

Aerobic degradation of methylene blue from colored effluents by *Ralstonia eutropha*

Habibi, A.* and Mehrabi, Z.

Faculty of Petroleum and Chemical Engineering, Razi University, Kermanshah, Iran

Received: 23 Nov. 2016

Accepted: 30 Jan. 2017

ABSTRACT: The present paper has examined the degrading ability of phenol-oxidizing bacterium, *Ralstonia eutropha*, for biological removal of methylene blue (MB) from aqueous solutions under aerobic conditions. Results show that MB has been extensively eliminated as a co-metabolism in the presence of supplementary carbon (glucose) and nitrogen (yeast extract and peptone) sources and the experimental observations indicate that MB is initially adsorbed on the cell's surface, in accordance to Langmuir Theory, then to be degraded by the cell. The type of nitrogen source, initial pH, aeration rate, and the presence of CaCl_2 are all influential factors in the process of MB removal. The biodegradation kinetics modeling has determined that while playing an uncompetitive role, MB inhibits its biodegradation at high concentrations. According to the best fit Han-Levenspiel Model, the maximum MB specific biodegradation rate (r_{max}), half-saturation concentration of MB (K_S), maximum allowable MB concentration (S_m), and the shape factors (n and m) have been $7.37 \text{ mg g}_{\text{cell}}^{-1} \text{ h}^{-1}$, 32.13 mg/L , 158.8 mg/L , 0.27 , and 0.76 , respectively.

Keywords: aerobic cell, biodegradation, dyestuff decolorization, kinetic modeling, wastewater treatment.

INTRODUCTION

Methylene blue (MB), 3, 7-bis (dimethyl amino)-phenothiazin-5-ium chloride, is one of the common dyestuffs, applied in industries such as textile, leather, paper, plastics, and paint, in which large amounts of MB, usually with a concentration of 10-200 mg/L, are discharged into the environment (Akhtar et al., 2005). These colored effluents are famous for their unfavorable impacts on natural ecosystems and aquatic life. At first, the quality of water's appearance is significantly affected by minute amount of MB (even less than 1 ppm), which manages to obstruct light

penetration (Garg et al., 2004). It also decreases the amount of oxygen, dissolved in aqueous media, for its high reduction potential. The aromatic structure of MB has demonstrated mutagenic effects on living cells (Eskizeybek et al., 2012). European Communities regulation for discharge into surface waters is 0.2 mg of MB in each liter (European Communities, 1989). As a result, this dye-containing industrial wastewater must be treated before being discharged so that its impacts on the environment can be minimized.

Recently, the population growth has led to an increase in industrial activities. It is, therefore, necessary to improve and found new processes, capable of efficiently treating

* Corresponding Author Email: a.habibi@razi.ac.ir

effluents in industrial facility units. As such, there have been several methods used to treat colored wastewaters such as adsorption, precipitation, ion exchange, photocatalytic degradation, reverse osmosis, flocculation, coagulation, and biodegradation, with the latter having technical advantages in comparison to others in terms of its lower operational costs, less water usage, and complete conversion of the dye to non-toxic mineral compounds (Saratale et al., 2011; Chatterjee et al., 2012; Noraini et al., 2012). In the last decade, several microbial processes for MB decolorization have been developed, using anaerobic active sludge or simultaneous aerobic/anaerobic microbial consortiums (Sponza & Isik, 2002; Ong et al., 2005; Cetin & Donmez, 2006; Jonstrup et al., 2011). However, a few report are available for biodegradation of MB under aerobic conditions (Noraini et al., 2012).

The use of non-pathogenic soil bacterium, *Ralstonia eutropha*, as an efficient degrader of phenolic compounds, has been reported in previous works (Nickzad et al., 2012; Motamedi et al., 2014; Habibi & Sharifi, 2016). To our knowledge, the literature contains no reports related to the degradative ability of *R. eutropha* toward MB in aqueous media under aerobic conditions. Hence the present research mainly focuses on studying the effects of environmental parameters such as initial pH, type and concentration of nutrients in the medium, and aeration on the biological decolorization process. The biosorption capacity of MB on the cell's surface as well as the biodegradation rate has been experimentally determined and then modeled by the relevant appropriate mathematical models.

MATERIAL AND METHODS

All chemicals, used, were analytical grade from Merck (Darmstadt, Germany), purchased from local supplier. The fine chemicals, used for media preparation, were bacteriological grade and were obtained from Merck.

The bacterium *R. eutropha* (PTCC 1615) was purchased from Persian-Type Culture Collection in lyophilized form and was then maintained on nutrient agar medium. Acclimation of *R. eutropha* to phenol was carried out, using glucose-phenol (GP) growth medium, containing (g/L): KH_2PO_4 , 1.0; K_2HPO_4 , 1.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 and glucose and phenol as carbon sources (Nickzad et al., 2012). During adaption protocol, glucose concentration gradually declined and simultaneously phenol concentration rose in the GP medium. Adaptation was completed, once the test bacterium was able to use phenol as a sole carbon source. The adaptation protocol induced the aerobic enzymatic system for cleavage of aromatic ring in MB along with monooxygenases in the test bacterium (van Schie & Young, 2000).

The effect of the composition of the cultivation medium was studied in 250 mL Erlenmeyer flasks with 100 mL of the media, containing the ingredients according to Table 1. In each experiment, after adjusting the initial pH to 6.5, the medium was sterilized in an autoclave at 121°C for 20 min. It was inoculated with 10% of 24-h grown culture of the phenol-adapted *R. eutropha* that provided an initial cell density of about 0.2 ± 0.02 g/L. The cultivation temperature of 30 °C and agitation speed of 180 rpm were constant during the experiments.

The abiotic tests were conducted to determine the possibilities of physico-chemical interactions of additional chemicals with MB.

The effect of initial pH was also studied in 250 mL Erlenmeyer flasks with 100 mL of decolorization culture medium (DCM), including 10 mg/L of MB along with the following ingredients (g/L): glucose, 3; yeast extract, 3; peptone, 3; $(\text{NH}_4)_2\text{SO}_4$, 1; KH_2PO_4 , 1; K_2HPO_4 , 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; and CaCl_2 , 0.1. The initial pH was

Table 1. The experiments to study the effect of nutrients on the MB decolorization performance of *R. eutropha*

Experiment name	MB (10 mg/L)	KH ₂ PO ₄ (1 g/L)	K ₂ HPO ₄ (1 g/L)	MgSO ₄ ·7H ₂ O (0.05 g/L)	Glucose (3 g/L)	(NH ₄) ₂ SO ₄ (1 g/L)	Peptone (3 g/L)	Yeast extract (3 g/L)	CaCl ₂ (0.1 g/L)
A	+	+	+	+	-	-	-	-	-
B	+	+	+	+	+	-	-	-	-
C	+	+	+	+	-	+	-	-	-
D	+	+	+	+	+	+	-	-	-
E	+	+	+	+	+	+	+	-	-
F	+	+	+	+	+	+	-	+	-
J	+	+	+	+	+	+	+	+	-
H	+	+	+	+	+	+	+	+	+

measured at 5.5, 6.5, 7.0, 7.5, and 8.5 under the cultivation temperature of 30°C, agitation speed of 180 rpm, and initial biomass of 0.2±0.02 g/L.

The decolorization yield (Y_{dye}) and average decolorization rate (R) during 72 h of the treatments were reported as the responses:

$$Y_{dye} (\%) = \frac{S_i - S_f}{S_i} \times 100 \quad (1)$$

$$R (\text{mg h}^{-1}) = \frac{S_i - S_f}{t} \quad (2)$$

where, S_i and S_f are the initial and final concentrations of MB (mg/L), and t is the treatment time (h).

In order to estimate the biosorption capacity of MB on cell's surface, initially 100 mL of the grown culture of test bacterium with biomass concentration of about 2.3±0.1 g/L was inactivated by heating in an autoclave for 20 min at 121°C. Then, 10 mL of the heat-killed culture was added into 90 mL of MB solutions with initial concentrations of 10, 15, 25, 50, 75, 100, 125, and 150 mg/L. The samples were incubated in a shaker incubator at 30°C and 180 rpm for 5 h. Then, the biomass was separated through centrifugation at 6000×g for 6 min. The adsorbed dye on the sediments were extracted with acetone-methanol (3:1 v:v) solutions. The cell was harvested by

centrifugation and blue color supernatant was picked out. The dyestuff extraction and cell separation steps were repeated until the entire blue color was removed from the cell. Concentration of MB in the supernatant was determined and the adsorption capacity of MB on cell (q_e , $\text{mg}_{\text{MB}} \text{g}_{\text{cell}}^{-1}$) was estimated by means of the Equation (3).

$$q_e (\text{mg g}_{\text{cell}}^{-1}) = \frac{(S_i - S_e)V}{M} \quad (3)$$

where q_e is the amount of MB, adsorbed at an equilibrium state; S_i and S_e are initial and equilibrium MB concentrations in the biosorption medium (mg/L) respectively, V is the volume of the biosorption medium (L), and M is the amount of dry cell biomass (g).

The experiments had use of a 2 L flat-bottom flask with lateral equipment (materials inlet tube, inlet valve, and sampler valve). Temperature was kept constant at 30±2°C during the process with a thermostatic chamber. The bioreactor was aerated, using a diffuser as well as an ordinary air-supplied pump. The Inlet blast was sterilized by being filtered through a 0.2 µm pore-size disposable sterile filter unit. A mechanical agitation at 180 rpm managed to mix an amount of 1.5 L of the DCM medium in the reactor. The initial pH was adjusted at 7.0 prior to sterilization. The reactor and medium were sterilized in

an autoclave at 121°C for 20 min and inoculation was done by 10% volume of the 24-grown culture of *R. eutropha* on the GP medium.

In order to evaluate the impact of aeration on the decolorization process, the performance of aerated reactor at 0.11, 0.22, 0.33, and 0.55 vvm were compared with a non-aerated reactor.

The kinetics of MB biodegradation were determined at different initial MB concentrations, ranging from 10 to 150 mg/L, where the reactor operated at an aeration rate of 0.55 vvm. In accordance with the experiments, the initial specific MB biodegradation rates (r) were determined during the first eight hours as follows.

$$r(\text{mg g}_{\text{cell}}^{-1} \text{h}^{-1}) = \frac{1}{X_i} \frac{S_i - S_R}{t} \quad (4)$$

where S_i and X_i are the initial concentrations of MB (mg/L) and cell biomass (g/L), respectively. S_R is the residual MB concentration (mg/L) after eight hours of treatment time.

At regular time intervals, 10 mL of the DCM medium were collected. The absorbance at 600 nm for the collected sample, containing the cell along with dyestuff, was measured prior to centrifugation.

$$OD_{600\text{nm}}^{\text{cell+dye}} = OD_{600\text{nm}}^{\text{dye}} + OD_{600\text{nm}}^{\text{cell}} \quad (5)$$

Afterwards the sample was centrifuged at 6000×g for 6 min and the supernatant as evaluated, using a UV-Vis scanning spectrophotometer at maximum MB absorbance ($\lambda_{\text{max}} = 665 \text{ nm}$). The residual MB concentration was determined from a calibration curve against standard MB solutions.

In order to assess the cell biomass concentration, the absorbance of the supernatant was also measured at 600 nm.

$$OD_{600\text{nm}}^{\text{sup}} = OD_{600\text{nm}}^{\text{dye}} \quad (6)$$

By subtracting of Equation (6) from Equation (5) and using a calibration curve that related the dry cell weight to $OD_{600\text{nm}}^{\text{cell}}$, the biomass concentration was determined.

Every bar represents the mean of three replicates±standard error.

All regression analysis were carried out by means of nonlinear least-squares technique (Levenburg-Marquardt method), available in GraphPad Prism software for windows, version 5.00. Goodness of fit for regression of the experimental data (r_{exp}) by the model (r_{pred}) was determined on the basis of the coefficient of determination (R^2) and the standard deviation of residuals ($Sy.x$) as bellows:

$$R^2 = 1 - \frac{\sum (r_{\text{exp}} - r_{\text{pred}})^2}{\sum (r_{\text{exp}} - \bar{r})^2} \quad (7)$$

$$Sy.x = \frac{\sqrt{\sum (r_{\text{exp}} - \bar{r})^2}}{N - p} \quad (8)$$

where, \bar{r} , N and p were the mathematical average value, the number of experimental data, and the number of parameters fit, respectively.

RESULTS AND DISCUSSION

Figure 1 illustrates the decolorization performance of the biological process before and after adding nutrients. In the biological experiment “A” and in the absence of additional chemicals, only about 2.8% of the initial MB was mainly removed due to dye adsorption on cell’s surface. Once glucose was added in the biological experiment “B”, the removal yield ascended to 6.8%. However addition of ammonium sulphate in the biological experiment “C” did not change the removal yield in comparison to the biological experiment “A”. The simultaneous addition of glucose and ammonium sulphate in the biological experiment “D” resulted to the removal of the initial MB by 29.8%. In the experiment, the biomass concentration increased during the process. These observation indicated that the decolorization process of MB dye could be performed as a non-growth substrate in the presence of supplementary substrates by the growing cells via co-metabolism approach (Balapure et al., 2014). Once both yeast extract and peptone

were added, as organic nitrogen sources, to the biological experiments “E” and “F”, the decolorization yield soared to 52.2% and 41.2%, respectively. The decolorization yield further zoomed to 82.8% with simultaneous addition of ammonium sulfate, yeast extract, and peptone (Biological experiment “J”). The findings agreed with previous reports, concerning the importance of nitrogen source as an essential factor for effective removal of colorant substances (Chen et al., 2003; Mathew & Madamwar, 2004; Kolekar et al., 2013). In the present study, application of yeast extract increased the specific degradation rate from $0.067 \text{ mg g}_{\text{cell}}^{-1} \text{ h}^{-1}$ to $0.083 \text{ mg g}_{\text{cell}}^{-1} \text{ h}^{-1}$, and the simultaneous addition of yeast extract and peptone led to a specific degradation rate of about $0.101 \text{ mg g}_{\text{cell}}^{-1} \text{ h}^{-1}$. The results indicated the important role of the organic nitrogen source on generation of the dye-reducing agents such as NADH^+ along with the enhancement of the degradative activity of the mono- and di-oxygenases, participated in the biological MB decomposition (Ong et al., 2005; Tan et al., 2013; Palanivelan et al., 2014). As shown in Figure 1, the decolorization yield in the biological

treatment also depended on the presence of CaCl_2 salt and the decolorization performance of the test cell was improved 15.2% by adding CaCl_2 . In fact, the variation of cell wall inflexibility has a significant effect on the biological processes. The presence of CaCl_2 could induce osmotic stress in the cell, subsequently increasing the permeability of cell wall for excretion of the preplasmic enzymes (Gomaa et al., 2013). High concentration of CaCl_2 leads to the cell wall's inflexibility. In this study, a suitable level for CaCl_2 concentration in the DGM medium was 0.1 g/L , with higher concentrations resulting in less decolorization yield. Ambatkar and Mukundan (2014) reported that an optimum concentration of CaCl_2 for azo dyestuff decolorization by crude peroxidase from *Armoracia rusticana* was 25 mM . Figure 1 compares the abiotic tests, indicating that the presence of the reductive chemicals such as glucose and organic nitrogen sources had a minor influence on MB decolorization (less than 10%). Thus, the biological degradation was a probable mechanism for MB decolorization in the present study.

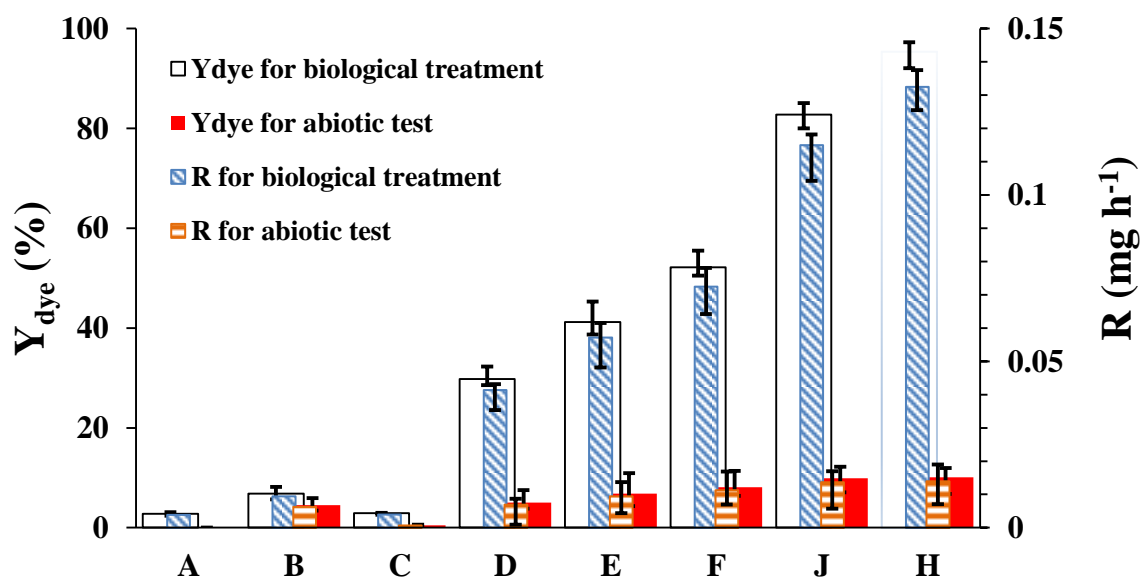


Fig. 1. Comparison of the effect of adding nutrients in the abiotic and biological processes

Figure 2 illustrates the MB absorbance spectrum. The absorption peak at 665 nm for MB was decreased gradually as the biological treatment time rose. By applying the biological process, the absorbance peak shifted from 665 nm to 655 nm, as shown

in Figure 2. The reduction in absorbance was likely due to the degradation of MB chromophore, with the peak shift showing the occurrence of de-methylation reaction by the cell (Matsuo et al., 2004).

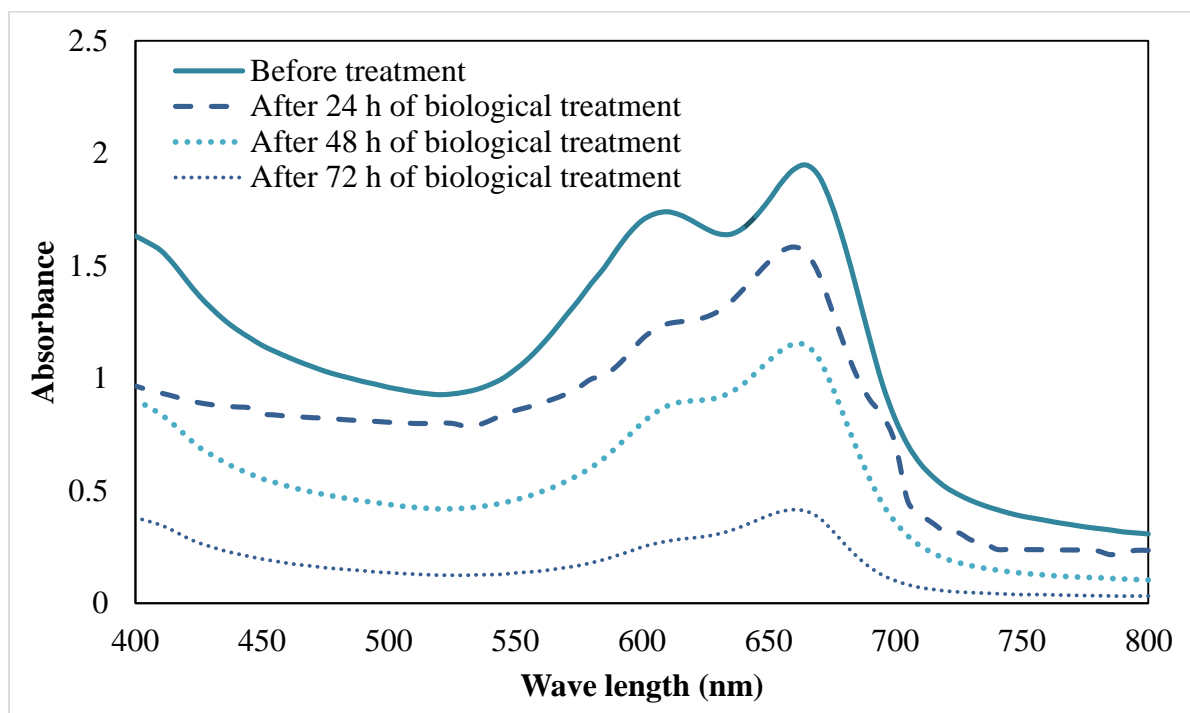


Fig. 2. Absorption spectral changes and biological degradation of MB aqueous solution by *R. eutropha* cell

Biosorption studies are often carried out to better understand the adsorption process and determine the capacity of adsorbents. Previously, Noraini et al. (2012) reported that a biological removal of MB from aqueous waste streams took place in two stages: Initially, the dyestuff was quickly adsorbed to cell surface via either physical interactions or chemical reactions and was then degraded by enzyme-catalytic reactions in the next step. In order to quantify the MB adsorption on the cell's surface, the equilibrium communication between the amount of adsorbed MB on cell surface (q_e , $\text{mg g}_{\text{cell}}^{-1}$) and the remained dye in the culture medium (S_e , mg/L) at 30°C was experimentally determined. Afterwards, by using non-linear regression analysis, the relevant Langmuir isotherm's

constants were estimated. On the best-fit Langmuir isotherm, the maximum of the equilibrium adsorption capacity (q_{max}) and Langmuir isotherm constant (k_L) were $0.617 \text{ mg g}_{\text{cell}}^{-1}$ and $0.0304 \text{ g}_{\text{cell}}^{-1} \text{ L}$, respectively. Figure 3 compares the experimental bioprotion data and Langmuir isotherm. According to the results, the Langmuir isotherm agreed well with the experiment data. It was previously reported for adsorption of the reactive red 198 on *Aspergillus parasiticus* cell (Tunali Akar et al., 2009). The Langmuir Model is only applicable in adsorbents whose surface is homogeneous with the assumption that the maximum adsorption occurs in a monolayer coverage on the adsorbent surface (Hameed et al., 2008).

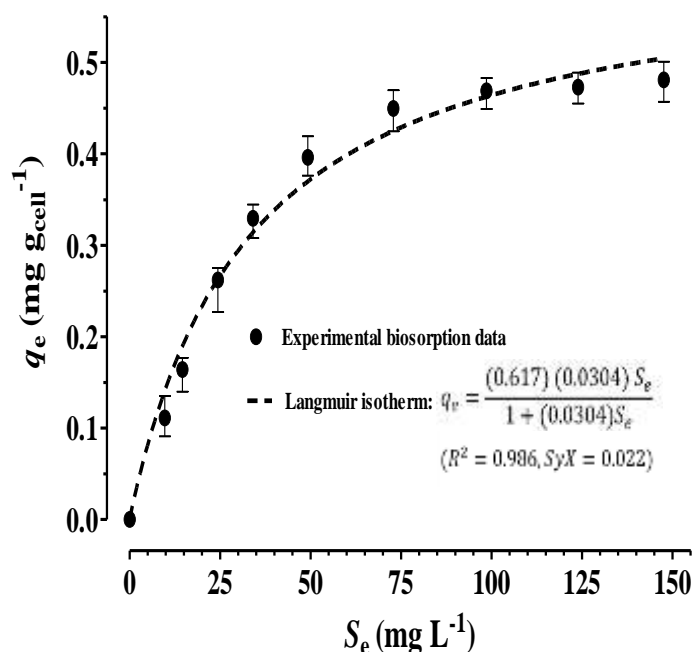


Fig. 3. Fitting of the Langmuir isotherm to the experimental MB adsorption on the cell’s surface

In the absence of water, Naumann et al. (1991) proposed that five major absorbance regions in Fourier Transform Infrared Spectroscopy (FTIR) spectra should be analyzed to identify the cell wall in bacteria: the 3000–2800 cm⁻¹ spectral region is the fatty acidic region (Region I); 1700-1500 cm⁻¹ one contains amide I and II bands of proteins and peptides (Region II); 1500-1200 cm⁻¹ is a mixed region of fatty acidic bending vibrations, proteins, and phosphate-carrying compounds (Region III); 1200-900 cm⁻¹ contains absorption bands of the carbohydrates in microbial cell walls (Region IV); and 900-700 cm⁻¹ is the ‘fingerprint region’ that contains weak but very unique absorbances, characteristic of

specific bacteria (Region V). These absorbance regions are obviously detected in FTIR spectrum of *R. eutropha* in Figure 4, which also provides a comparison between the FTIR spectrums of the cell wall before and after MB adsorption test. The comparison was used to analyze the changes in functional groups of cell wall after the adsorption of MB. Shifting the peaks after the adsorption of the dyestuff with cell wall is listed in Table 2. Significant changes were observed in the peak values, indicating the existence of physical interaction between cell wall and MB. A similar shifting was recorded in adsorption of MB by chitin nanoparticles (Dhananasekaran et al., 2016).

Table 2. FTIR analysis peaks of *R. eutropha* cell wall and shifting the peak values after adsorption of MB

Vibration modes	Before adsorption	After adsorption
N-H stretching	3291	3285
C-H stretching	2930	2928
C-H bending	1731	1653
C=O stretching	1656	1601
N-O stretching	1540	1533
S=O stretching	1127	1132
C=C bending	978	951
C-X stretching (X= Halo compound)	618	616

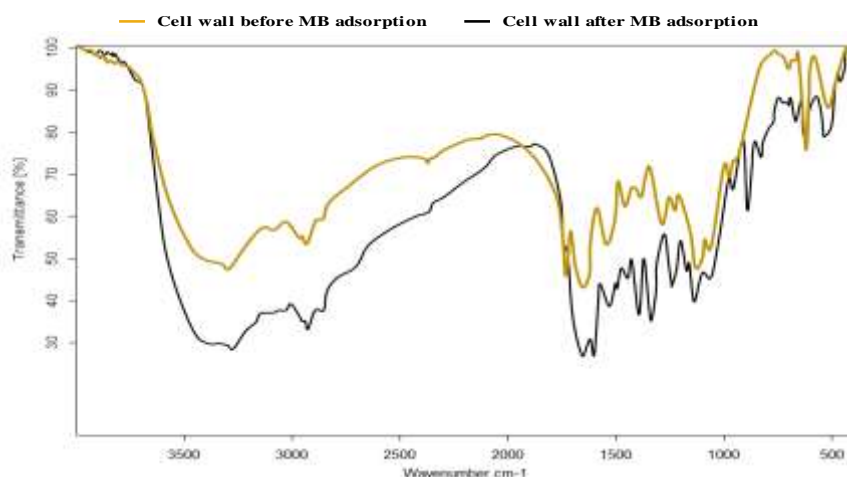


Fig. 4. FTIR analysis of *R. eutropha* cell wall prior and after MB adsorption

Nonetheless, the low value of q_{max} in the present study indicated that the cell as sorbent could not significantly remove MB from the medium, thus the decolorization observed in the present study was performed mainly due to an enzymatic degradation.

Nutrients' expulsion from cell membrane as well as catalytic activities of the enzymatic systems are influenced by pH. Figure 5 shows the effect of initial pH on MB removal by *R. eutropha*. The maximum MB removal occurred at an

initial pH of 7.0 and the performance obviously declined when initial pH was changed from 7.0 to either acidic or alkali conditions. Sharma et al. (2009) found an optimum pH at about 7.0 for yellow 211 dye decolorization by *Bacillus subtilis*. Wang et al. (2009) reached a maximum reactive dye decolorization efficiency, using *Citrobacter sp.* at pH= 7.0 and Mohana et al. (2008), reported an optimal condition for degradation of direct black 22 dye with a bacterial consortium at pH= 7.

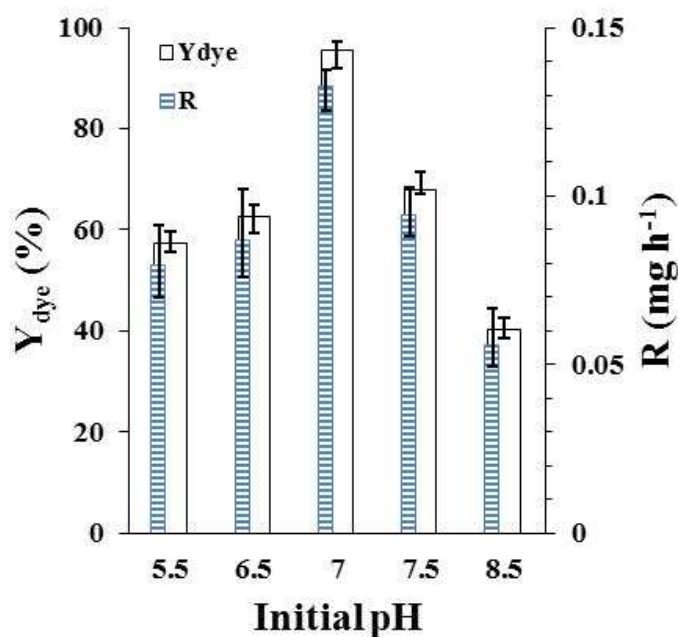


Fig. 5. Impact of initial pH on decolorization performance of the cell

In aerobic microorganisms, aeration is necessary not only for cell growth but also for maintaining the metabolic activities. Oxygen availability for cell depends on the rate of oxygen transfer from air bubble (gas phase) to the culture medium (liquid phase) and then delivering the active center of the respiration reaction within the cell. Here Figure 6 demonstrates the effect of aeration rate on the MB removal. Results show that the cell growth was enhanced in the aerated reactors (Fig. 6a). With aerating the reactor at 0.11 and 0.55 vvm, about 92.3% and 98.3% of the initial MB (10 mg/L) were respectively removed after 12 h and 8 h from the beginning of the process (Fig. 6b). A comparison between the decolorization performances indicated that the specific decolorization rates in the aerated reactor were increased 4 to 6 times higher than the non-aerated reactor, revealing the importance of bioavailability of the dissolved oxygen for the catalytic activities of mono- and di-oxygenases, participating in MB degradation. In the anaerobic processes, previously reported for MB decolorization, the effluent was exposed to air, and thus the reduced form of MB (leucomethylene blue) was re-oxidized by oxygen and the blue color reappeared gradually (Isik & Sponza, 2003). In the present study, the efficiency of the MB decolorization was increased in the aerated reactor; therefore, the irreversible decolorization of MB confirms the fact that the chemical structure of MB was disrupted by the cell. The decolorization activity was increased as the aeration rate rose, reaching a specific removal rate of about $0.614 \text{ mg g}_{\text{cell}}^{-1} \text{ h}^{-1}$ in aeration rate of 0.55 vvm. An aeration rate above 0.55 vvm was not favorable for foam formation and operational instability in the reactor.

Figure 7 shows the temporal variations of residual MB concentration. Based on the results, more than 98% of MB was rapidly removed in 8 h when initial concentration was equal to 10 mg/L. However, further

increase of MB concentration up to 15 and 25 mg/L, raised the degradation time for a complete degradation (more than 95% removal) to 16 and 48 h respectively.

Figure 8 demonstrates the initial specific MB biodegradation rate (r) as a function of initial MB concentration (S_i). The trend shows that the MB biodegradation rate is a function of the initial MB concentration. Decreasing “ r ” at higher concentrations either indicates inhibitory effect of MB or produces intermediates on the degradative activities of the cell. To determine the kinetic constants, involved in the MB biodegradation, the namely Haldane (Eq. 9) and Han-Levenspiel (Eq. 10) Models, developed on the basis of enzymatic-inhibition mechanisms, were used.

$$r = \frac{r_{\max} S_i [1 + (S_i / S_m)]^n}{S_i + K_S [1 + (S_i / S_m)]^m} \quad (9)$$

$$r = \frac{r_{\max} S_i}{K_S + S_i [1 + (S_i / K_I)]} \quad (10)$$

where, r_{\max} is the maximum specific MB degradation rate in the absence of inhibitory effect of MB ($\text{mg g}_{\text{cell}}^{-1} \text{ h}^{-1}$), K_S is the half-saturation concentration of MB (mg/L), K_I is the substrate inhibition concentration, and S_m is the maximum MB concentration, above which the biodegradation is completely inhibited (mg/L). Both n and m are empirical constants, defining the mechanisms of inhibitor actions in Han-Levenspiel Model (-).

Figure 8 compares the values, predicted by the models, as well as the experimental data. Results show that Han-Levenspiel Model provides a good mathematical description of the process, compared to Haldane Model. On the best fit models (Table 3), the maximum specific MB biodegradation rate were estimated 7.37 and 26.00 $\text{mg g}_{\text{cell}}^{-1} \text{ h}^{-1}$ by the Han-Levenspiel and Haldane models respectively. The estimated half-saturation concentration by the Haldane Model was

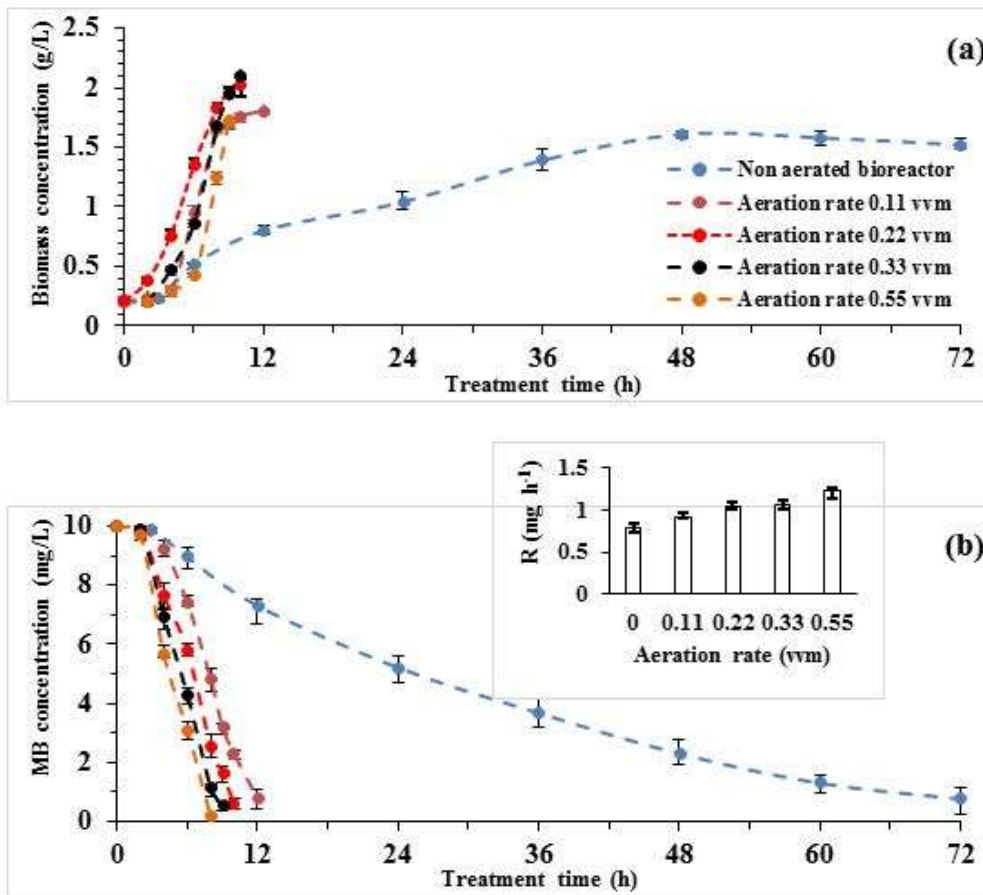


Fig. 6. Temporal variations of the cell biomass (a), and residual MB concentration (b) as a function of aeration rate on stirred tank bioreactor

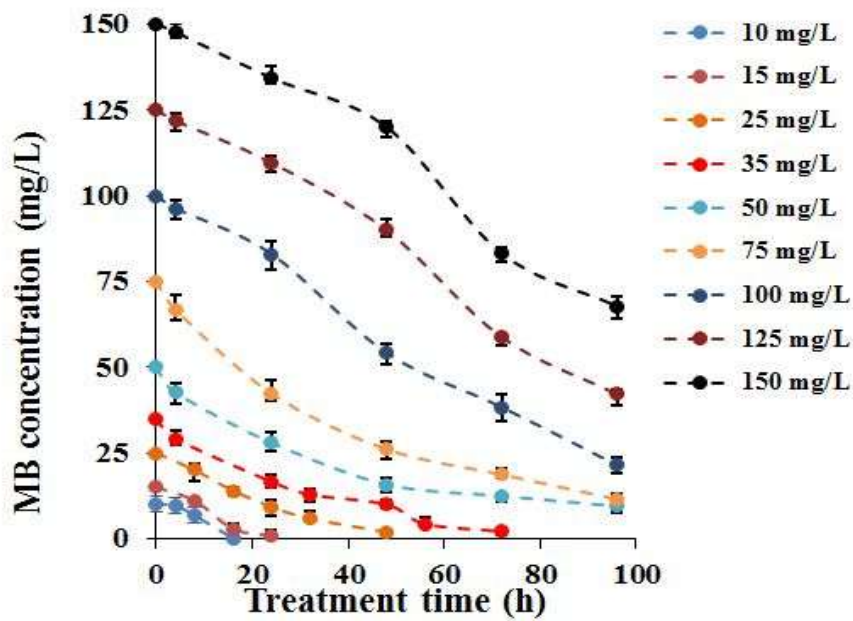


Fig. 7. The variation of MB concentration in aerated stirred tank bioreactor (0.55 vvm) at different initial MB concentrations

higher than the one, predicted by the Han-Levenspiel Model (150.4 mg/L in Haldane versus 32.13 mg/L in Han-Levenspiel). The kinetic constants reported in other microbial MB degradation processes are compared with this study in Table 3. Further test of the Han-Levenspiel equation showed the importance of the predicted S_m value relative to the experimental results, obtained in this study, where 158.8 mg/L was the highest estimated MB concentration degradable by *R. eutropha* cell. The Haldane Model predicted a comparative K_I value of about 31.62 mg/L. An inhibition concentration about 21.23 mg/L was previously reported for MB

biodegradation by an activated sludge (Ma et al., 2011). Ultimately, the obtained values of shape factors in the Han-Levenspiel Model were equal to 0.27, and 0.76, which represent a generalized uncompetitive inhibition role of MB on its biodegradation in the present study (Han & Levenspiel, 1988). Based on the extensive studies performed by Houas et al. (2001), phenolic components such as benzene sulfonic acid, and phenol have been found to be the main metabolites, resulting from MB decomposition. High concentrations of these phenolic compounds could harm the bacterium cell at the high initial MB concentrations.

Table 3. Comparison between the kinetic constants, obtained in this study and other MB kinetic studies

Microorganism	Mathematical model	Kinetic constants						Goodness of fit*		
		r_{max} (mg g _{cell} ⁻¹ h ⁻¹)	K_S (mg/L)	K_I (mg/L)	S_m (mg/L)	n	m	R ²	Sy.x	Ref.
<i>R. eutropha</i>	$r = \frac{r_{max} S_i}{K_S + S_i + \left(\frac{S_i^2}{K_I}\right)}$	26.00	150.4	31.64	-	-	-	0.958	0.365	This study
<i>R. eutropha</i>	$r = \frac{r_{max} S_i [1 + (S_i / S_m)]^n}{S_i + K_S [1 + (S_i / S_m)]^m}$	7.37	32.13	-	158.8	0.76	0.27	0.989	0.251	This study
Activated sludge	$r = \frac{r_{max} S_i}{K_S + S_i + \left(\frac{S_i^2}{K_I}\right)}$	184.15	209.96	21.23	-	-	-	0.98	-	Ma et al., 2011
<i>Phanerochaete chrysosporium</i>	$r = \frac{r_{max} S_i}{K_S + S_i}$	2.10	132.8	-	-	-	-	-	-	Radha et al., 2005

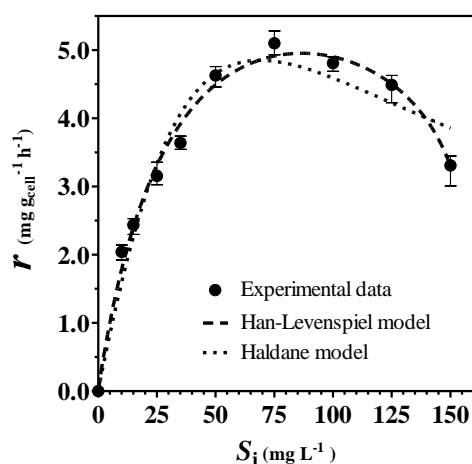


Fig. 8. Comparison of the initial specific MB biodegradation rate and predicted values by the substrate-inhibition models at different initial MB concentrations

CONCLUSIONS

This study focused on the degradative ability of *R. eutropha* towards decolorization of MB from synthetic wastewaters under aerobic conditions. Results indicated that the test bacterium was extensively capable of decolorizing MB as a non-growth substrate in the presence of glucose and organic nitrogen sources. The experiments in the aerated stirred tank reactor showed the MB degradation rate was 4 to 6 times higher than the non-aerated reactor. The kinetic studies on initial specific MB biodegradation rate indicated MB had a uncompetitive inhibitory role on its degradation at high concentrations. The high degradative activities of *R. eutropha* in this study showed a high potential application of the bacterium in biological MB decomposition.

REFERENCES

- Akhtar, S., Ali Khan, A. and Husain, Q. (2005). Partially purified bitter melon (*Momordica charantia*) peroxidase catalyzed decolorization of textile and other industrially important dyes. *Bioresour. Technol.*, 96(16), 1804-1811.
- Ambatkar, M. and Mukundan, U. (2014). Calcium salts enhance activity and azo dye decolorization capacity of crude peroxidase from *Armoracia rusticana*. *Am. J. Plant Sci.*, 5(2), 212-218.
- Balapure, K.H., Jain, K., Chattaraj, S., Bhatt, N.S. and Madamwar, D. (2014). Co-metabolic degradation of diazo dye- reactive blue 160 by enriched mixed cultures BDN. *J Hazard. Mater.*, 279, 85-95.
- Cetin, D. and Donmez, G. (2006). Decolorization of reactive dyes by mixed cultures isolated from textile effluent under anaerobic conditions. *Enzyme Microb. Technol.*, 38(7), 926-930.
- Chatterjee, S., Kumar, A., Basu, S. and Dutta, S. (2012). Application of response surface methodology for methylene blue dye removal from aqueous solution using low cost adsorbent. *Chem. Eng. J.*, 181, 289-299.
- Chen, K.C., Wu, J.Y., Liou, D.J. and Hwang, S.C.J. (2003). Decolorization of the textile dyes by newly isolated bacterial strains. *J. Biotechnol.*, 101(1), 57-68.
- Dhananasekaran, S., Palanivel, R. and Pappu, S. (2016). Adsorption of methylene blue, bromophenol blue, and coomassie brilliant blue by α -chitin nanoparticles. *J. Adv. Res.*, 7(1), 113-124.
- Eskizeybek, V., Sari, F., Gulce, H., Gulce, A. and Avcı, A. (2012). Preparation of the new polyaniline/ZnO nanocomposite and its photocatalytic activity for degradation of methylene blue and malachite green dyes under UV and natural sun lights irradiations. *Appl. Catal. B*, 119, 197-206.
- European Communities Regulations (1989). Quality of Surface Water Intended for the Abstraction of Drinking Water, S.I. No 294 of 1989.5.
- Garg, V.K., Amita, M., Kumar, R. and Gupta, R. (2004). Basic dye (methylene blue) removal from simulated wastewater by adsorption using Indian rosewood sawdust: a timber industry waste. *Dyes Pigm.*, 63(3), 243-250.
- Gomaa, O.M., Selim, N.S. and Linz, J.E., (2013). Biochemical and biophysical response to calcium chloride stress in *Aspergillus niger* and its role in malachite green degradation. *Cell Biochem. Biophys.*, 65(3), 413-423.
- Habibi, A. and Sharifi, S. (2016). Kinetic modelling for simultaneous biodegradation of phenol and formaldehyde at inhibitory concentrations by *Ralstonia eutropha*. *Clean Soil Air Water*, 44(9), 1113-1122.
- Hameed, B.H., Mahmoud, D.K. and Ahmad, A.L. (2008). Equilibrium modeling and kinetic studies on the adsorption of basic dye by a low-cost adsorbent: Coconut (*Cocos nucifera*) bunch waste. *J. Hazard. Mater.*, 158(1), 65-72.
- Han, K. and Levenspiel, O. (1988). Extended Monod kinetics for substrate, product, and cell inhibition. *Biotechnol. Bioeng.*, 32(4), 430-43.
- Houas, A., Lachheb, H., Ksibi, M., Elaloui, E., Guillard, C. and Herrmann, J.M. (2001). Photocatalytic degradation pathway of methylene blue in water. *Appl. Catal. B*, 31(2), 145-157.
- Isik, M. and Sponza, D.T. (2003). Effect of oxygen on decolorization of azo dyes by *Escherichia coli* and *Pseudomonas* sp. and fate of aromatic amines, *Process Biochem.*, 38(8), 1183-1192.
- Jonstrup, M., Kumar, N., Murto, M. and Mattiasson, B. (2011). Sequential anaerobic-aerobic treatment of azo dyes: decolourisation and amine degradability. *Desalination*, 280, 339-346.
- Kolekar, Y.M., Konde, P.D., Markad, V.L., Kulkarni, S.V., Chaudhari, A.U. and Kodam, K.M. (2013). Effective bioremoval and detoxification of textile dye mixture by *Alishewanella* sp. *KMK6. Appl. Microbiol. Biotechnol.*, 97(2), 881-889.
- Ma, D.Y., Wang, X.H., Song, C., Wang, S.G., Fan, M.H. and Li, X.M. (2011). Aerobic granulation for

methylene blue biodegradation in a sequencing batch reactor. *Desalination*, 276, 233-238.

Mathew, S. and Madamwar, D. (2004). Decolorization of ranocid fast blue dye by bacterial consortium SV5. *Appl. Biochem. Biotechnol.*, 118, 371-381.

Matsuo, S., Sakaguchi, N., Yamada, K., Matsuo, T. and Wakita, H. (2004). Role in photocatalysis and coordination structure of metal ions adsorbed on titanium dioxide articles: a comparison between lanthanide and iron ions. *Appl. Surf. Sci.*, 228, 233-244.

Mohana, S., Shrivastava, S., Divecha, J. and Madamwar, D. (2008). Response surface methodology for optimization of medium for decolorization of textile dye Direct Black 22 by a novel bacterial consortium. *Bioresour. Technol.*, 99(3), 562-569.

Motamedi, M., Habibi, A., Maleki, M. and Vahabzadeh, F. (2015). Experimental investigation and kinetic modeling of p-nitrophenol and phenol by kissiris-immobilized *Ralstonia eutropha* in a batch reactor. *Clean Soil Air Water*, 43(2), 237-243.

Naumann, D., Helm, D. and Labischinski, H. (1991). Microbiological characterizations by FT-IR spectroscopy. *Nature*, 351, 81-82.

Nickzad, A., Mogharei, A., Monazzami, A., Jamshidian, H. and Vahabzadeh, F. (2012). Biodegradation of phenol by *Ralstonia eutropha* in a kissiris-immobilized cell bioreactor. *Water Environ. Res.* 84(4), 626-634.

Noraini, C.H.C., Morad, N., Norli, I., Teng, T.T. and Ogugbue, C.J. (2012). Methylene blue degradation by *Sphingomonas paucimobilis* under aerobic conditions. *Water, Air, Soil Pollut.*, 223(8), 5131-5142.

Ong, S.A., Toorisaka, E., Hirata, M. and Hano, T. (2005). Biodegradation of redox dye methylene blue by up-flow anaerobic sludge blanket reactor. *J. Hazard. Mater.* 124, 88-94.

Palanivelan, R., Rajakumar, S. and Ayyasamy, P.M. (2014). Effect of various carbon and nitrogen sources on decolorization of textile dye remazol golden yellow using bacterial species. *J. Environ. Biol.*, 35(5), 781-787.

Radha, K.V., Regupathi, I., Arunagiri, A. and Murugesan, T. (2005). Decolorization studies of synthetic dyes using *Phanerochaete chrysosporium* and their kinetics. *Process Biochem.*, 40(10), 3337-3345.

Saratale, R.G., Saratale, G.D., Chang, J.S. and Govindwar, S.P. (2011). Bacterial decolorization and degradation of azo dyes: a review. *J. Taiwan Inst. Chem. Eng.*, 42(1), 138-157.

Sharma, P., Singh, L. and Dilbaghi, N. (2009). Optimization of process variables for decolorization of disperse yellow 211 by *Bacillus subtilis* using Box-Behnken design. *J. Hazard. Mater.*, 164, 1024-1029.

Sponza, D.T. and Isik, M. (2002). Decolorization and azo dye degradation by anaerobic/aerobic sequential process. *Enzyme Microb. Technol.*, 31, 102-110.

Tan, L., Ning, S., Zhang, X. and Shi, S. (2013). Aerobic decolorization and degradation of azo dyes by growing cells of a newly isolated yeast *Candida tropicalis* TL-F1. *Bioresour. Technol.*, 138, 307-313.

Tunali Akar, S., Akar, T. and Cabuk, A. (2009). Decolorization of a textile dye, reactive red 198 (RR198), by *Aspergillus parasiticus* fungal biosorbent, *Braz. J. Chem. Eng.*, 26(2), 399-405.

van Schie, P.M. and Young, L.Y. (2000). Biodegradation of phenol: mechanisms and applications. *Bioremediation J.*, 4(1), 1-18.

Wang, H., Su, J.Q., Zheng, X.W., Tian, Y., Xiong, X.J. and Zheng, T.L. (2009). Bacterial decolorization and degradation of the reactive dye reactive red 180 by *Citrobacter* sp. CK3. *Int. Biodeter. Biodegr.*, 63(4), 395-399.

