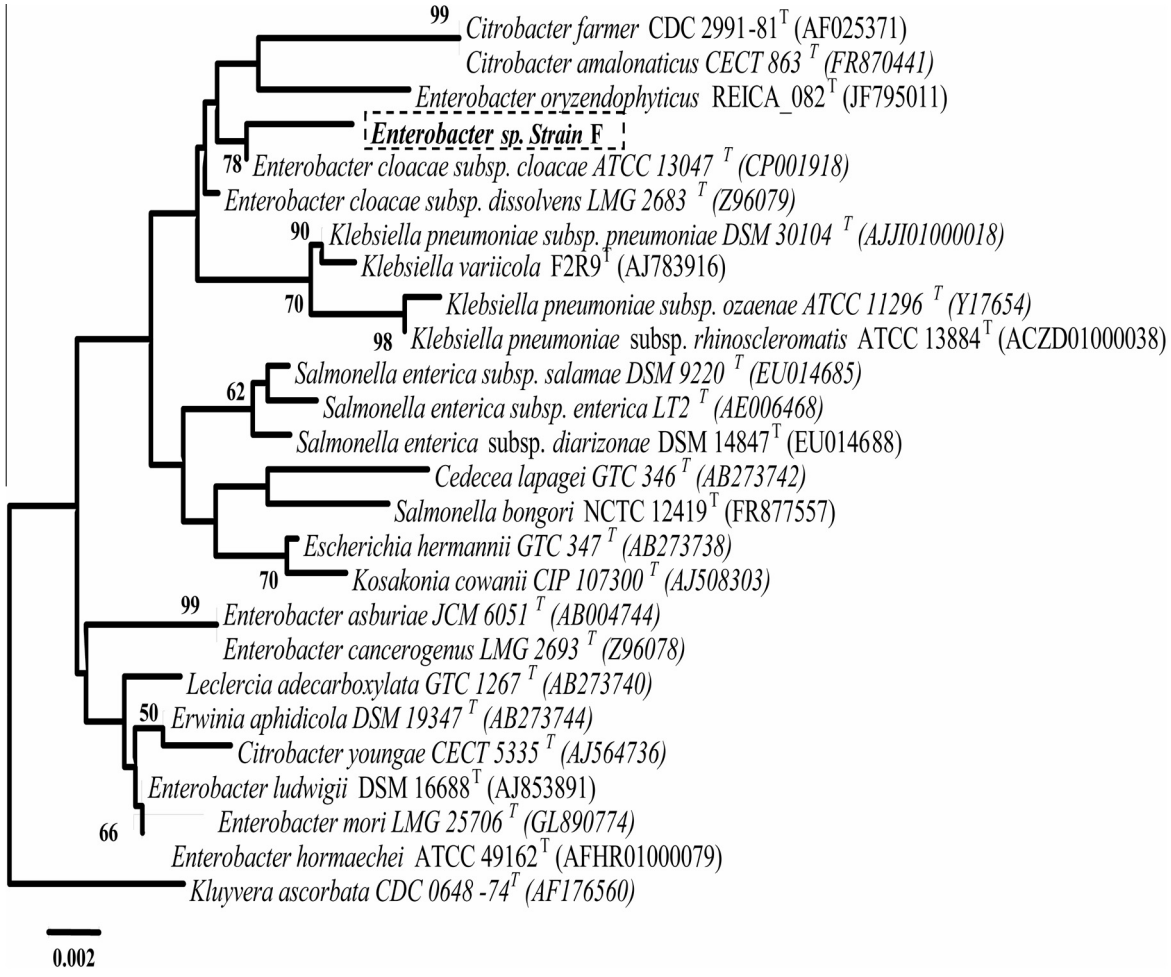

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing relationships of *Enterobacter* sp.F and other species of genus *Enterobacter*. Numbers at nodes shows the level of bootstrap support based on data for 1000 replications. Bar, 0.002 substitutions per nucleotide position and numbers in bracket represent GenBank accession numbers.

in NCIM Resource Centre, CSIR-NCL, Pune with accession number of NCIM 5545. The earlier report shows other species of Enterobacter *cloacae* have role in biodegradation of dye (Wang et al., 2009a).

4.2. Decolorization of Reactive Blue 19 under aerobic and anaerobic conditions

Controlled condition (abiotic) resulted into 25% decolorisation over period of 108 h under aerobic condition (shaker speed of 150 rpm). The growth of *Enterobacter* sp.F was greater under aerobic as compared to anaerobic conditions (Fig. 2a), while decolorisation of Reactive Blue 19 was more than 90% under anaerobic conditions and only 47% under aerobic conditions (shaker speed of 150 rpm) (Fig. 2b). This result suggested that oxygen was favorable to the growth of the bacterium but detrimental for the synthesis of the enzyme responsible for the process of the dye degradation. It is speculated that under anaerobic condition, the oxygen of carbonyl group in the anthraquinone dye is a preferable terminal electron receptor in the absence of oxygen in extracellular environment (anaerobic conditions). Hence, in metabolic pathway, *Enterobacter* sp.F consume the oxygen of carbonyl group in the anthraquinone dye RB19 (Wang et al., 2009a), resulting into more decolorisation under anaerobic condition compared to aerobic condition. So, anaerobic condition appears to be necessary for bacterial decolorization, though the cell growth was poorer than that under aerobic condition. Statistical analysis conducted using "*t*" test also showed a significant difference (p-value = 0.0038) under the aerobic and anaerobic decolorisation. Therefore, anaerobic condition was subsequently adopted to investigate bacterial decolorisation.

4.3. Effects of pH on decolorisation

The pH tolerance of decolorizing bacteria is important because RB19 dye binds to cotton fibers under alkaline conditions and hence the effluent is usually alkaline with pH greater than eight (Aksu et al., 2007). Therefore, decolorisation was carried out at different pH values. *Enterobacter* sp.F showed a high decolorisation rate (90%) at pH 10 after 48 h of incubation. This could be due to the fact that the optimum pH for the growth of *Enterobacter* sp.F was in the basic medium. Ninety percent decolorisation efficiency was observed at pH 5.0 and pH 7.0 after 74 and 60 h of incubation period respectively (Fig. 2c). Though the decolorisation profile as shown in Fig. 2c at different pH looks similar, they are statistically different. It was confirmed by One-Way ANOVA and Tukey–Kramer multiple comparisons test. Statistical analysis conducted using One-Way ANOVA showed a significant difference (p-value < 0.0006) in the decolorisation when carried out at different pH namely 5, 7 and 10. Further, Tukey–Kramer multiple comparisons test showed significantly higher decolorisation at pH 10.0 as compared to that at other pH 7 (p-value = 0.042) and pH 5 (p-value = 0.0006). Therefore, pH 10 was adopted to investigate bacterial decolorisation in the subsequent experiments. Thus, *Enterobacter* sp.F shows the ability to decolorize dye (RB19) at pH 10, which is significant for its possible commercial utilization

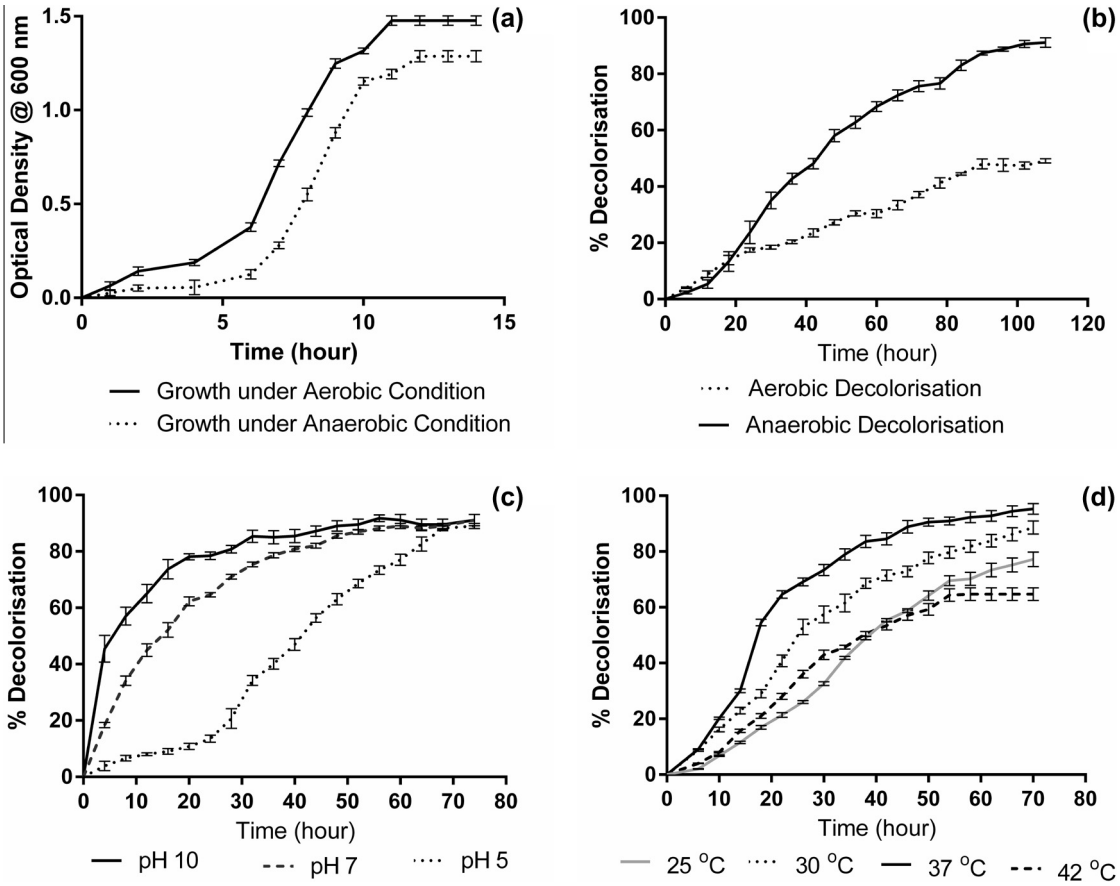

Fig. 2. (a) Growth of *Enterobacter* sp.F cultures at OD_{600} and (b) dye decolorisation in aerobic and anaerobic conditions (pH = 7; temperature 30 °C; [RB19] = 50 mg/L; 8 g/L glucose; glucose to microbe weight ratio = 100). (c) Effect of pH on the extent of decolorisation (Temperature 37 °C; [RB19] = 50 mg/L; 8 g/L glucose; glucose to microbe weight ratio = 100). (d) Effect of temperature on decolorisation efficiency (pH = 10; [RB19] = 75 mg/L; 8 g/L glucose; glucose to microbe weight ratio = 100).

in the treatment of cotton textile industry wastewater containing RB19 dye and having pH of 9–10 (Paul et al., 2012).

4.4. Effects of temperature on decolorisation

It was found that with an increase in temperature from 25 to 37 °C the decolorisation rate increased (maximum observed was at 37 °C) and then a further increase in temperature to 42 °C drastically affected decolorisation activity of *Enterobacter* sp.F (Fig. 2d). The optimum temperature for decolorisation was found to be 37 °C. We are unsure about the decrease in decolorizing activity when temperature was increased to 42 °C. The possible reason behind it may be due to the loss of cell viability or deactivation of the enzymes responsible for the decolorisation at 42 °C (Dafale et al., 2008; Cristovao et al., 2009). One-Way ANOVA showed a significant difference (p-value < 0.0001) in the decolorisation at different temperatures. Further Tukey–Kramer multiple comparisons test also showed significantly higher decolorisation at 37 °C as compared to that at other temperatures (p-value < 0.0001). Therefore, temperature of 37 °C was adopted as an optimum to investigate bacterial decolorisation in the subsequent experiments.

4.5. Study of kinetic parameters RB19 decolorisation to determine decolorisation rate constant

Michaelis–Menten type rate model (Cristovao et al., 2009) has been widely used for the kinetics of substrate conversion by living cells. 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 relating V_{max} and temperature. Michaelis–Menten type rate model neglects the temperature effect on K_m (Shuler and Kargi, 1992). Hence, to analyse the temperature effect on the decolorisation rate constant, a general kinetic model (Eq. (2)) of dye decolorisation has been given as

$$\frac{dA}{dt} = -k_1 M^m A^n \tag{2}$$

where t is the time (h), M and m the cell mass concentration (MLSS, mg/L) and its reaction order (first order), respectively, A and n are the dye concentration (mg/L) and its reaction order (first order), respectively.

Since, cell growth or death was not observed in the nutrient containing test solution in 24 h, the MLSS concentration in the decolorisation experiment rate can be assumed to be constant. For the particular case of a first-order reaction with respect to dye concentration ($n = 1$) at constant MLSS (M^m = constant), Eq. (2) can be simplified to Eq. (3)

$$\frac{dA}{dt} = -k_{pfo} A^n \tag{3}$$

where, $k_{pfo} = k_1 M^n$ = pseudo first order rate constant

$$\frac{A_t}{A_0} = e^{-k_{pfo} t} \tag{4}$$

$$\ln\left(\frac{A_0}{A_t}\right) = k_{pfo} t \tag{5}$$

In order to approximate the reaction by first-order kinetics with respect to the dye concentration, the values of $\ln [A_0/A_t]$ were plotted against time, using experimental data of decolorisation. Each data point represented the mean value of at least three values obtained from triplicate tests. A high-degree of linearity ($R^2 > 0.90$) between the $\ln [A_0/A_t]$ and time was observed, giving a first-order reaction ($n = 1$). The reaction rate constant is obtained graphically by plotting $\ln [A_0/A_t]$ versus t (Fig. 3a). First-order kinetics with respect to dye concentration has also been reported by several researchers (Dafale et al., 2008; Wuhrmann et al., 1980) and the observation in this work is consistent with earlier reported work.

4.6. Activation energy (E_a) determination during anaerobic decolorization

The anaerobic decolorization of RB19 by anaerobically acclimatized bacterium followed a first-order kinetic with respect to dye concentration. Thus, the pseudo first-order constant k_{pfo} (h^{-1})

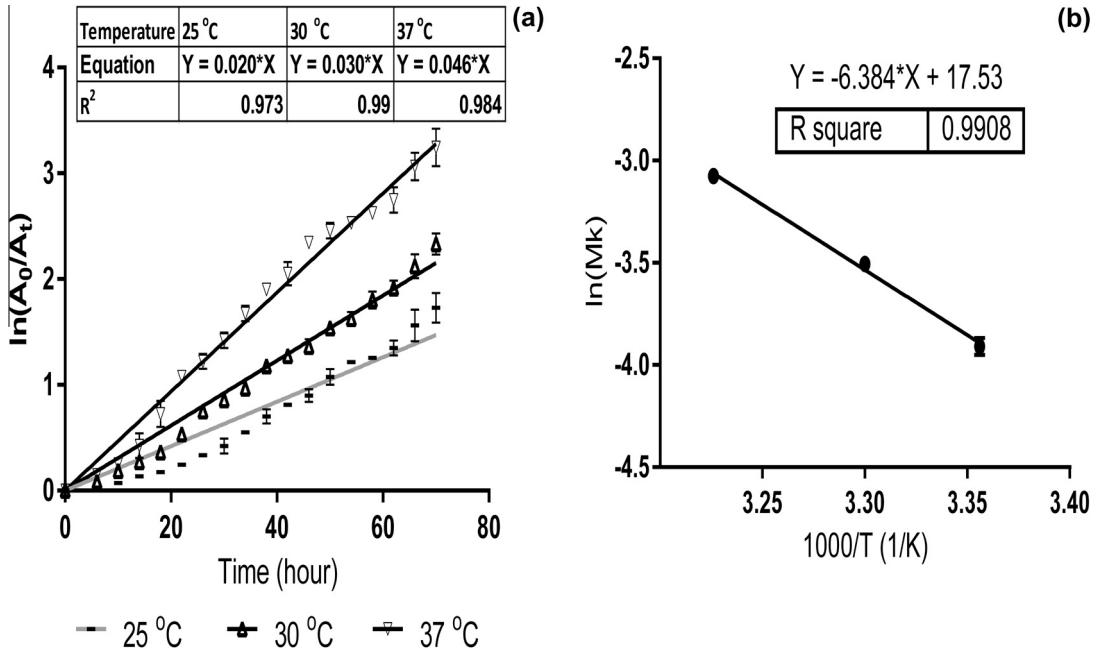

Fig. 3. (a) Calculation of the pseudo first-order constant k_{pfo} (h^{-1}) at each temperature. (b) Estimation of activation energy of RB19 decolorisation by Arrhenius equation: (pH = 10; [RB19] = 75 mg/L; 8 g/L glucose; glucose to microbe weight ratio = 100).

was determined at each temperature tested (Fig. 3a). In order to calculate E_a values ln (k_1M) versus 1000/T was plotted, and the slope E_a/R was obtained by linear regression. This slope was multiplied by the universal gas constant (R = 8314 JK^{-1} mol^{-1}) to obtain the E_a value. A significant effect of temperature on decolorization of the anthraquinone dye (RB19) by anaerobically activated bacterium was obtained. The decolorization increased from temperature 25–37 °C and declined above 40 °C. The activation energy of the reaction as given by Arrhenius equation

$$K_{pfo} = -K_0 e^{\left(\frac{-E_a}{RT}\right)} \quad (6)$$

where K_0 is the frequency factor and has the same unit as k_{pfo}, E_a the activation energy (cal K^{-1} mol^{-1}), R the gas constant and T is the temperature (K). Eq. (7) gives the overall relationship among dye concentration, temperature and cell mass concentration.

$$\ln\left(K_{pfo}\right) = \ln(K_0) - \frac{E_a}{RT} \quad (7)$$

The high-degree of linearity ($R^2 > 0.90$) between two variables gives reliable estimations of the activation energy (E_a) and frequency factor (K_0), which was usually within 4–20 kcal mol^{-1} range, mostly about 11 kcal mol^{-1} (Shuler and Kargi, 1992). Fig. 3b represents the Arrhenius equation, which could describe the anaerobic decolorisation of RB19 by bacterium at different temperatures, which was used to calculate E_a, and K_0 values and were found to be 12.62 kcal/mol and 4.1×10^7 mg L^{-1} h^{-1} respectively. Relatively small E_a indicate typical bio-chemical phenomenon. Activation energy indicates the energy required for the activation of the enzyme stroke. The changes in the enzyme or substrate (glucose) concentration is unlikely to change the activation energy as the biological action of the enzyme remains the same.

4.7. Effects of initial glucose concentration and glucose to microbe weight ratio on decolorisation

Decolorizing bacteria obtain energy from a variety of carbon sources and thereby supplementary carbon sources enhance the decolorizing performance of the biological system. As synthetic dyes are deficient in carbon content, biodegradation of dyes without any added carbon sources is reported to be difficult (Saratale et al., 2011; Pearce et al., 2003). Of various carbon sources, glucose was found to be an ideal candidate in optimizing both bacterial biomass and decolorization efficiency (Moosvi et al., 2005). Therefore, number of batch studies with different glucose concentration and glucose to microbe weight ratio were performed. The correlation coefficients of all these studies were determined and were observed to follow the first-order kinetics. In experiments with glucose supplementation, results suggested that *Enterobacter sp.* F exhibited strong decolorizing activity with decolorisation rate constant of 0.08 h^{-1} and 0.113 h^{-1} for glucose concentration of 6 g/L (Fig. 4a) and glucose to microbe weight ratio of 40 (Fig. 4b) respectively. With increased glucose concentration (12 g/L) and glucose to microbe weight ratio (100), low decolorisation rate constant was obtained, similar to the result obtained with glucose concentration of 2 g/L and glucose to microbe weight ratio of 30. The possible reason for getting such results is that the lower decolorisation rate constant in the presence of lower glucose concentration may cause substrate limitation for the growth of the bacteria.

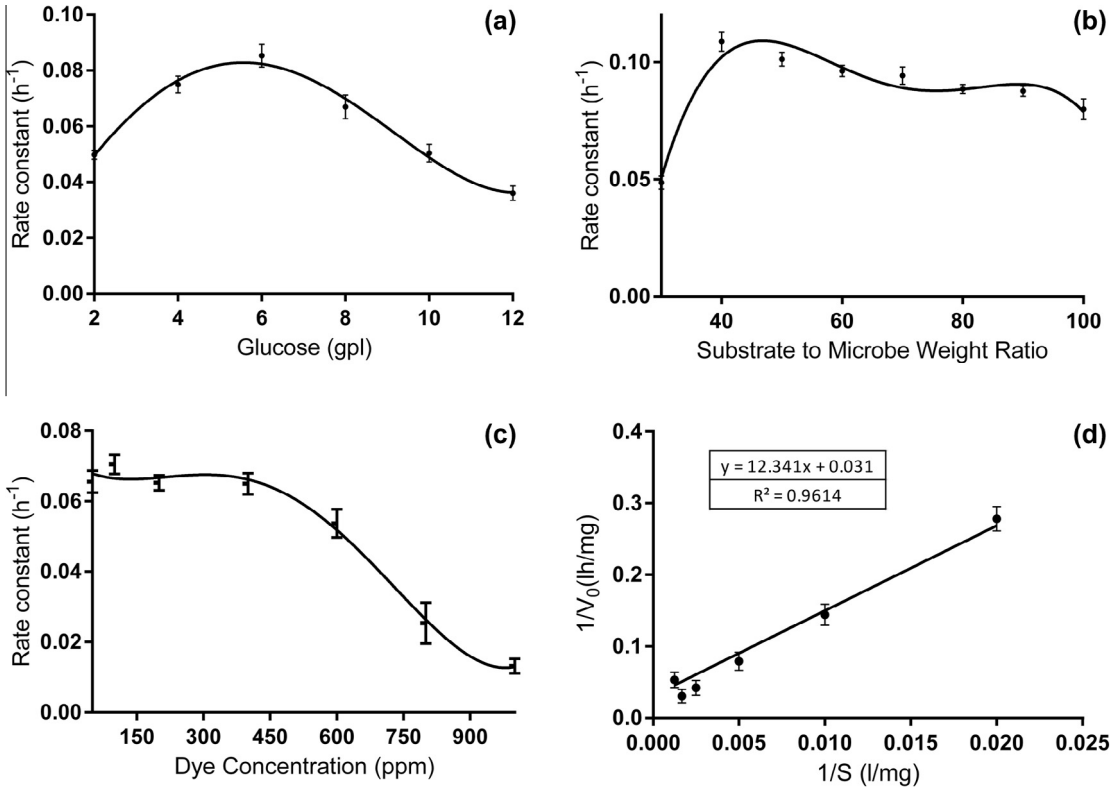

Fig. 4. (a) Effect of glucose concentration on decolorisation rate constant (pH = 10; temperature 37 °C; [RB19] = 75 mg/L; glucose to microbe weight ratio = 100). (b) Effect of glucose to microbe weight ratio on decolorisation rate constant (pH = 10; temperature 37 °C; [RB19] = 75 mg/L; 6 g/L glucose). (c) Effect of initial dye concentration on decolorisation rate constant (pH = 10; temperature 37 °C; 6 g/L glucose; glucose to microbe weight ratio = 40). (d) The double reciprocal plot of initial decolorisation rate (mg L^{-1} h^{-1}) against RB-19 concentration (mg/L) to calculate V_{max} and K_m.

When the glucose concentration was much higher, such as 8 g/L and 12 g/L, the bacteria could utilize glucose preferentially for their growth and not for the synthesis of the decolorisation related enzymes which resulted into lower decolorisation rate constant (Saratale et al., 2011;Wang et al., 2009b). It was evident from the significant microbial growth rate.

Statistical analysis using One-way ANOVA showed that there was a significant difference (p-value < 0.0003) in decolorisation obtained using different initial glucose concentration. Further, Tukey–Kramer multiple comparisons test showed significantly higher decolorisation at initial glucose concentration of 6 gpl as compared to that at other initial glucose concentrations like 2, 8, 10 and 12 (p-value < 0.038). By Tukey–Kramer test, there was no significant difference in decolorisation at initial glucose concentration of 6 gpl as compared to that at 4 gpl initial glucose concentration (p-value > 0.05). But, decolorisation rate constant was about 15% higher for 6 gpl initial glucose concentration as compared to that at 4 gpl initial glucose concentration. Therefore, an initial glucose concentration of 6 gpl was adopted to investigate bacterial decolorisation in the subsequent experiments.

One way ANOVA followed by Tukey–Kramer multiple comparisons test showed that there was a significant difference (p-value < 0.0012) in decolorisation in the presence of different glucose to microbe weight ratio, with ratio of 40 resulting in the highest decolorisation as compared to other glucose to microbe weight ratio of 30, 90 and 100 (p-value < 0.013). By Tukey–Kramer test, there was no significant difference in decolorisation at glucose to microbe weight ratio of 40 as compared to that at other glucose to microbe weight ratio of 50, 60 and 70 (p-value > 0.05). But, decolorisation rate constant was high at glucose to microbe weight ratio of 40(0.113 h^{-1}) as compared to that at other glucose to microbe weight ratio of 50(0.09 h^{-1}), 60(0.095 h^{-1}) and 70(0.098 h^{-1}). Therefore, glucose to microbe weight ratio of 40 was adopted to investigate bacterial decolorisation in the subsequent experiments.

4.8. Effect of initial dye concentration on dye decolorisation rate constant

The concentration of dye substrate can influence the efficiency of dye removal through a combination of factors including the toxicity of the dye at higher concentrations and the ability of the enzyme to recognize the substrate (dye) efficiently at very low concentrations. Dye concentrations of 10 ppm up to 250 ppm have been cited as being present in dye house effluent (Neill et al., 1999). In this study, the effect of different concentrations of RB19 dye on decolorisation was observed by taking strong concentration of 50, 100, 200, 400, 600, 800 and 1000 mg/L levels. The decolorisation rate constant obtained was 0.068, 0.071, 0.0677, 0.0679, 0.053, 0.02 and 0.015 h^{-1}, respectively (Fig. 4c). Higher concentrations of RB-19(beyond 400 mg/L) repress the growth and the extent of decolorisation (lower decolorisation rate). The dye decolorisation was strongly inhibited at 1000 mg/L of dye concentration in the medium. Similar results have been also reported in the literature for azo reactive dyes (Wang et al., 2009a; Moosvi et al., 2005, 2007). One way ANOVA followed by Tukey–Kramer multiple comparisons test showed that there was a significant difference (p-value < 0.0001) in decolorisation with varying dye concentration, when experimented with dye concentrations of 50, 100, 200 and 400 mg/L resulting in much higher decolorisation as compared to higher dye concentrations namely 600, 800 and 1000 mg/L (p-value < 0.0003).

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

The maximum decolorisation rate of RB19 was measured by using test solution containing 100 mg/l dye, ≈1400 ± 50 mg/l MLSS (mixed liquid suspended solid) and growth medium, incubated under anaerobic conditions at 37 °C for 24 h. Michaelis–Menten type rate model, equation Eq. (8) has been widely used for the kinetics of substrate conversion by enzyme and or by living cells. The primary function of enzymes is to enhance the rate of reaction so that they are compatible with the need of the microorganism (Cristovao et al., 2009).

To understand how the system functions we need a kinetic description of their activity and is described by the equation:

$$V_0 = -\frac{d[RB-19]}{dt} = \frac{V_{\max}[RB-19]}{[RB-19]+K_m} \quad (8)$$

The Michaelis constant, K_m and the maximum decolorisation rate, $V_{\max}$ can be readily derived from the rates of catalytic reaction measured at a various substrate (RB19) concentrations. The K_m values for a system depends upon the specific dye (RB19) and on environmental conditions viz. pH, temperature, etc. A double reciprocal or$1/[V_0]$ versus $1/[S]$, yields a straight line with an intercept of $1/V_{\max}$ and a slope of $K_m/V_{\max}$. The decolorization of RB-19 by acclimatized bacterium was studied at different dye concentration ranging from 50 to 1000 mg/L in proposed nutrient medium at optimum environmental conditions (Fig. 4d). The double reciprocal plot of decolorisation rate (mg L^{-1} h^{-1}) against RB19 concentration mg/L was used to calculate $V_{\max}$ and K_m. The values obtained were $V_{\max}$ = 32.25 h^{-1}, K_m = 398 mg/L.

The maximum decolorisation rate, $V_{\max}$ of 32.25 h^{-1} was reached, when all the active sites on bacterium were saturated with the RB-19 dye. This would happen, when RB19 dye concentration was greater than K_m (398 mg/L). Looking at Eq. (8) at very low dye concentration, when [RB19] $\ll K_m$, $V_0 \sim$ [RB-19]$V_{\max}/K_m$, that is, the decolorisation rate is directly proportional to concentration of RB-19. At high [S], when [RB-19] > K_m (398 mg/L) $V_0 = V_{\max}$ and hence independent of concentration of RB19.

4.10. Effect of repeated additions of dye aliquots on continuous decolorisation

The dye industry wastes are periodically discharged (batch operations) from the dyeing and textile industry, therefore the consequence of repeated addition of dye addition on the possibility of continuous decolorisation was investigated. For repeated addition of glucose; the bacteria utilize glucose preferentially for their growth and not for the synthesis of the related enzymes responsible for decolorisation. Hence, consecutive cycles of dye decolorisation were studied by the repeated additions of Reactive Blue 19 samples containing concentration of 50 mg/L without repeated addition of glucose in the medium. It showed the effective dye decolorisation up to 6 cycles. In first cycle 90% decolorization was obtained within 24 h. The subsequent addition of similar levels of dye results in faster rate of decolorization process till the 6th cycle (Fig. 5a). Maximum decolorization rate (4.7 mg l^{-1} h^{-1}) was obtained at the 6th cycle of decolorization process (Fig. 5b). The observed increase in the decolorization rate up to sixth addition was due to an increase in the microbial mass in anaerobic bottle with the addition of each new installment of dye, which increased from $12 * 10^9$ to $9 * 10^{13}$ cells ml^{-1} in the system (Fig. 5b). After 6th cycle, the decolorization efficiency of organism gradually decreased and required more time for the decolorisation of RB19 dye. It was due to the decrease in the number of viable cell in systems (Fig. 5b). Another possible reason for the observed reduction in the decolorisation rate after 6th cycle might be the nutrients depletion in the medium and inhibition of enzyme responsible for the decolorisation. Similar observations have been recorded previously for the decolorisation of reactive dye like Red BLI, Reactive Red 141 and Navy blue HER (Kalyani et al., 2008;Telke et al.,

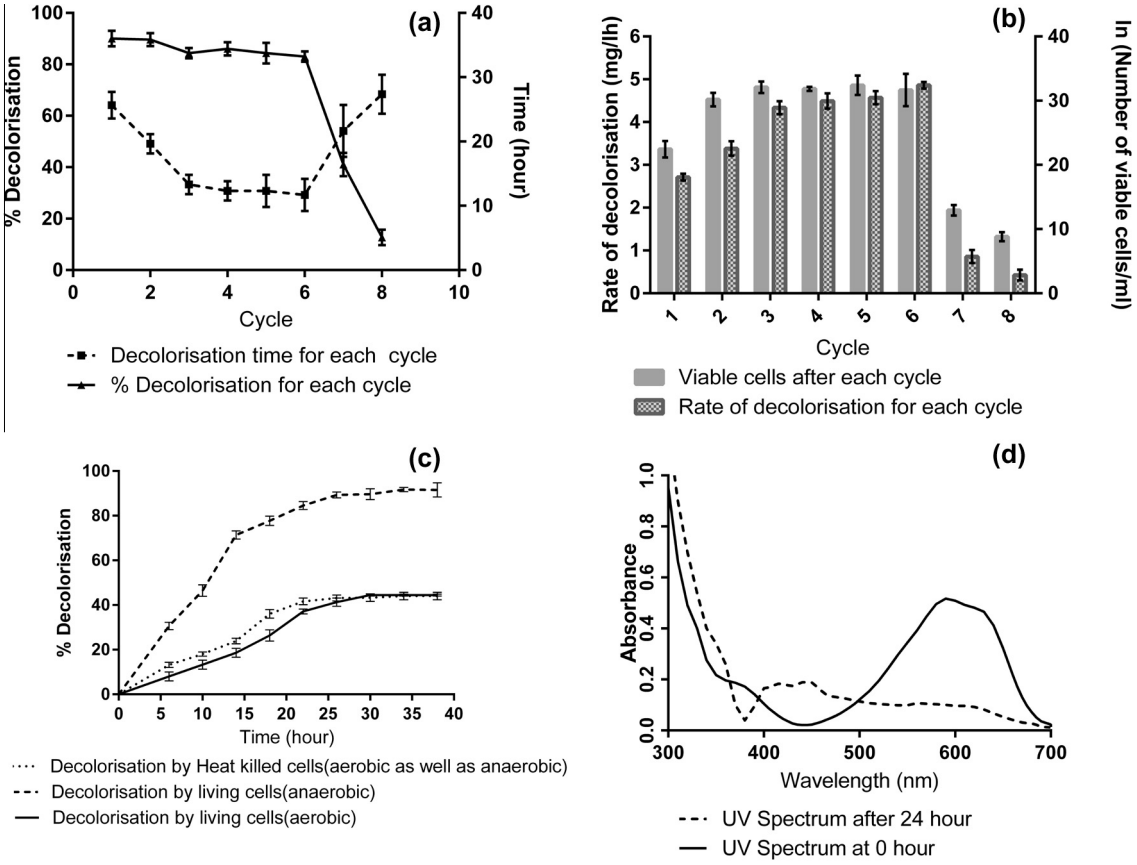

Fig. 5. (a) RB-19 dye decolorisation percentage in fed batch process (pH = 10; temperature 37 °C; 6 g/L glucose; glucose to microbe weight ratio = 40; [RB19] = 50 mg/L). (b) Rate of decolorisation for each cycle and number of viable cells after each cycle in fed batch process (pH = 10; temperature 37 °C; 6 g/L glucose; glucose to microbe weight ratio = 40;[RB19] = 50 mg/L). (c) Decolorisation of Reactive Blue 19 by living and heat-killed cells under aerobic as well as anaerobic condition (pH = 10; temperature 37 °C; [RB19] = 100 mg/L; 6 g/L glucose; glucose to microbe weight ratio = 40) (d) UV–Vis spectra of RB19 decolorisation at 0 h, 24 h.

2008;Saratale et al., 2009).Thus *Enterobacter* sp.F shows the ability to decolorize repeated addition of dye aliquots, which is noteworthy for its possible commercial exploitation.

5. Decolorisation mechanism of *Enterobacter* sp.F against Reactive Blue 19

Decolorisation of dye solution by bacteria could be due to adsorption to (a) microbial cell or (b) biochemical reaction or both leading to biodegradation. In adsorption, examination of the absorption spectrum would reveal that all peaks decreased approximately in the same proportion to each other. If dye removal is attributed to biochemical reaction, either the major visible light absorbance peak should have completely disappeared or a new peak would have appeared (Ayed et al., 2010).

In the cultures with the addition of heat-killed bacterial cells, only 45% decolorization was recorded after 108 h of incubation under aerobic as well as anaerobic condition (Fig. 5c), which might only be due to the physical adsorption of dye by the dead bacterial cells and this was also confirmed by the recovery of blue colored cell pellets obtained after centrifugation. In the presence of living cell under aerobic condition and with optimized parameters, decolorization efficiency was similar to the decolorisation using heat killed cells (Fig. 5c), which must be only due to the adsorption of dye by the living bacterial cells. Adsorption of RB19 under aerobic decolorisation was also confirmed by the recovery of blue colored cell pellets obtained after centrifugation. In the presence of living cell and under anaerobic condition, 90% decolorisation was achieved within 24 h with optimized parameters (Fig. 5c). The cell pellets were also lightly pigmented with yellow color (instead of blue pellets obtained earlier). These light yellow pigmented cells indicate the biodegradation of RB19 dye on the surface of bacterial cells. So, it can be concluded that this observed 90% decolorisation can be attributed to biodegradation. Statistical analysis using One-way ANOVA showed that there was a significant difference (p-value < 0.0007) in decolorisation obtained by using heat killed cells and living cells(under aerobic and anaerobic condition). Further, Tukey–Kramer multiple comparisons test showed significantly higher decolorisation in the presence of living cells under anaerobic condition as compared to that in the under aerobic condition and heat killed cells (p-value < 0.0033). By Tukey–Kramer test, there was no significant difference in decolorisation in the presence of living cells under aerobic condition as compared to that in the presence of heat killed cells (p-value > 0.05) confirming the earlier hypothesis of only physical adsorption by dead or living cells under aerobic condition.

Meanwhile, the blue color of RB19 was caused by anthraquinone (chromophore). An UV–vis spectral scan (300–700 nm) of the supernatants after decolorisation showed that the maximum absorbance wavelength shifted from 592 to 448 nm in living cell cultures (Fig. 5d). So, it could be presumed that the anthraquinone group cleaved during the reaction which indicated that the primary chromophore was destroyed. Due to the destruction of chromophore, the significant changes occurred in UV–vis spectra. The change occurring in UV–vis spectra indicated that the molecular

structure of RB-19 evidently changed after decolorisation. The absorbance peak at UV spectra did not disappear in the end of decolorisation, which indicated that RB19 was not completely mineralized while some new metabolites were formed in the culture. These results indicate that the color removal by *Enterobacter* sp.F has a definite bio-chemical origin or a reason.

6. Biodegradation analysis

The degradation was confirmed from FTIR analysis. Comparison of FTIR spectrum of control dye with extracted metabolites after complete decolorisation, clearly indicated the biodegradation of the parent dye compound by *Enterobacter* sp.F.

The FTIR spectra revealed that the ring structures of RB-19 were definitely degraded because the peaks of the wavenumber between 670 and 870 cm^{-1}, which denote aromatic rings, which was also disappeared (Fanchiang and Tseng, 2009). Moreover, the cleavage of anthraquinone rings was also evidenced by the peak becoming weaker near 1590 cm^{-1}, which is responsible for the combination of stretching vibration of C═O conjugated with C═C (Yuen et al., 2005). Apart from the destruction of the aromatic structures, the functional groups attached to them were also transformed by *Enterobacter* sp.F. The fact of this phenomenon was due to the observance of strong peaks of C-NH_2 around 1040 and 1118 cm^{-1} with reduced intensity (Fanchiang and Tseng, 2009). In addition, the broad peak around 3420 cm^{-1}, which is assigned to N—H vibration (Memon and Memon, 2012), disappeared after decolorisation. The peaks around 2920 and 2850 cm^{-1} rep-resent the C—H asymmetric stretching and C—H symmetric stretching of CH_2, respectively (Yuen et al., 2005) and did not disappear after decolorisation. This suggested that cleavage of the vinyl sulfonyl group (—SO_2—CH_2—CH_2—OSO_3Na) did not occur during decolorisation. Thus, it may be concluded that *Enterobacter* sp.F effectively decolorizes Reactive Blue 19 due to biodegradation.

7. Conclusions

The optimal conditions for the decolorizing activity of *Enterobacter* sp.F were anaerobic conditions with 6 g/L glucose supplementation, 40 as glucose to microbe weight ratio, pH of 10, and temperature of 37 °C. *Enterobacter* sp.F showed decolorizing activity for RB 19 dye degradation through bio-chemical mechanism, and it could tolerate up to 400 mg/L of RB 19 without experiencing significant toxicity.

Because of degradative and decolorizing activity against anthraquinone reactive dye commonly used in the textile industries, it is proposed that *Enterobacter* sp.F has a practical application in the biotransformation of anthraquinone based reactive dye effluents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.09.108.

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