Original Article

Decolorization of the benzidine-based azo dye Congo red by the new strain *Shewanella xiamenensis* G5-03

Descoloração do corante azo derivado da benzidina vermelho Congo por uma nova linhagem de *Shewanella xiamenensis* G5-03

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Abstract

Shewanella xiamenensis G5-03 was observed to decolorize the azo dye Congo red in synthetic wastewater. The influence of some factors on the dye decolorization efficiency was evaluated. The optimal decolorization conditions were temperature 30-35 °C, pH 10.0, incubation time 10 h, and static condition. The kinetic of Congo red decolorization fitted to the Michaelis–Menten model (V_{max} = 111.11 mg L^{-1} h^{-1} and K_m = 448.3 mg L^{-1}). The bacterium was also able to degrade benzidine, a product of azo bond breakage of the Congo red, which contributed to reduce the phytotoxicity. The ability of *S. xiamenensis* G5-03 for simultaneous decolorization and degradation of Congo red shows its potential application for the biological treatment of wastewaters containing azo dyes.

Keywords: kinetic study, bacterium, bioremediation, phytotoxicity.

Resumo

Shewanella xiamenensis G5-03 foi capaz de descolorir o corante azo vermelho Congo em água residuária sintética. A influência de alguns fatores na eficiência da descoloração do corante foi avaliada. As condições ótimas de descoloração foram temperatura de 30-35 °C, pH 10,0 e condições estáticas. A cinética de descoloração do vermelho Congo se ajustou ao modelo de Michaelis–Menten (V_{max} = 111,11 mg L^{-1} h^{-1} and K_m = 448,3 mg L^{-1}). A bactéria também foi capaz de degradar a benzidina, um produto da quebra da ligação azo do vermelho Congo, o que contribuiu para a redução da fitotoxicidade. A habilidade da *S. xiamenensis* G5-03 em simultaneamente descolorir e degradar o vermelho Congo demonstra seu potencial de aplicação no tratamento de águas residuárias contendo corantes azo.

Palavras-chave: estudo cinético, bactéria, biorremediação, fitotoxicidade.

1. Introduction

The textile industry produces large volumes of effluents containing residues of dyes, metals, COD, BOD, total solids, and variable pH (Holkar et al., 2014). Because of that, they are the cause of serious environmental concerns. The discharge of textile-dye-containing effluent in aqueous ecosystems affects the aesthetic merits and transparency of water, diminishes the photosynthetic activity by reduction of sun penetration, and reduces oxygen transference into the water, which causes acute toxic effects in fish and other animals (Vikrant et al., 2018). Besides, many textile dyes and some of their metabolites are toxic, mutagenic, and carcinogenic (Saratale et al., 2011). Thus, they must be removed from the effluent before its disposal.

Several physicochemical effluent treatment processes, including coagulation, electrocoagulation, adsorption, ozonation, and photon-Fenton oxidation, have been reported for the removal of dyes (Castro et al., 2017).

Nevertheless, these methods possess inherent limitations such as high cost, the formation of sludge containing the dyes or their metabolites, and intensive requirements of energy (Cossolin et al., 2019). On the order hand, biotechnologies approaches using microorganisms for the remediation of effluents have been of considerable interest since they are cost-effective, environmentally friendly, and produce less sludge (Vikrant et al., 2018).

Microorganisms are the primary degraders of nature and have been applied to remediate different pollutants. Studies have reported different groups of decolorizing microorganisms, including bacteria, filamentous fungi, yeast, and algae (Chia et al., 2014; Holkar et al., 2014; Song et al., 2017). White-rot fungi are the most common microorganisms for this approach, because of their ability to produce some extracellular lignin enzymes, which are responsible for decolorizing dyes. However, a

long growth cycle limits the performance of the fungal decolorization system (Stolz, 2001). Bacteria can degrade and even mineralize many textile dyes under certain specific optimum cultural conditions. The features like omnipotence, faster growth, facultative nature, and high adaptability are the desirable qualities of the bacterial community for the textile dye bioremediation (Gomare et al., 2009).

In the past decade many bacterial strains belonging to different genera such as *Klebsiella* (Franciscon et al., 2009), *Brevibacillus* (Gomare et al., 2009), *Comamonas* (Jadhav et al., 2011), *Pseudomonas* (Hussain et al., 2013), *Shewanella* (Babu et al., 2013), *Enterobacter* (Holkar et al., 2014), and *Acinetobacter* (Anwar et al., 2014) have been reported to degrade azo dyes, the most important and most abundant class of synthetic dyes used in commercial applications (Saratale et al., 2011). Azo dyes are aromatic compounds with one or more azo bond (–N=N–). The bacterial degradation of azo dyes often is initiated by reductive cleavage of the azo bond, which results in the formation of colorless aromatic amines (Stolz, 2001). Azoreductase is the key enzyme responsible for this process, which occurs mainly in anaerobic conditions (Saratale et al., 2011).

In this study, Congo red, an azo dye also called Direct Red 28, was used as a model compound in a biological decolorization process using the bacteria *Shewanella xiamenensis* G5-03. Congo red is a recalcitrant compound and known to metabolize to benzidine, a human carcinogen. Nevertheless, it has been used in textile, paper, rubber, and plastics industries (El Messaoudi et al., 2016).

The genus *Shewanella* is widely distributed in nature and has been studied in applied and environmental microbiology (Venkateswaran et al., 1999). Bacteria of this genus are known for their respiratory diversity, which allows them to couple the degradation of organic compounds to the reduction of the various terminal electron acceptors (Fredrickson et al., 2008). This characteristic of *Shewanella* strains has attracted many interests for their application in the bioremediation of hazardous compounds, including azo dyes (Babu et al., 2013).

The purpose of this study was to investigate the potential of *Shewanella xiamenensis* G5-03 for decolorizing azo dye Congo red in synthetic wastewater. The influence of various environmental conditions on the dye decolorization process, such as pH, temperature, dye concentration, static, and agitated incubation, was studied to achieve maximum dye degradation. Besides, the decolorization kinetic, benzidine from dye degradation, and their toxicity were also assessed.

2. Material and Methods

2.1. Dye and chemicals

Congo red (CR) was purchased from VETEC – Sigma-Aldrich, Brazil. A sterile stock solution of the dye (1.0 g L^{-1}) was prepared using distilled water, and the sterilization was made by filtration through a 0.22 µm membrane filter. All other chemicals used were of analytical grade.

2.2. Culture media and synthetic wastewater

Tryptic soy broth (TSB) used in bacterial inoculum production was purchased from Himedia Labs. Agar was added in TSB (1.5% w/v) to prepare a solid media used in stock cultures of the bacterial strain.

CR sterile stock solution was diluted in rich mineral medium, consisted of (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 (Franciscon et al., 2009), at required concentration to create the synthetic dye loaded wastewater.

2.3. Decolorizing bacterium and identification

Details of the isolation and identification of decolorizing bacterium were described in our previous study (Cossolin et al., 2019). The identification was based on the sequencing of the 16S ribosomal DNA (rDNA) gene, which was compared with the known sequences available in the GenBank using the BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted by carrying out the multiple alignments using Clustal W (Thompson et al., 1994) and processing the data using the neighbor-joining method (Saitou and Nei, 1987). The 16S rDNA gene sequence of the *Shewanella xiamenensis* G5-03 has been deposited in GenBank under the accession number MN005116.

2.4. Inoculum preparation and decolorization experiments

A loopful of bacterial strain grown on the TSA plate (24 h, 35 ºC) was inoculated in Erlenmeyer flask containing 100 mL TSB. The flask was incubated at 35 °C in a rotary shaker (150 rpm) until the optical density (OD_{600}) of 1.0 was reached. This biomass was used as inoculum (5% v/v) for all decolorization experiments. Decolorization experiments were carried out in Erlenmeyer flask containing 100 mL of synthetic dye loaded wastewater. Effects of temperature (25, 30, 35, 40 °C), initial pH (5, 7, 10, adjusted by the addition of 0.1 M HCl or 0.1 M NaOH solutions), dye concentration (50-750 mg L^{-1}), and static and agitated incubation were evaluated. Dye concentration was monitored spectrophotometrically at λ_{max} of 497 nm (UV–Vis spectrophotometer Hach). The cells were removed by centrifugation (3600 rpm, 15 min) before dye concentration determination. Uninoculated Erlenmeyer flasks were included to estimate the abiotic decolorization. The decolorization percentage was calculated according to Equation 1:

$$Decolorization\,(\%) = \frac{C_i - C_f}{C_i} \, x100 \tag{1}$$

where C_i is the initial concentration of dye (mg L^{-1}), and C_f is the concentration of dye (mg L^{-1}) at reaction time t (hour).

2.5. Measurement of biomass

The bacterium biomass was determined by measuring the OD_{600} during the decolorization (Holkar et al., 2014): $OD_{600} = OD_{sample} - OD_{supernatant}$.

2.6. Benzidine quantification

Benzidine released from the reduction of CR was quantified colorimetrically according to the method described by Oren et al. (1991). In 0.2 mL of decolorized synthetic wastewater, 0.8 mL of ultrapure water, 0.05 mL of 1 M HCl, 3 mL of ethanol, 0.5 mL of 5% p-dimethylaminobenzaldehyde in ethanol, and 0.5 mL of 15.7% citric acid in 6% NaOH were added. After 10 min, 2.5 mL of ultrapure water was added to the tubes, and benzidine was measured spectrophotometrically at λ_{max} of 440 nm (UV–Vis spectrophotometer Hach).

2.7. Phytotoxicity studies

Phytotoxicity tests using *Ocimum basilicum* and *Eruca sativa* seeds were performed in order to assess the toxicity of the original dye and its metabolites obtained after decolorization. For this, synthetic wastewater containing 50 mg L^{-1} of CR was submitted to decolorization by *S. xiamenensis* G5-03 for 120 h. Then, ten seeds of each plant were grown in Petri plates with a supply of 5 mL of synthetic wastewater obtained at different time intervals during decolorization (0, 10, and 120 h) and incubated at 23 ± 1 °C in the dark. Distilled water was used as control. After 7 days, germination (%) and radicle lengths (cm) were recorded. The germination index (GI) was calculated according to Equation 2 (Gomare et al., 2009):

$$GI\,(\%) = \frac{Ls\,x\,\%Gs}{Lc\,x\,\%Gc}\,x\,100 \qquad (2)$$

where *Ls* and *Lc* are the length of radicles (mm) in seeds treated with synthetic wastewater sample and distilled water (control), respectively. *Gs* and *Gc* are the numbers of seeds that germinated in synthetic wastewater sample and distilled water (control), respectively.

2.8 Statistical analysis

The results presented in this study are the mean of triplicate ± standard deviations (SD). For the comparison of two levels of a single factor, the data were analyzed by the Student *t* test. 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 multiple comparisons test. The *p*-values of less than 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. Decolorizing bacterium

The multiple alignments revealed that the 16S rDNA gene sequence of G5-03 showed 99.51% similarity to that of *Shewanella xiamenensis*. The phylogenetic relationship of the strain with other *Shewanella* species found in the GenBank is shown in Figure 1.

3.2. Effect of pH and temperature on CR decolorization

The pH and temperature play a crucial role in the optimal physiological performance of microorganisms during the decolorization process. Therefore, these factors were studied in detail during the CR decolorization. *S. xiamenensis* G5-03 showed the highest decolorization rates at pH 10.0 (90.2%) and pH 7.0 (71.7%) after 10 h
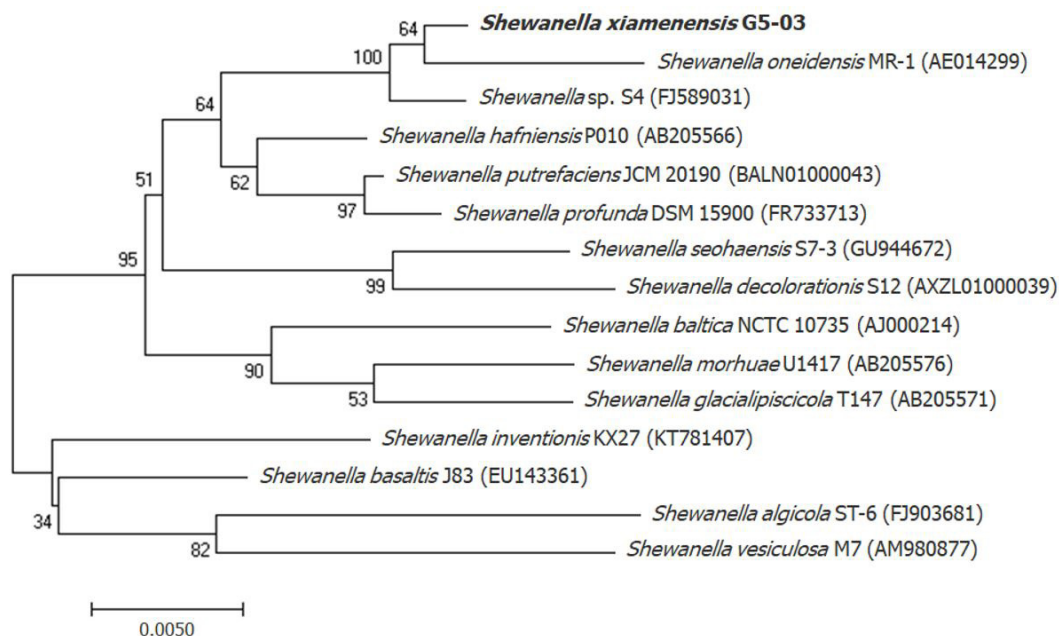

Figure 1. Neighbor-joining phylogenetic tree based on 16S rDNA gene sequences showing the relationships of strain G5-03 with other *Shewanella* species found in the GenBank. Numbers at nod shows the percentage of 1000 bootstrap replicates. The bar indicates 0.005 substitutions per nucleotide position.

of incubation, while at pH 5.0, the decolorization rate reached 24.1% (Figure 2a). Statistical analysis conducted using One-Way ANOVA showed a significant difference ($p<0.00001$) in the decolorization when carried out at different pH values. Further, Tukey multiple comparisons test showed that decolorization at pH 5 was significantly lower when compared to those at pH 7 ($p=0.0011$) and pH 10 ($p=0.0001$). There was no significant difference in the decolorization between pH 7 and 10. Considering that many dyes bind to the textile fibers by addition or substitution mechanisms under alkaline conditions, and hence the pH of effluent is usually higher than eight (Aksu and Dönmez, 2003), pH 10 was adopted to investigate bacterial decolorization in the subsequent experiments.

When the decolorization of CR by *S. xiamenensis* G5-03 was studied at different temperatures, it was found that the highest decolorization rates were obtained at 30 °C (97.5%) and 35 °C (90.2%) (Figure 2b). A further increase in temperature to 40 °C resulted in a marginal reduction in the decolorization activity (34.8%), probably because of the slower growth rate and deactivation of enzymes responsible for degradation at such high temperatures. One-Way ANOVA showed a significant difference ($p<0.00001$) in the decolorization at different temperatures. The Tukey's multiple comparisons test showed there was no significant difference between decolorization rates obtained at 30 and 35 °C. However, they were statistically higher than those decolorization rates found at 25 and 40 °C.
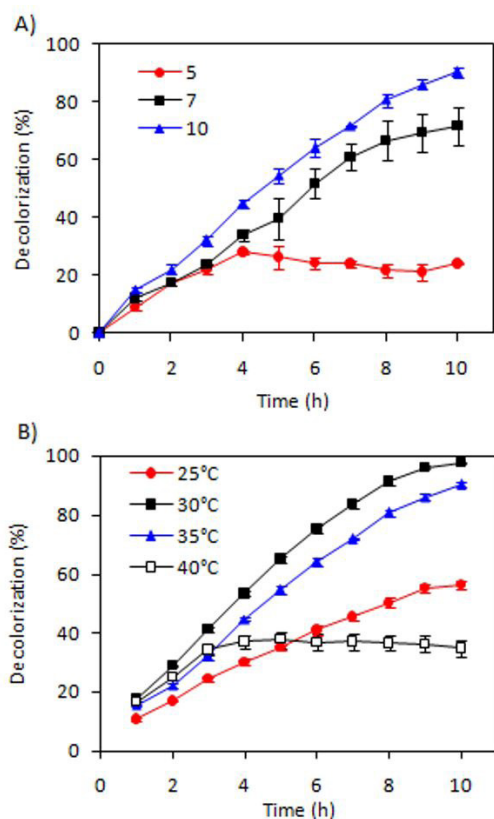
3.3. Effect of static/agitated incubation on CR decolorization

It was found that the growth of G5-03 strain was higher under agitated incubation (aerobic condition) as compared to static incubation (microaerophilic condition) (Figure 3). On the other hand, the agitation had a negative impact on the CR decolorization (14.1% after 10 h), while 90.2% of CR was decolorized in static condition. Statistical analysis conducted using the Student t test showed a significant difference ($p<0.00001$) between static and agitated incubation, indicating the inhibitory effect of oxygen on decolorization. The azo dye decolorization initiates with the reduction of azo bonds by azoreductases in a process that uses NADH as the electron donor. In aerobic conditions, the oxygen uses the electrons donated from NADH to form ATP depriving the azoreductase of obtaining electrons to decolorize the azo dyes (Chang et al., 2001). Some studies have reported that dye decolorization under static/microaerophilic conditions is more efficient if compared to agitation/aerobic conditions. *Comamonas* sp. UVS exhibited dye-decolorizing activity only when incubated under static conditions (Jadhav et al., 2011). *Micrococcus glutamicus* NCIM-2168 was able to completely decolorized the dye Reactive Green 19A after 42 h under static condition, whereas only 30% of the dye was decolorized under aerobic condition (Saratale et al., 2009). Only 20% of Remazol Red dye was decolorized by *Lysinibacillus* sp. RGS after 6 h in agitated condition while the dye color was completely removed in static condition (Saratale et al., 2013).

3.4. Decolorization kinetic model

Michaelis-Menten model has been widely used to describe the dye decolorization kinetics by enzymes or living cells (Holkar et al., 2014). The two model parameters (V_{max} and K_m) are calculated according to Equation 3:

$$V_0 = \frac{V_{max} S}{K_m + S} \tag{3}$$

where S is the substrate (dye) concentration (mg L^{-1}), V_{max} is the maximum specific decolorization rate (mg L^{-1} h^{-1}), and

Figure 2. Effect of pH (A) and temperature (B) on the CR decolorization by *S. xiamenensis* G5-03.
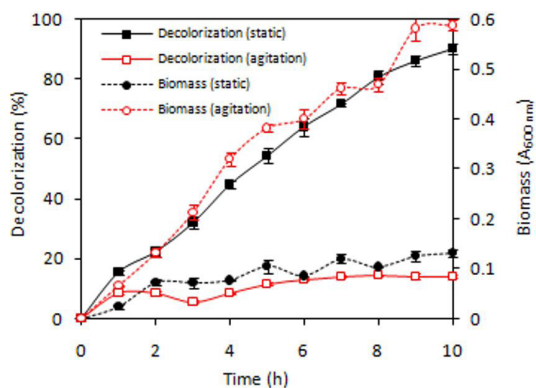

Figure 3. Effect of agitated and static incubation on CR decolorization by *S. xiamenensis* G5-03.

K_m is the half-saturation constant (mg L^{-1}). The Michaelis-Menten equation was transformed into the double reciprocal form: $1/V_0 = K_m/(V_{max} S) + 1/V_{max}$, to determine V_{max} and K_m. A plot of $1/V_0$ versus $1/S$ yields a straight line with a slope of K_m/V_{max} and an intercept of $1/V_{max}$.

When the initial CR concentration increased from 50 to 750 mg L^{-1}, the CR degradation rate significantly increased from 19.9 to 55.8 mg L^{-1} h^{-1} (Figure 4a). The corresponding linear regression equation from the plot of $1/V_0$ versus $1/S$ is y = 4.035x + 0.009 (R^2= 0.949) (Figure 4b). Thereby, the obtained constants were V_{max} = 111.11 mg L^{-1} h^{-1} and K_m = 448.3 mg L^{-1}.

3.5. Benzidine quantification

Degradation of CR usually proceeds by reduction of its azo bonds, resulting in the release of benzidine and other aromatic amines. Figure 5 shows the benzidine concentration in synthetic wastewater during CR decolorization by *S. xiamenensis* G5-03. The concentration of benzidine decreased from 17.2 mg L^{-1} (at 10 h) to 5.3 mg L^{-1} (at 120 h), indicating that the bacterium was not only able to break the azo bonds but also degrade the benzidine.

3.6. Phytotoxicity studies

Two agriculturally valuable plant seeds, i.e., *Ocimum basilicum* and *Eruca sativa*, were used for the phytotoxicity
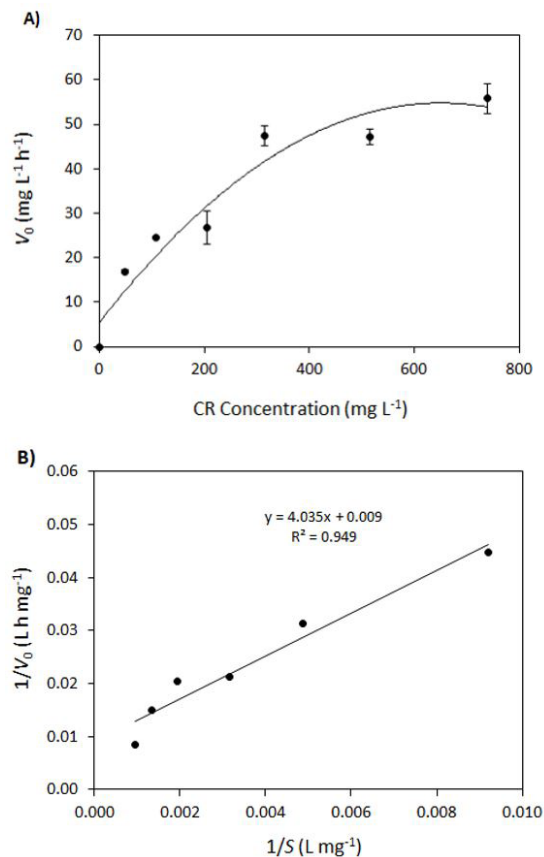

Figure 4. (A) Effect of CR concentration on specific decolorization rate; (B) The double reciprocal plot of initial decolorization rate against CR concentration.

assessment of the CR and its decolorization products. As shown in Figure 6, the germination indexes (GI) of *O. basilicum* and *E. sativa* that were grown in untreated synthetic wastewater were 74.0 and 88.3%, respectively, and after 10 h of decolorization, decreased to 38.1 and 41.9%. These results indicated that the decolorization products, including benzidine formed during the cleavage of azo bonds, are more toxic than the CR. With an extended decolorization time (120 h), the GI increased to 58.0 and 72.6% for *O. basilicum* and *E. sativa*, respectively, presumably due to the degradation of benzidine and other toxic compounds. As indicated by Nouren & Bhatti (2015), GI values lower than 50% represents high phytotoxicity, values falling between 50–80% represent moderate phytotoxicity, and values above 80% represent nontoxic effects. Therefore, phytotoxicity tests indicated that CR decolorization by *S. xiamenensis* G5-03 led to partial detoxification of the dye.

4. Conclusion

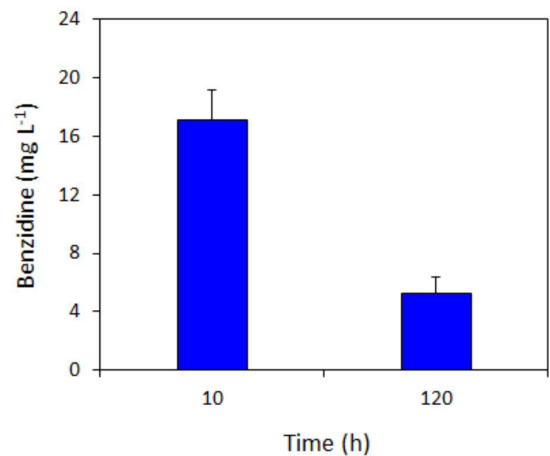The strain *S. xiamenensis* G5-03 can decolorize the azo dye Congo red and degrade the benzidine, a product of

Figure 5. Changes in the concentration of benzidine during decolorization of CR by *S. xiamenensis* G5-03.
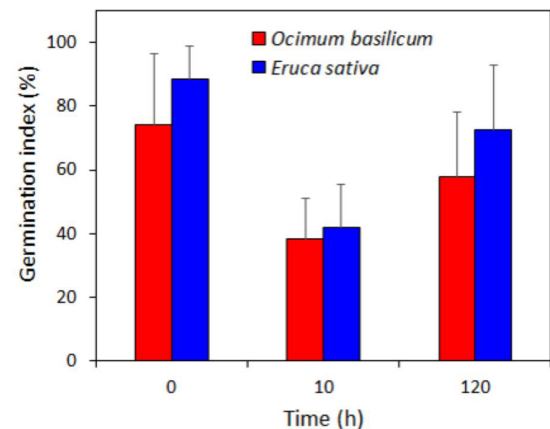

Figure 6. Evolution of phytotoxicity (germination index) during the CR decolorization by *S. xiamenensis* G5-03.

the cleavage of the dye. The optimal conditions for the decolorizing activity were static incubation at pH 10.0, and 30-35 °C. Considering that many textile wastewaters are usually alkaline with pH higher than eight, this strain could be useful for the biological treatment of this kind of wastewater. The decolorization kinetic could be described with the Michaelis-Menten model, and the V_{max} and K_m were found to be 111.11 mg L^{-1} h^{-1} and 448.3 mg L^{-1}, respectively. According to these findings, *S. xiamenensis* G5-03 might be valuable for the bioremediation of Congo red dye.

Acknowledgements

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