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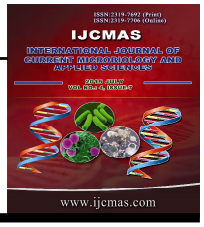

Original Research Article

Decolorization of Black B azo dye by *Pseudomonas aeruginosa*

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Keywords

Pseudomonas aeruginosa, decolorization, Remazol Black B

A B S T R A C T

The azo dye Black B from textile industries was investigated the biodegradation process by *Pseudomonas aeruginosa* UCP1567 (clinical isolate after eliminating resistance to antibiotics) using a 2^3 factorial design. The independent variables were used aeration/static condition, concentrations of azo dye and inoculum size, on the variable response, the decolorization of the azo dye. The results showed higher decolorization by *P. aeruginosa* of 85-94.4% under static condition, 52% under aeration of 100 rpm and 45% at aeration of 200 rpm. The static condition assays were observed the pH range varied from 5.8 to 8.5, and the bioassay using *Artemia salina* showed higher mortality (> 80%) compared with control, indicating recalcitrance compound was formed after decolorization/biodegradation. The results showed that the three variables static/aeration, azo dye concentration and inoculum size are significant in the process of Remazol Black B removal. In studies with *P. aeruginosa*, the colour of the black B azo dye was removed 85to 94.4%, respectively, after 5 days of static incubation, and the colour removal efficiencies were observed under microaerophilic conditions, as co-metabolism of azo dye degradation.

Introduction

Due to their synthetic nature and complex chemical structure, the molecules of dyes remain for long periods of time in nature. Azo dyes constitute the largest class of synthetic dyes, and are characterized by their typical binding l-N = N- (Manu;Chaudhari, 2003). These recalcitrant compounds can cause serious environmental problems due to the formation of carcinogenic or mutagenic compounds (Balan, 2009; Gupta et al., 2015).

Their high solubility in water sees to it that azo dyes are not degraded by conventional sewage treatment plants (Stloz et al.,1999). Thus, colored wastewater containing reactive azo dyes has caused serious environmental problems, mainly because these compounds, when present in water are highly visible, since they affect the transparency and aesthetics of the body of water (Stloz et al., 2002; Strehaiano et al.,2004; Gupta et al., 2015).

Thus, it is important to investigate microbial strains that have high potential for decolorization, by means of the reductive cleavage of the azo group in the molecule of textile dyes. In recent years, several researchers have demonstrated the ability of various microorganisms to transform azo compounds into colorless products as well as to bring about complete mineralization, under certain environmental conditions (Marinho,2010).

Bacteria have been cited as a promising application when treating textile effluents, due to the investment costs being five to twenty times lower than in some other chemical treatment processes, such as ozone or hydrogen peroxide (Saum et al. 1997; Pandey; Upadhyay,2010; Godlewska et al., 2014).

Aerobic biotransformation of azo dyes by fungi and bacteria has been extensively studied. According to Pandey e Upadhyay,2010, aerobic treatment is more attractive as a method for biodegrading these compounds. In this process, aerobic reductive metabolism requires specific enzymes (aerobic azoredutases) that catalyze the reduction of the azo compound by using NAD-dependent ones (Abraham et al.,2003). The aerobic azoredutases (monomeric flavins) isolated from Pseudomonas K22 and KF46 use NADPH and NADH as co-factors in the reduction of various sulfur dyes (Zimmermann et al., 1984).

Given that the decolorization of azo dyes can be influenced by several factors which have varying degrees of importance, factor models have been used as an important tool for both conducting research and for applied work (Chang; Kuo,2000; Mielgo et al.,2001; Chen;Yao, 2000). Therefore, they should be regarded as significant parameters for a given process, in addition to which the interaction between them enables optimization processes in the decolorization processes to be achieved. This study set out to evaluate the potential for decoloring Remazol Black by Pseudomonas aeruginosa (UCP 1567) under different culture conditions, using a complete factorial design 2^3.

Material and Methods

Microorganism

Pseudomonas aeruginosa UCP 1567 was provided by the Culture Bank at the Center for Research in Environmental Sciences NPCIAMB, UNICAP, Recife, Brazil, registered with the World Federation for Culture Collection, WFCC and maintained in nutrient agar at 4ºC.

Dye

The azo dye used was reactive Remazol Black B (CI 17095) obtained from Sigma (Sigma-Aldrich Corporation, St. Louis, Missouri, USA).

Decolorization of Remazol Black by *P. aeruginosa*

Pre-inoculum

Pseudomonas aeruginosa was previously grown in 100 mL of Luria Bertani broth

(LB) (g L^{-1}): tryptone 10g, yeast extract 5g, NaCl 10g, 5g of glucose added distributed in 250 mL Erlenmeyer flasks and incubated for 12 h at an agitation rate of 150 rpm at 37°C, resulting in a culture of 10^8 CFU/mL.

Kinetics of decolorisation

Then, inoculum of 1.5 and 3.75 mL were transferred to 250 mL Erlenmeyer flasks, containing 50 mL of LB medium supplemented with glucose, pH 7.0 ± 0.2.

The assays were performed according to the factorial design 2^3, for 120 h at 37°C. Samples were taken every 24 hours to determine the kinetics of decolorization.

Analytical methods

Determining pH

The pH of the cell-free medium was measured using an Orion pH meter, model 310.

Decolorization of Remazol Black

The removal of the dye was measured after centrifugation (10,000 gx 8 min.), at a temperature of 28°C, by a spectrophotometric reading of 597 nm. To calculate the percentage of the decolorization, the following equation was used:

$$\text{Remoção da cor (\%)} = \frac{(A) - (B)}{(A)} \times 100$$

(Eq.1)

Removal of the color (%)

where, (A) indicates the absorbance of the non-inoculated liquid medium and (B) indicates the absorbance of the residual liquid medium containing Remazol Black.

Toxicity tests

The biological assay was performed using the McLaughlin et al.(1995), method in assays 1, 2, 3 (greatest decolorization). About 10 brine shrimp (*Artemia salina*) larvae were transferred to flasks containing the test sample (decolored material) with different concentrations (25, 50, 75%), diluted in 5 mL of artificial sea water. The control test was conducted using only seawater. The tests were performed in triplicate. The count of dead and living shrimp was made after 24 h of exposure.

Factorial design

In the planning matrix, the lower and upper levels were replaced by -1 and +1, respectively, which makes the values of all the levels at the midpoint equal to zero. The complete factorial design 2^3 to examine the effects and interactions of the independent variables: volume of the inoculum, concentration of the dye and agitation, on the response variable, percentage of decolorization after 120 h. All results were analyzed using STATISTICA software version 5.0, Statsoft, USA (Table 1).

Results and Discussion

Decolorization of azo dye

The initial reaction to the decolorization of azo dyes is the reductive cleavage of the azo group. However, under anaerobic conditions, these reactions can be catalyzed by several biological systems, leading to the accumulation of aromatic amines (Amoozegar et al.,2010).

In recent years, several studies have reported some factors that can affect the removal of synthetic dyes in the process of microbial decolorization. Among them, nutritional

factors (carbon and nitrogen sources) and physical factors (temperature, agitation and pH, etc.) can influence the degradation of azo compounds (Chang; Kuo,2000; Mielgo et al.,2001; Marinho,2010).

Therefore, experiments were performed with *P. aeruginosa* (UCP 1567) for 120 h using different combinations of the three chosen variables (agitation, dye concentration and inoculum size) as per factorial design 2^3. Thus, it was observed that the best rate of decolorization of the dye was obtained when all the independent variables were regulated at their lowest level (assay 1), which prompted a decolorization of 94% and suggested the cleavage of the azo dye (Table 1).

When the independent variables were at their higher level (assay 8), the rate of decolorization decreased noticeably to only 38%. However, when the flasks were subjected to moderate agitation (100 rpm), the rate of decolorization was higher (52%) (Table.1). In the assays with greatest decolorization, there was an increase in pH, suggesting the presence of amines, arising from the cleavage of the dye and recognized by its alkalinity (Table 1). To Padmavathy et al.(2003), the decolorization of the reactive azo dyes Red RB and Reamzol Red using glucose as co-substrate, contributed to a decolorization of 91-94%. In a study of *Lysinibacillus sp* dye mixture in textile discoloration observed a percentage discoloration around 87% after 48 hours (Saratale et al. 2013) .

The color of the dyes Congo red and DB 38 was removed by up to 98% and 72%, respectively, by *Escherichia coli* under anaerobic conditions and no decolorization occurred during aerobic incubation (Isik; Sponza,2000). According to Chang *et al.*(2000), the presence of oxygen does not directly inhibit the azoredutase activity, this inhibition probably being an event dependent on microbial metabolism. Bromley-Challenor *et al.* 2003, reported that occasional gentle agitation promotes uniformity of the dye, by reducing the limitations of diffusion. In study by Kurode et al. 2011, evaluating discoloration parameters and physical-chemical of a dye mixture, using strain of Bacillus sp, there was the efficiency of the micro-organisms in removing 71% of the coloring, in period 24h under optimum temperature 40 °

Silveira *et al.(*2009), in a study on the Selection of Pseudomonas for decolorizing industrial textile dyes observed decolorization of 98% for methyl-orange after 48 h and 78% after 56 h, respectively, under anaerobic conditions. On studying the textile dye Acid Blue 92, they showed that the two strains were able to decolor concentrations below 30 mg L^{1}, with decolorization reaching 98% decolorization in *P. oleovorans* and 94% for *P.aeruginosa*. However, decolorization of the dye by *P. aeruginosa* decreased rapidly at a concentration of 70 mg/ L to 50%, while *P. Oleovans* was able to remove 90% of the color of the dye at a concentration of 60 mg L^{1} and up to 76% at 90 mg L^{1}.

Chang and Kuo (2000), on studying the decolorization of the reactive dye Red 22, using *Escherichia coli* under anoxic and aerobic conditions, also observed that the level of dissolved oxygen significantly inhibited the removal of color. According to Isik and Sponza (2003), the decolorization of the azo dyes Congo Red and Reactive Black 38 in cultures of *Pseudomonas sp*. was 100 and 83%, respectively, after five days of anaerobic incubation. However, under microaerophilic conditions, only decolorization of 76 and 74%, respectively, was observed. No decolorization occurred under aerobic conditions.

Figure 1 Pareto chart showing the main effects and interactions of the independent variables in the process of decolourization Remazol Black B by *Pseudomonas aeruginosa* UCP 1567, after 120 h of culture growth at 37°C. (1) Inoculum size. (2) Dye concentration and (3) Range of agitation

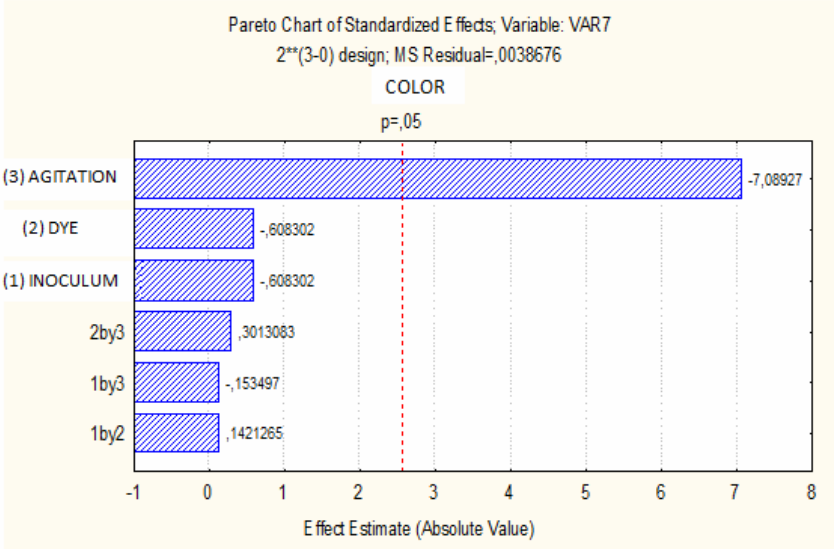

Figure 2 Evaluation of the toxicity of metabolites formed during the process of decolourization by *Artemia salina* Remazol Black B by *Pseudomonas aeruginosa* (UCP 1567), after 120 h of cultivation at 37^0C

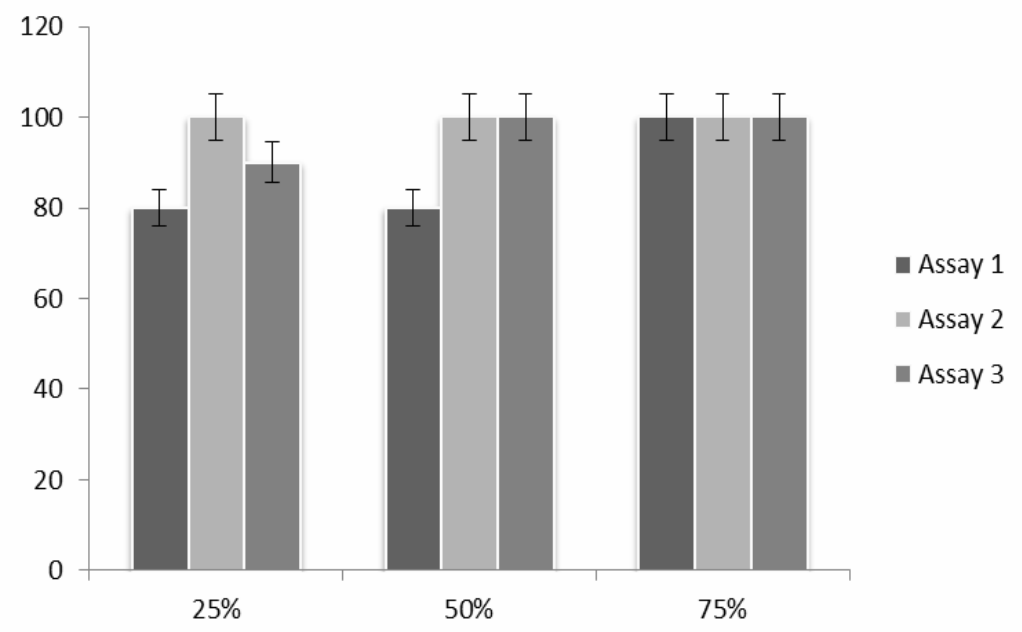

Table 1.Matrix encoded 2^3 factorial design in relation to the response of decoloring Remazol Black B and pH by *Pseudomonas aeruginosa* UCP 1567 after growing the culture for 120h at 37^0C

Sample	Level of the Factors [a]			Decolourization (%) [b]		Final pH [c]
	Inoculum	Dye	Agitation	24h	120h	
1	-1	-1	-1	**14.3**	**94.4**	**8.2**
2	+1	-1	-1	**7.0**	**89.1**	**8.0**
3	-1	+1	-1	**9.0**	**86.9**	**8.2**
4	+1	+1	-1	**11.0**	**85.0**	**7.9**
5	-1	-1	+1	**9.4**	**45.0**	**7.6**
6	+1	-1	+1	**5.8**	**40.2**	**8.0**
7	-1	+1	+1	**7.0**	**43.0**	**8.2**
8	+1	+1	+1	**7.1**	**38.0**	**8.2**
9	0	0	0	**12.0**	**52.6**	**8.0**
10	0	0	0	**9.7**	**53.4**	**8.3**
11	0	0	0	**6.0**	**52.3**	**7.9**
12	0	0	0	**8.4**	**52.2**	**8.0**

[a]Levels of the factors, coded as values of -1 and +1 and 0 (midpoint) in the table, as follows: Inoculum (10^8): 2.5 mL for level -1; 3.75 mL level 0; 5 mL level +1; dye concentration: 1 mM to level -1; level 0 1.5 mM level 0; 2.0 mM level +1; Agitation: 0 rpm to level -1; 100 rpm level 0; 200 rpm level +1. [b] Response obtained with each assay regarding decoloration of Remazol Black B by *Pseudomonas aeruginosa*. [C] Final pH of the medium in the end of the experiments.

In accordance with the Pareto diagram (Figure 1), it is observed that independent variables (inoculum size, dye concentration and agitation) exerted a negative effect on the decoloring process of the dye. However, only agitation showed a statistically significant effect, suggesting that this variable is a parameter of great importance in the process of decolorization of Remazol Black by *P. aeruginosa.*

Toxicity

Toxicity assays using *A. salina* in the decolored samples (assays 1, 2 and 3) were conducted in order to determine the acute toxicity of the dye after treatment with *P. aeruginosa.*

The results of the toxicity tests are shown in Figure 2. The percentage of mortality was compared with the control, which did not show any toxic substance. Among the samples tested, assay 1 showed the lowest toxicity (80%). The other assays showed high mortality (> 80%).

According to Amoozegar et al.(2010), and Evangelista et al.(2004), the mortality observed may be suggested by the presence of SO_3H groups, which often resist biodegradation or are only partially degraded.

Thus, it was observed that degradation of the dye formed metabolites, which were shown to be toxic for *A. salina.* The presence of sulphanilic acid in the environment may have contributed to the toxicity presented. According to Sponza and Isikil,2004, toxicity toxicity tests using *D. magna* showed that the non-degradable portion of the dye was responsible for the toxicity.

Determining pH

The pH of the medium during culture tended to alkaline range (8.0) (Table 1). This fact can be explained by the production of intermediate metabolites (aromatic amines), as a result of the biodegradation of the compound. The pH decreased after 24h (5.7), tending to the alkaline range at the end of culture growth (8.2) The initial decrease in pH is explained by the accumulation of organic acids, resulting from the degradation of glucose (Ambrosio,2004). According to Adebayo et al.,2004 the total decolouration of the dye Methyl Red (5mg L-1) using a bacterial consortium, was observed after 24 h at pH 7 and 8, and of only 82 and 65% at pH 5 and 6, respectively.

In conclusion, under the conditions studied, *P. aeruginosa* after removal of the frequency of plasmid resistance effectively removes the azo dye Remazol Black in conditions of co-metabolism. The application of factorial design shows that of the three variables used, only one (agitation), seems to affect significantly the process of decolorization of Remazol Black by using *P. aeruginosa*.

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