Decolorization of textile azo dye Reactive Red 239 by the novel strain *Shewanella xiamenensis* G5-03 isolated from contaminated soil

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ABSTRACT

Shewanella xiamenensis G5-03 isolated from contaminated landfill soil efficiently decolorized five textile azo dyes under static conditions. One of them, Reactive Red 239 (RR239), was completely decolorized at a pH range of 7 to 11 (at 35°C) within 3-6 h. The bacterium was also efficiently decolorized RR239 in a wide temperature range of 25-40°C (at pH 8). The kinetics of RR239 decolorization by G5-03 fitted to the Michaelis–Menten Model ($K_m = 443.3$ mg L^{-1}, $V_{max} = 166.7$ mg L^{-1} h^{-1}). The decolorization of RR239 was monitored by UV-Vis and FTIR spectroscopy, which showed significant changes in peak positions when compared to the dye spectrum. Overall, the ability of *S. xiamenensis* G5-03 to decolorize textile azo dyes in a wide range of temperatures and pH (neutral-alkaline) indicate that this strain is a potential candidate for treating dye-containing effluent.

Keywords: bacterium, biodegradation, FTIR, kinetic study, Reactive Red 239.

Descoloração do corante têxtil azo Reactive Red 239 por uma nova linhagem *Shewanella xiamenensis* G5-03 isolada de solo contaminado

RESUMO

Shewanella xiamenensis G5-03 isolada de solo contaminado de aterro sanitário foi eficiente em descolorir cinco corantes têxteis azos em condições estáticas. Um deles, Reactive Red 239 (RR239), foi completamente descolorido na faixa de pH de 7 a 11 (a 35°C) dentro de 3-6 h. A bactéria também foi eficiente em descolorir RR239 em uma ampla faixa de temperatura de 25-40°C (em pH 8). A cinética de descoloração do RR239 pela G5-03 se ajustou ao modelo de Michaelis-Menten (K_m = 443,3 mg L^{-1}, V_{max} = 166,7 mg L^{-1} h^{-1}). A descoloração do RR239 foi monitorada por espectroscopia UV-Vis e FTIR, que mostrou mudanças significativas nas posições dos picos quando comparadas ao espectro do corante. No geral, a capacidade de *S. xiamenensis* G5-03 para descolorir os corantes têxteis azo em uma ampla faixa de temperatura e pH (neutro-alcalino) indica que esta cepa é uma candidata em potencial para o tratamento de efluente contendo corante.

Palavras-chave: bactéria, biodegradação, estudo cinético, FTIR, Reactive Red 239.

1. INTRODUCTION

Textile dyes are recalcitrant compounds, and most of them and some of their metabolites are toxic, mutagenic, and carcinogenic. During the dyeing process, around 15% of the dyes are lost in wastewater (Vikrant *et al.,* 2018), and this is the cause of serious environmental and health concerns. Textile dyes in aqueous ecosystems may affect aesthetic qualities, reduce light penetration, which is essential for photosynthesis, and make oxygen transference into the water difficult, causing acute toxic effects on aquatic organisms (Saratale *et al.,* 2011).

Various conventional physico-chemical wastewater treatment processes including flocculation, chemical coagulation, precipitation, ozonation, photocatalysis, color irradiation, and adsorption have been applied for textile effluent decolorization. These techniques, however, have limitations such as high cost, the formation of large quantities of sludge containing the dyes and their metabolites and intensive energy requirements (Castro *et al.,* 2017). On the other hand, bioremediation is considered an efficient and economical technique widely studied in recent years to treat several kinds of industrial effluents. This technology is based on biological processes in which microorganisms can degrade and even mineralize hazardous compounds, including textile dyes, with low environmental impact, costs, and energy requirements (Saratale *et al.,* 2011).

Azo dyes are characterized by one or more chromophore (azo) group (−N=N−) in their chemical structures and constitute the largest group used in textile dyestuffs, representing about 60-70% by weight. According to the number of chromophore groups in the molecule, azo dyes are classified as monoazo, diazo or triazo, and are available in six application categories: acid, basic, disperse, direct, azoic, and pigments (Meng *et al.,* 2014). Studies have shown that several bacterial genera can decolorize azo dyes: *Alcaligenes* (Shah *et al.,* 2012), *Pseudomonas* (Hussain *et al.,* 2013), *Enterobacter* (Holkar *et al.* 2014), *Aeromonas*, *Exiguobacterium* (Hsueh *et al.,* 2016), and *Halomonas* (Guadie *et al.,* 2018). Despite the diversity of bacteria that can decolorize azo dyes, the mechanism of decolorization is similar for all of them and involves the reductive cleavage of the azo bond by azoreductases in anaerobic conditions. Posteriorly, the end products of this reaction are degraded by hydrolases and oxygenases produced by bacteria in aerobic conditions (Vikrant *et al.,* 2018).

In this study, textile azo dyes were decolorized by a novel strain, *Shewanella xiamenensis* G5-03, isolated from contaminated landfill soil. Considering that several environmental

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conditions influence the dye-decolorization process, variables including pH, temperature, dye concentration, aerobic, and microaerophilic incubation were studied to achieve maximum dye degradation. The confirmation of dye degradation was carried out using UV visible spectroscopy and Fourier transform infrared spectroscopy (FTIR) analysis.

2. MATERIALS AND METHODS

2.1. Dyes, chemicals and culture media

Textile azo dyes used, i.e., Reactive Red 239 (RR239), Reactive Yellow 176 (RY176), Reactive Black B (RBB), Direct Blue 85 (DB85), and Direct Orange 39 (DO39) were obtained from Têxtil São João (São João da Boa Vista, SP, Brazil), and used as received without further purification. Sterile stock solutions of the dyes were prepared by dissolving the powdered dyestuff in distilled water and sterilized by filtration (Millipore cellulose filters Ø 0.22 µm), and the other concentrations were obtained by diluting these stock dye solutions. All other chemicals used in this study were of analytical grade.

Tryptic soy broth (TSB) was purchased from Himedia Labs and consisted of (g L^{-1}): pancreatic digest of casein 17.0, papaic digest of soybean meal 3.0, sodium chloride 5.0, dextrose 2.5, dipotassium hydrogen phosphate 2.5, pH 7.3 ± 0.2. Agar was added in TSB (1.5% w/v) to prepare tryptic soy agar (TSA) used in the stock culture of the bacterial strain. Rich mineral medium (MMR) used in all decolorization experiments contained (g L^{-1}): K_2HPO_4 1.6, KH_2PO_4 0.2, $(NH_4)_2SO_4$ 1.0, $MgSO_4.7H_2O$ 0.2, $FeSO_4.7H_2O$ 0.01, NaCl 0.1, $CaCl_2.H_2O$ 0.02, glucose 3.0 and yeast extract 1.0.

2.2. Decolorizing bacterial strain

The stock cultures were maintained by periodic subculture on TSA slants at 4°C. This bacterial strain was isolated from contaminated landfill soil in Cuiabá, Brazil. The identification of the strain was based on the analysis of 16S ribosomal RNA (rRNA) gene. After the cells were cultured overnight in TSB, the genomic DNA was extracted using a Bacterial Genomic Miniprep Kit from Sigma Aldrich. The 16S rRNA gene fragment of the strain was then amplified by PCR amplification using forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492r (5'-GGTTACCTTGTTACGACTT-3'). The 16S rRNA PCR product was purified using GFX™ PCR Purification Kit (GE Healthcare) and sequenced by GenOne (Rio de Janeiro, Brazil) using the Sanger method. The 16S rRNA sequence of the decolorizing bacterial strain was compared with the 16S rRNA sequence data from the reference and type strains available in the public database GenBank using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Inoculum preparation

To inoculum preparation used in decolorization experiments, a loopful of the decolorizing bacterial strain grown on TSA plate (24 h, 35ºC), was inoculated in an Erlenmeyer flask containing 100 mL MMR (pH 7.0). The flask was incubated at 35°C in a shaking incubator (150 rpm) until the exponential phase of bacterial growth was reached. A microbial culture with an optical density of 1.0 (at 600 nm) was used as inoculum (5% v/v).

2.4. Decolorization studies

Decolorization experiments were carried out in an Erlenmeyer flask (250 mL) containing 100 mL MMR supplemented with dye (50 mg L^{-1}). Five azo dyes were studied individually (RR239, RRB, RY176, DB85, and DO39). The flasks were incubated at 35°C under static condition. Dye concentration was measured following the colorimetric procedure of using curves prepared at λ_{max} of 542 nm (RR239), 597 nm (RRB), 418 nm (RY176), 590 nm (DB85), and 420 nm (DO39), using UV–Vis spectrophotometer Hach DR6000. To avoid biomass

interference during dye concentration, determination cells were removed by centrifugation (6,000xg, 15 min). All decolorization experiments were performed in triplicate. Uninoculated controls were always included to estimate the abiotic decolorization. The percentage of decolorization was calculated according to Equation 1:

$$Decolorization\,(\%) = \frac{C_{(0\,h)} - C_{(t)}}{C_{(0\,h)}}\,x\,100 \qquad (1)$$

Where $C_{(0\,h)}$ is the initial concentration of dye, and $C_{(t)}$ is the concentration of dye at reaction time t (hour).

2.5. Analysis of color removal in MMR containing a mixture of dyes

Decolorization of a mixture of all dyes, each at a concentration of 30 mg L^{-1}, was evaluated in a MMR. The mixture of dyes did not have a well-defined peak at the visible absorption spectra. Therefore, the actual color level was measured using the American Dye Manufacturers Institute (ADMI) weighted-ordinate spectrophotometric method (method 2120F from Standard Methods for the Examination Water and Wastewater) (APHA *et al.*, 2012). The percentage of decolorization was calculated according to Equation 2:

$$ADMI\,removal\,ratio\,(\%) = \frac{Initial\,ADMI_{(0\,h)} - Observed\,ADMI_{(t)}}{Initial\,ADMI_{(0\,h)}}\,x\,100 \qquad (2)$$

Where ADMI$_{(0\,h)}$ and ADMI$_{(t)}$ are the initial ADMI value (at 0 h) and the ADMI value after a particular reaction time (t), respectively.

2.6. Effects of different parameters on RR239 decolorization

RR239 was used to study the effects of different environmental parameters on decolorization since it was required less time for its complete decolorization. Temperature (25-45°C), pH (4.0-12.0), static/agitated incubation, and dye concentration (50-800 mg L^{-1}) were evaluated. Repetitive decolorization capacity of the decolorizing bacterial strain by addition of RR239 (50 mg L^{-1}) at medium without supplementing any additional nutrient was also studied. Since dye decolorization may take place by biosorption or biodegradation, dead biomass was obtained by samples autoclaving (121°C, 20 min) and was used for estimation of biosorption.

2.7. Measurement of biomass

The biomass was determined by measuring the optical density (OD) at a wavelength of 600 nm during decolorization (Holkar *et al.,* 2014):

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

2.8. Analysis of metabolites obtained after RR239 decolorization

FTIR analysis of metabolites obtained after RR239 decolorization was done on a Shimadzu IRAffinity-1 spectrophotometer (Model: IRAffinity-1; Catalogue Number: 206-73500-38; Serial Number: A21374902249S1; Brand: Shimadzu Corporation spectrophotometer). Decolorized samples were centrifuged at 10,000×g for 15 min, and the extraction of metabolites was carried out from the supernatant using an equal volume of ethyl acetate. The extracts were dried over anhydrous Na$_2$SO$_4$ and evaporated to dryness in a rotary evaporator. The samples were mixed with spectroscopically pure KBr (0.0005 g sample : 0.1 g KBr), pellets were fixed in a sample holder, and then analyzed. The qualitative analyses were carried out using the following parameters: Measured Mode (% Transmittance), Apodization (Happ_Genzel), Number of Scans (200), Resolution (16), Range (400-4000 cm^{-1}), Gain (1). The background obtained from KBr disks was automatically subtracted from the samples disks spectra.

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3. RESULTS AND DISCUSSION

3.1. Identification of decolorizing bacterial strain

The colony of the decolorizing bacterial strain was circular, flat, smooth, and reddish-brown. The bacterium was observed to be Gram-negative. Sequence analysis of the 16S rRNA gene showed that the strain had the highest similarity with the species *Shewanella xiamenensis* (99.51%). The sequence of the bacterial strain was submitted to GenBank with the accession number MN005116.

3.2. Decolorization performance of azo dyes

The decolorization of the azo dyes RR239, RY176, RBB, DB85 and DO39 (50 mg L^{-1} each) by *S. xiamenensis* G5-03 was evaluated in MMR within 5 h, 35°C, pH 7.0 and static condition. The results are presented in Table 1, showing that *S. xiamenensis* G5-03 decolorized all of them, with faster decolorization rates of RR239 (complete decolorization) and DB85 (97%). *S. xiamenensis* G5-03 also decolorized the MMR with the mixtures of the dyes (74% of color removal in terms of ADMI value). After 24 h, the color removal in terms of ADMI was 98% (data not shown), indicating that the bacterium represents a promising tool for the color removal of various azo dyes from textile dye effluent. RR239 was selected for further studies because of its complete decolorization.

Table 1. Decolorization performance of azo dyes by *S. xiamenensis* G5-03.

Dye	Chemical class	Decolorization (%)
RR239	Reactive monoazo	CD
RY176	Reactive monoazo	80 ± 1.4
RRB	Reactive diazo	86 ± 0.7
DO39	Direct monoazo	75 ± 0.6
DB85	Direct triazo	97 ± 0.6
Mixture of dyes[*]		74 ± 0.2 [**]

CD – Complete decolorization
[*] 30 mg L^{-1} each dye; [**] color removal in terms of ADMI value
Data are shown as mean ± std derived from triplicates.

3.3. Effect of pH and temperature on RR239 decolorization

The effect of pH and temperature on the decolorization of RR239 by *S. xiamenensis* G5-03 was studied in detail, since these factors play a crucial role in the optimal physiological performance of microbial culture. *S. xiamenensis* G5-03 revealed effective removal of RR239 color at a wide range of pH. The dye was completely decolorized at pH 7, 8, and 9 in 5, 3, and 4 h, respectively, and 6 h were necessary to remove color at pH 10 and 11 (Table 2). In the decolorization system without bacteria (control), the decolorization rates varied between 3.7-5.4% at pH 7-11, and at other pH values, the decolorization was insignificant (data not shown). Studies have demonstrated that microorganisms have higher decolorization efficiency under neutral conditions than acidic or alkaline (Shah *et al,.* 2012; Hussain *et al.,* 2013). On the other hand, it has been reported that some alkali-stable azoreductase were found with bacteria (Tan *et al.,* 2013). *Brevibacillus laterosporus* exhibited the ability to decolorize Disperse Red 54 dye within the pH range of 7-11 (Kurade *et al.,* 2016). *Enterobacter* sp. F NCIM 5545 showed significantly higher decolorization of Reactive Blue 19 at pH 10 when compared to that at pH 5 and 7 (Holkar *et al.,* 2014). The pH tolerance of decolorizing bacteria is important because reactive dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions (Aksu, 2003). Thus, the ability of *S. xiamenensis* G5-03 to decolorize reactive dyes in alkaline pH make it suitable for application in biotreatment of textile industry effluent.

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When the decolorization of RR239 by *S. xiamenensis* G5-03 was studied at various temperatures, it was observed that the highest decolorization rate was obtained at 35°C (complete decolorization in 5 h) (Table 2). At 25, 30, and 35°C, high decolorization rates were also observed. When the temperature was increased to 45°C, a marginal reduction in the decolorization activity was observed (9%). Probably this reduction is due to the loss of cell viability and deactivation of the enzymes responsible for decolorization at such high temperature (Holkar *et al.,* 2014). Whereas the optimal decolorization rates were observed at pH 8.0 and 35°C, these conditions were used in subsequent studies.

Deactivated cells of *S. xiamenensis* G5-03, reached by sterilization at 121°C for 20 min, did not show any significant color removal of RR239, suggesting that the decolorization was mainly attributed to biodegradation rather than biosorption.

Table 2. Effect of pH and temperature on the decolorization of RR239.

Parameters	pH (Temperature 35°C)						Temperature (°C) (pH 7.0)				
	6	7	8	9	10	11	25	30	35	40	45
Decolorization (%)	55±1.9	CD	CD	CD	CD	CD	88±1.3	94±0.9	CD	92±2.9	9±0.7
Time for decolorization (h)	24	5	3	4	6	6	6	6	5	7	24

CD – Complete decolorization.

3.4. Effect of static/agitated incubation

The growth of *S. xiamenensis* G5-03 was higher under aerobic conditions (agitation, 100 rpm) than microaerophilic (static) conditions (Figure 1). On the other hand, the agitation had a negative impact on the RR239 removal (only 42% of dye was removed after 5 h), while 96% of RR239 was removed in low oxygen. This result suggested that molecular oxygen inhibits azoreductase activities responsible for the process of the dye decolorization, although the cell growth is stimulated. This inhibition has been attributed to a predominant competition for NADH utilization by aerobic respiration, which triggers an electron transfer from NADH to oxygen to form ATP and deprive the azoreductase of obtaining electrons to decolorize azo dyes (Chang *et al.,* 2001).
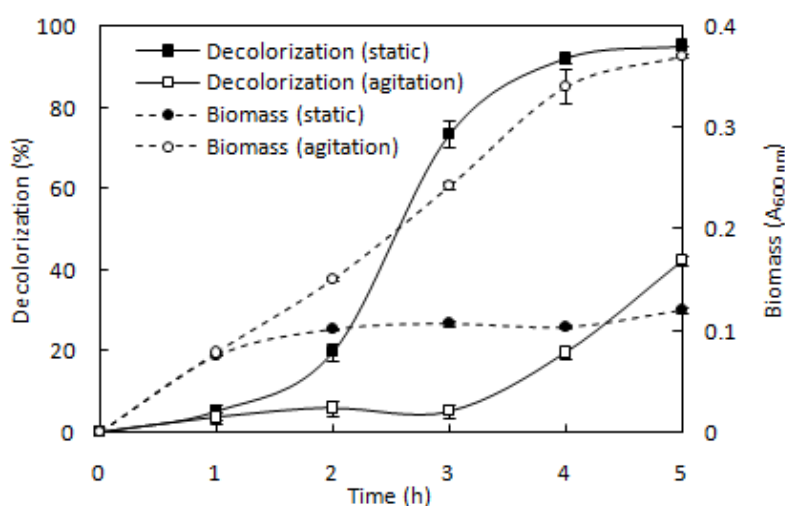

Figure 1. Effect of static and agitated incubation on RR239 decolorization.

Several studies have reported efficient dye decolorization under static/microaerophilic conditions when compared with agitation/aerobic conditions. *Alcaligenes faecalis* PMS-1

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completely removed Reactive Orange 13 color after 16 h under anoxic conditions, whereas only 35% decolorization was observed under aerobic conditions (Shah *et al.,* 2012). *Pseudomonas* sp. RA20 had a better decolorizing potential of Reactive Black B under static incubation as compared to that of under shaking incubation. However, the growth of RA20 was relatively more under the shaking incubation as compared to that of static incubation (Hussain *et al.,* 2013). *Halomonas* sp. strain A55 completely decolorized Reactive Red 184 only when incubated under anaerobic and anoxic conditions (Guadie *et al.,* 2018).

3.5. Effect of initial RR239 concentration on decolorization

When studying the effect of increasing concentrations of RR239 on the decolorization performance exhibited by *S. xiamenensis* G5-03, an inverse relationship was observed. As the dye concentration increased from 50 to 800 mg/L, the percentage of decolorization rate was found to decrease from 100% to 48.8% after 3 h (Figure 2). Reduction in the decolorization rates by *S. xiamenensis* G5-03 at higher dye concentration is related to the toxic effect of dye on the bacteria or inadequate biomass concentration for the uptake of higher concentrations of dye (Saratale *et al.,* 2011).
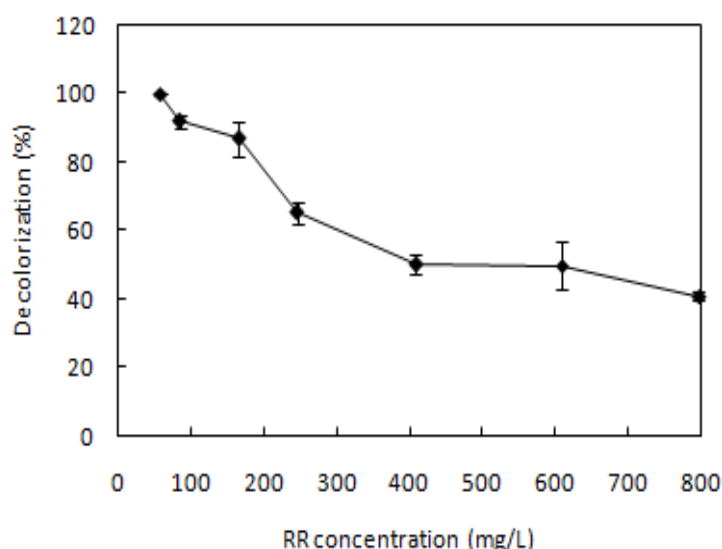

Figure 2. Effect of RR239 concentration on decolorization efficiency.

3.6. Kinetic study of RR239 decolorization

To determine the maximum decolorization rate of RR239, experiments with different dye concentrations (50-800 mg L^{-1}), incubated under static conditions at 35ºC, were performed. Figure 3a shows a dependence of specific decolorization rate to the concentration of RR239, and these experimental data were fitted quite well to the Michaelis-Menten model (Equation 3).

$$V = \frac{V_{max}\, S}{K_m + S} \tag{3}$$

Where V is the specific decolorization rate (mg L^{-1} h^{-1}), V_{max} is the maximum specific decolorization rate (mg L^{-1} h^{-1}), S is the substrate concentration (mg L^{-1}), K_m is the Michaelis-Menten constant (mg L^{-1}). K_m is equal to the concentration of the substrate (RR239) when the reaction rate is half of the maximum velocity. To determine V_{max} and K_m, the Michaelis-Menten equation was transformed by a double-reciprocal approach as follows: $1/V = Km/(V_{max}\, S) + 1/Vmax$. A plot of $1/V$ versus $1/S$ yields a straight line (Figure 3b) with an interception of $1/V_{max}$ and a slope of K_m/V_{max}. Thus, the values for V_{max} and K_m were 166.7 mg L^{-1} h^{-1} and 443.3 mg L^{-1}, respectively.
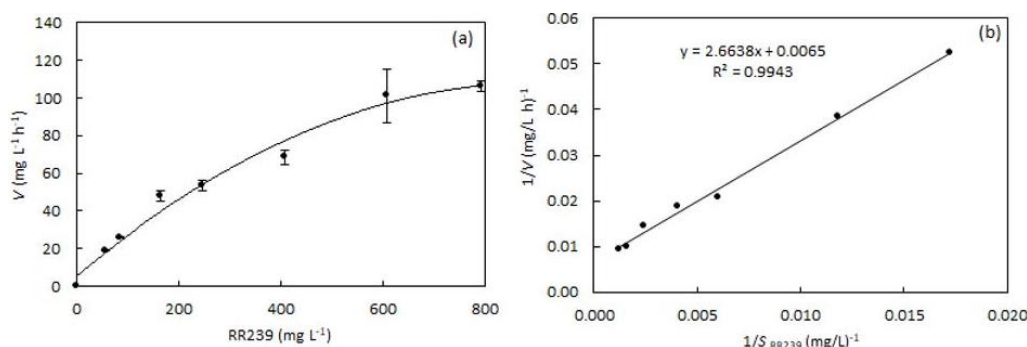
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Figure 3. (a) Effect of RR239 concentration on specific decolorization rate. (b) The double reciprocal plot of initial decolorization rate against RR23 concentration.

3.7. Decolorization of repeated additions of dye aliquots

This study was carried out to test the ability of *S. xiamenensis* G5-03 to decolorize repeated addition of RR239 aliquots (50 mg L^{-1}) after every 12 h at static condition. There was complete decolorization for first two dye aliquot additions, and high decolorization activity (>78%) was observed up to the seventh cycle within 84 h (Figure 4). These results indicate that the G5-03 strain holds reusability and persistence in repetitive decolorization operation. Decrease in decolorization after the eighth cycle might be due to the decrease in viable cells, exhaustion of nutrients, and accumulation of toxic compounds in the medium.
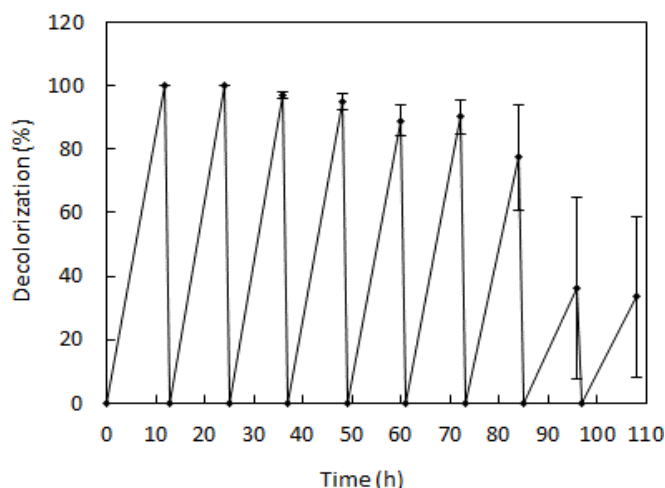

Figure 4. Repetitive decolorization capacity of the *S. xiamenensis* G5-03 by addition of RR239 (50 mg L^{-1}) at medium without supplementing any additional nutrients.

3.8. Analysis of metabolites resulting from decolorization of RR239

Comparison of FTIR spectrum of control RR239 (before degradation) with FTIR spectrum of extracted metabolites after decolorization clearly indicated the biodegradation of the dye by *S. xiamenensis* G5-03 (Figure 5). The FTIR spectrum of the control RR239 (Figure 5a) showed a peak at 3456.44 cm^{-1} for N–H stretching vibrations of the amino group coupled with stretching of O–H group. Peak at 1620.21 cm^{-1} was attributed to the N=N stretching vibrations of the azo bonds. The peaks at 1550.77 cm^{-1} for C=N stretching and 1496.76 cm^{-1} for aromatic C=C stretching supported the aromatic structure of the dye. The stretching vibration between –N–C= was reported at 1411.89 cm^{-1}. The peaks at 1319.31 and 1141.86 cm^{-1} were attributed to stretching vibrations of S=O and at 617.22 cm^{-1} to C–S, indicating sulfur-containing nature of the dye. Peak at 1049.28 cm^{-1} corresponded to stretching vibrations of the C–OH. The peaks

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between 670 and 850 cm^{-1} also supported the aromatic nature of the dye (Shah *et al.,* 2012; Khan *et al.,* 2014; Kurade *et al.,* 2016). Absence of the peak 1620.21 cm^{-1} for N=N stretching vibrations in FTIR spectrum of extracted metabolites confirming the cleavage of azo bonds, that would be an essential step for color removal. The decrease in the intensities of the peaks at the low-frequency region of spectra (670-850 cm^{-1}) suggested the fission of aromatic rings. A new peak at 1651.07 cm^{-1} represented the formation of charged aromatic amine derivatives, whereas peak at 1303.88 cm^{-1} for C–N stretching vibration support formation of aromatic primary amines. Further, new peaks around 2954-2924 cm^{-1} represented the C–H stretching vibrations of CH$_2$, which could be related to aromatic ring opening. Thus, it may be concluded that *S. xiamenensis* G5-03 decolorizes RR239 due to biodegradation (Khan *et al.,* 2014; Pérez-Calderón *et al.*, 2018).
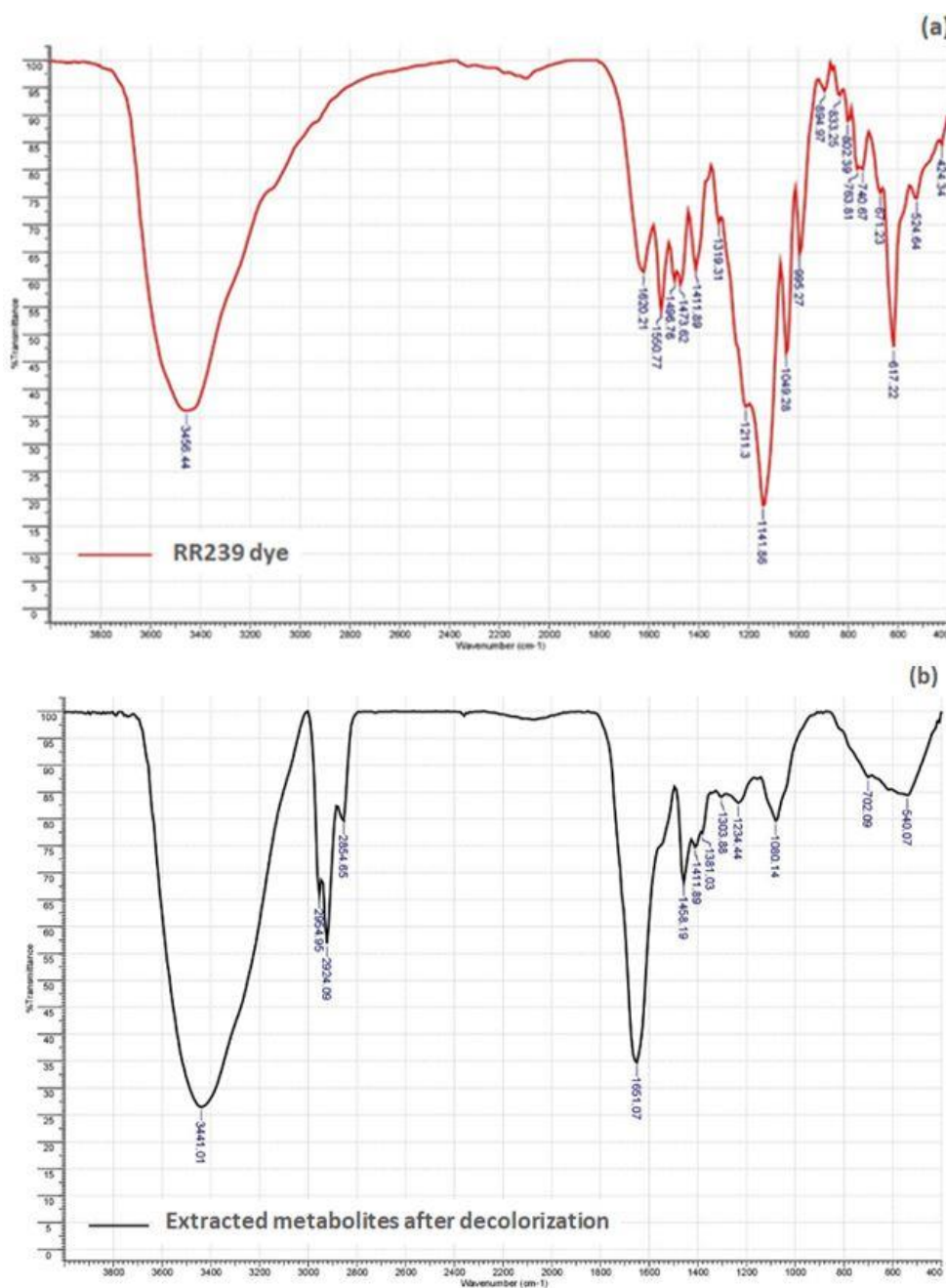

Figure 5. The FTIR spectra of RR239 dye (a) and extracted metabolites after decolorization (b).

4. CONCLUSION

Textile azo dyes were decolorized by the novel strain *S. xiamenensis* G5-03 isolated from contaminated landfill soil, in static (microaerophilic) condition. Most notably, G5-03 effectively decolorized Reactive Red 239 dye over wide pH (7-11) and temperature (25-40°C) ranges. The decolorization kinetics could be described by the Michaelis–Menten equation. The K_m and V_{max} were found to be 443.3 mg L^{-1} and 166.7 mg L^{-1} h^{-1}, respectively. FTIR analysis showed the decolorization is due to biodegradation. These results suggested that the *S. xiamenensis* G5-03 could be useful in the biological treatment of textile wastewater.

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