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Experimental design for decolourization of textile dye by *Pseudomonas oleovorans*

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ABSTRACT

The present study describes the optimization of environmental conditions in colour removal by *Pseudomonas oleovorans* of an industrial textile dye. The first, a factorial design (2^{n-2}) was performed to screen the most significant parameters and the second one, a full experimental design (2^4) was then carried out. Decolourization of the industrial dye was negatively influenced by tube volume and dye concentration, which is probably related to the maximal capacity of the strain to decolourize azo dyes. The decolourization process showed to be time-dependent. The best conditions for dye decolourization by *Pseudomonas oleovorans* based on the experimental design and economic factors were agitation speed, 0 RPM, pH 8.5, temperature 32°C, 25% of tube volume, initial culture size of 0.16 $g \cdot l^{-1}$, dye concentration of 50mg L^{-1}. Under such conditions, a near total decolourization (95.4%) was achieved after 32 hours.

KEYWORDS

Decolourization;
Industrial dye;
Pseudomonas oleovorans;
Experimental design;
Optimization.

INTRODUCTION

There are over 100,000 commercially available dyes with over 7×10^7 tons of dyestuff produced annually worldwide[1,2]. In Brazil alone, 26,500 tons are produced[3]. These dyes are widely used in a number of industries, such as textiles, food, cosmetics and paper printing, with the textile industry the largest consumer of dyes[4].

Considerable attention has been given to issues associated with the presence of coloured compounds in aqueous wastewater generated from textile industries. Colour is the first contaminant recognised in wastewater, however in addition to the aesthetic problem, the dyes obstruct light penetration and oxygen transfer in bodies of water. Without an adequate treatment, dyes are stable and can remain in the environment for an extended period of time[5].

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Currently, the most popular methods of colour removal from wastewaters involve physical and chemical processes that can be costly and usually involve the formation of a concentrated sludge that creates a secondary, highly significant disposal problem[6]. Alternative, biodegradative systems based on colour removal using whole bacterial cells have been shown to be highly effective[7].

Environmental biotechnology is constantly expanding efforts in the biological treatment of dye-contaminated wastewaters. Although numerous microorganisms are capable of decolourizing dyes, only a few are able to mineralise these compounds into CO_2 and H_2O [8]. Under aerobic conditions, azo dyes are not easily metabolized[1]. However, under anaerobic conditions, several bacterial strains, including *Pseudomonas oleovorans*, can enzymatically reduce the azo bond in the dye molecule to produce colourless by-products[5].

The statistical design of experiments is a widely used methodology in upstream and downstream processing[9-11], which differs from the classical optimization methods. The further method includes single factor variation, maintaining other factors constant. This method is not suitable for multifactor optimization not only because it is time-consuming but also because it is unable to detect the true optimum due to the interaction between factors[9].

In order to avoid multifactorial problems, the statistical design of experiments is used. These designs require a relatively small number of runs per factors allowing the identification of the most important variables and optimum ranges, as well as the experimental verification of this identified optimum, the designs can also indicate a promising direction for further experiments[12,13].

The application of experimental design and response surface methodology (RSM) in textile effluent treatment processes can lead to improved decolourization, reduced process variability, time and overall costs. Additionally the factor influencing the experiments can be identified, optimized and possible synergic or antagonistic interactions that may exist between factors can be evaluated[14]. This work describes the optimization of environmental conditions for the decolourization of industrial dye by *Pseudomonas oleovorans* in submerse cultures.

MATERIALS AND METHODS

Microorganisms

The microorganism was obtained from the Brazilian Collection of Industrial and Environmental Microorganism (CBMAI) of the University of Campinas, previously identified as *Pseudomonas oleovorans* (CBMAI 703).

The microorganism was preserved in cryotubes containing glass beads and 50% glycerol (v/v). Each cryotube was loaded from the same initial culture and had an average of 30 beads. Thus, it was possible to use the same cell generation for all experiments[5].

Dyes and ragents

Textile dye was obtained with the kind permission of Clariant of Brazil (Sao Paulo, Brazil). As the dye is for commercial use, the commercial name has been omitted in this study, receiving the following codename: B15 (C.I. 13390). The dye was filter-sterilised on a 0.2 μm filter (Millipore, USA) prior to addition to sterile culture medium. All other reagents were of analytical grade.

Pre-culture conditions

For each experiment, an Erlenmeyer flask containing 20ml of Nutrient Broth (meat extract $3g \cdot l^{-1}$ and peptone $5g \cdot l^{-1}$) was inoculated with a single glass bead from the same cryotube and incubated at 28°C for 24 h when an early stationary phase or final exponential phase was reached.

Statistical design of experiments

Optimization of the decolourization condition was firstly performed by a fractional statistical design of experiments (2^{n-2}), and the studied variables are listed in TABLE 1. According to the results obtained, a second statistical design of experiments (2^n) was performed aiming the best decolourization conditions.

All the experiments were performed in anoxic conditions (without aeration). The second experimental design was performed with an inoculum concentration of $0.16g \cdot l^{-1}$ and a reactor volume of 25%. The studied variables of the second design are listed in TABLE 2. The results obtained were analysed in Statistica 7.0 (Statsoft, Inc., USA) statistical package[12,15].

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TABLE 1 : Studied parameters on decolourization under submerse culture (2^{n-2})

Variables	Levels		
	-1	0	+1
Dye Concentration (mg l^{-1})	30	50	70
Agitation (RPM)	0	75	150
Initial pH	6.0	7.0	8.0
Temperature (°C)	27	32	37
Time (hours)	36	48	60
Volume (%)	25	50	75
Inoculum (mg l^{-1})	0.16	0.32	0.48

Decolourization cultures

The decolourization cultures were performed in 500 ml screw-topped bottles, containing 125 ml of Nutrient Broth (meat extract 3g·l^{-1}, peptone 5g·l^{-1} and adjusted pH = 8.5) supplemented with 50mg·l^{-1} of dye, and inoculated with 10ml of fresh 24-hours-old cultures (approx. 0.16g·l^{-1} of dry weight of cells). The bottles were incubated under static anoxic conditions away from light at 32°C for 36 hours. Control experiments were performed using the same medium without microorganisms or dyes.

Determination of cell growth and decolourization

The sample from the decolourization cultures were collected and analysed following the methodology described previously[5]. As all samples contained biomass and dye, biomass concentration (first and second step) and dye (third step) were evaluated as follows:

(1) $\mathbf{OD_{600nm}}$ of sample mixtures without centrifugation:

$$\mathbf{OD_{600nm}^{X+dye} = OD_{600nm}^{dye} + OD_{600nm}^{X}}$$

(2) $\mathbf{OD_{600nm}}$ of sample supernatant (sup) after centrifugation for 10 minutes at 10,000g:

$$\mathbf{OD_{600nm}^{sup} = OD_{600nm}^{dye}}$$ and

(3) $\mathbf{OD_{609nm}}$ of sample supernatant after centrifugation:

$$\mathbf{OD_{609nm}^{sup} = OD_{609nm}^{dye}}$$

The biomass produced was determined by subtracting the value obtained on the first step from the value obtained on the second. Colour removal efficiency was determined by the following equation:

$$\mathbf{Decolourization} = \frac{\mathbf{A_{\lambda initial} - A_{\lambda final}}}{\mathbf{A_{\lambda initial}}} \quad (1)$$

TABLE 2 : Studied parameters on decolourization under submerse culture (2^n)

Variables	Levels		
	-1	0	+1
Dye Concentration (mg l^{-1})	40	55	70
Time (hours)	24	36	48
Temperature (°C)	24	28	32
Initial pH	7.5	8.0	8.5

in which $A_{\lambda initial}$ represented the absorbance before the decolourization process and $A_{\lambda final}$ the value obtained during the third step. Each decolourization value was a mean of three parallel experiments.

Decolourization was also determined by wavescan, which was performed in an Ultrospec 3000 (GE Healthcare, USA). Samples were scanned from 240 to 790nm to measure the colour removal and aromatic compounds degradation by *Pseudomonas oleovorans*, culture medium without industrial dye was used as blank.

RESULTS AND DISCUSSION

Factorial experimental design (2^{7-2})

The responses obtained from the 35 runs of the factorial experimental design (2^{7-2}), and the summarizations of significance of the variables are listed in TABLE 3 and 4, respectively. Tube volume and dye concentration were the variables which suffered the greatest influence, showing negative responses; inoculum concentration showed a negative response as well, though not as high as the former variables. On the other hand, time, pH value and temperature showed a positive effect over the decolourization process of the industrial dye studied. Agitation showed a positive effect as well, though not significant.

The results for biomass production within the decolourization process showed that the temperature was the most influenced variable with a negative effect, as well as the concentration of inoculum. The pH value and e reactor volume showed a positive significant effect. Time, dye concentration and agitation showed no significance upon biomass production, and only the former variable had a positive effect.

Agitation had not shown significant increase during the decolourization process, and therefore was not studied any further in this work. The concentration of inoculum was fixed at 0.16g·l^{-1}, as well as the tube

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TABLE 3 : Conditions and results of the 2^{7-2}-factorial experimental design selected for industrial dye decolourization

Run	temp[a]	pH	dye[b]	agit[c]	vol[d]	time[e]	Inoc[f]	Decolour[g]	Biomass[h]
1	27	8	30	150	25	60	0.16	96.62	0.107
2	27	8	70	0	75	60	0.48	69.65	0.106
3	27	6	70	150	25	60	0.16	90.59	0.168
4	27	6	70	0	25	36	0.48	72.88	0.123
5	27	6	30	150	75	36	0.48	83.88	0.149
6	27	6	70	0	75	36	0.16	54.62	0.181
7	27	8	70	150	25	36	0.48	79.02	0.199
8	27	6	70	150	75	60	0.48	66.80	0.129
9	27	6	30	0	25	60	0.48	89.48	0.240
10	27	6	30	0	75	60	0.16	93.15	0.205
11	27	6	30	150	25	36	0.16	93.24	0.181
12	27	8	30	0	75	36	0.48	84.75	0.246
13	27	8	30	0	25	36	0.16	88.13	0.224
14	27	8	70	0	25	60	0.16	95.34	0.263
15	27	8	30	150	75	60	0.16	94.88	0.327
16	27	8	70	150	75	36	0.16	67.01	0.289
17*	32	7	50	75	50	48	0.32	89.01	0.089
18*	32	7	50	75	50	48	0.32	88.84	0.086
19*	32	7	50	75	50	48	0.32	88.45	0.085
20	37	8	30	150	25	36	0.16	94.31	0.088
21	37	6	30	150	75	60	0.16	90.44	0.100
22	37	8	70	0	25	36	0.48	95.68	0.075
23	37	6	70	150	75	36	0.16	52.85	0.178
24	37	8	30	150	75	36	0.48	89.67	0.088
25	37	8	30	0	25	60	0.48	98.36	0.101
26	37	6	70	150	25	36	0.48	94.39	0.102
27	37	6	70	0	75	60	0.48	62.13	0.091
28	37	8	30	0	75	60	0.16	96.43	0.089
29	37	6	70	0	25	60	0.16	98.10	0.066
30	37	6	30	0	25	36	0.16	95.85	0.085
31	37	8	70	150	25	60	0.16	97.67	0.073
32	37	6	30	150	25	60	0.48	96.14	0.043
33	37	8	70	0	75	36	0.16	79.45	0.089
34	37	6	30	0	75	36	0.48	78.09	0.084
35	37	8	70	150	75	60	0.48	73.79	0.082

[a]temperature (°C); [b]dye concentration (mg·l^{-1}); [c]agitation (RPM); [d]volume (%); [e]time (hours); [f]inoculum concentration (g·l^{-1}); [g]decolourization (%); [h]biomass (g·l^{-1}); *central point repetitions

volume, which was fixed at 25% based on the theory by TABLE 3.

Umbuzeiro et al.[16] and Chen et al.[17] in which the decrease of decolourization based on the volume and the dye concentration should be explained by the rate between overall dye mass presented in the medium and the initial biomass presented, which leads to protein production inhibition, due to dyes genotoxicity, or to the aromatic rings presented in the dye, which may affect the DNA synthesis and therefore inhibit cell growth.

TABLE 4 : Estimated effects of studied parameters (t distribution)

Parameter	Decolourization (%)	Biomass (g•l^{-1})
Temperature (°C)	46.5218[a]	-141.763[a]
Initial pH	52.6789[a]	24.6371[a]
Dye (mg•l^{-1})	-129.685[a]	-9.5515[a]
Agitation (RPM)	4.1846	0.3984
Volume (%)	-144.89[a]	27.569[a]
Time (hours)	63.5059[a]	-18.7553[a]
Inoculum (g•l^{-1})	-23.3211[a]	-406087[a]

[a]Statistically significant values (at the 95% confidence level)

TABLE 5 : Conditions and results of the 2^4-experimental design selected for industrial dye decolourization

Runs	Time[a]	Dye[b]	pH	Temp[c]	Decolour[d]	Biomass[e]
1	24	40	7.5	24	45.06	0.106
2	24	40	7.5	32	73.01	0.169
3	24	40	8.5	24	51.77	0.162
4	24	40	8.5	32	80.68	0.226
5	24	70	7.5	24	43.35	0.103
6	24	70	7.5	32	55.27	0.164
7	24	70	8.5	24	50.26	0.145
8	24	70	8.5	32	66.49	0.212
9*	36	55	8.0	28	69.26	0.204
10*	36	55	8.0	28	69.45	0.201
11*	36	55	8.0	28	69.75	0.200
12	48	40	7.5	24	57.82	0.140
13	48	40	7.5	32	85.77	0.274
14	48	40	8.5	24	75.15	0.224
15	48	40	8.5	32	85.69	0.304
16	48	70	7.5	24	50.78	0.145
17	48	70	7.5	32	87.78	0.292
18	48	70	8.5	24	61.10	0.258
19	48	70	8.5	32	90.50	0.348

[a]time (hours); [b]dye concentration (mg·l^{-1}); [c]temperature (°C); [d]decolourization (%); [e]biomass (g·l^{-1}); *central point repetitions

TABLE 6 : Estimated effects of studied parameters (t distribution)

Parameter	Decolourization (%)	Biomass (g•l^{-1})
Time (hours)	130.2372[a]	83.82709[a]
Dye (mg•l^{-1})	-50.0103[a]	7.445959[a]
pH	63.55008[a]	58.36671[a]
Temperature (°C)	192.1682[a]	84.78786[a]

[a]Statistically significant values (at the 95% confidence level)

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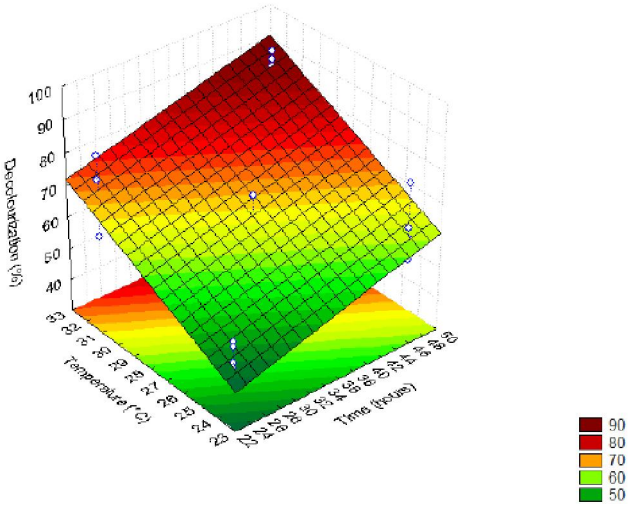

Figure 1 : Response surface of decolourization of B15 dye as a function of temperature and time

Full experimental design (2^4)

The responses obtained from 19 runs of the second experimental design (2^4), and the summarizations of the significance of variables are listed in TABLE 5 and 6, respectively. In this set of experiments, the temperature was the most influencing variable, with a positive effect over dye decolourization; time and pH also showed positive effects upon the decolourization process. Dye concentration was the only parameter which presented a negative effect, however all the parameters studied showed statistically significant values at a 95% confidence level.

All the variables studied presented a significant effect over biomass production. The temperature and process time were the variables which appeared to be more significant for biomass production, although pH and dye concentration showed to be significant as well.

Temperature also influenced biomass production negatively, this could be a result of the change from natural temperature for *P. oleovorans* incubation[5]. The decrease in temperature in this case was quite important based on economical issues, when decolourization was performed at temperatures near 28-32°C; there was no need to warm the medium prior to the decolourization process.

In many systems, the rate of colour removal increases with temperature, within a defined range depending on the system. The temperature required to produce the maximum rate of colour removal tends to correspond to the optimum cell culture growth temperature[18], in present case 28-32°C.

Culture time positively affected dye decolourization suggesting that the medium dissolved oxygen, as well as the available oxygen within the tube, exerting a limiting role at the beginning of the decolourization process, as the oxygen competes with the azo bound as an electron receptor. Once extinct, and when the redox potential was reached, the effective dye decolourization began[19].

During the cell growth stage, the oxygen present would have a significant effect upon the physiological characteristics of cells. During the dye reduction stage, if there is any oxygen dissolved in the cell environment, the oxygen would inhibit the dye reduction mechanism. This could be caused as the electrons released from the electron donors by the cells would preferentially used to reduce oxygen rather than dyes[20].

The pH results suggest that coloured wastewaters should be buffered to enhance colour removal. Biological reduction of the dyes can result in an increase in the pH due to the release of aromatic amines from the metabolism of dyes, which is more basic than the original dye[21]. Chang et al.[18] found that the dye reduction rate increased nearly 2.5-fold as the pH was raised from 5.0 to 7.0, whereas the rate became insensitive to pH in a range from 7.0 to 9.5.

Dye concentration can also influence the efficiency of colour removal through a combination of factors including dye toxicity at high concentrations, and the ability of the enzymes involved in the process to recognise the substrate efficiently at very low concentrations. Indeed, the kinetic model that governs the process efficiency of colour removal of dyes by whole bacterial cells can be described using a single-site binding model, such as the Michaelis-Menten Model:

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (2)$$

where; v is the observed velocity of the reaction at a given substrate concentration $[S]$, V_{max} is the maximum velocity at a saturating concentration of the substrate and K_m is the Michaelis constant[22].

Wuhrmann et al.[23] observed that after a rapidly reduction of colour, the rate of colour removal decreased more rapidly than would be predicted by a first order reaction. This should be attributed to the initial dye adsorption and mass transport within the biomass[5]. It has also been observed that the higher the dye concentration, the longer the time required to totally remove colour[24].

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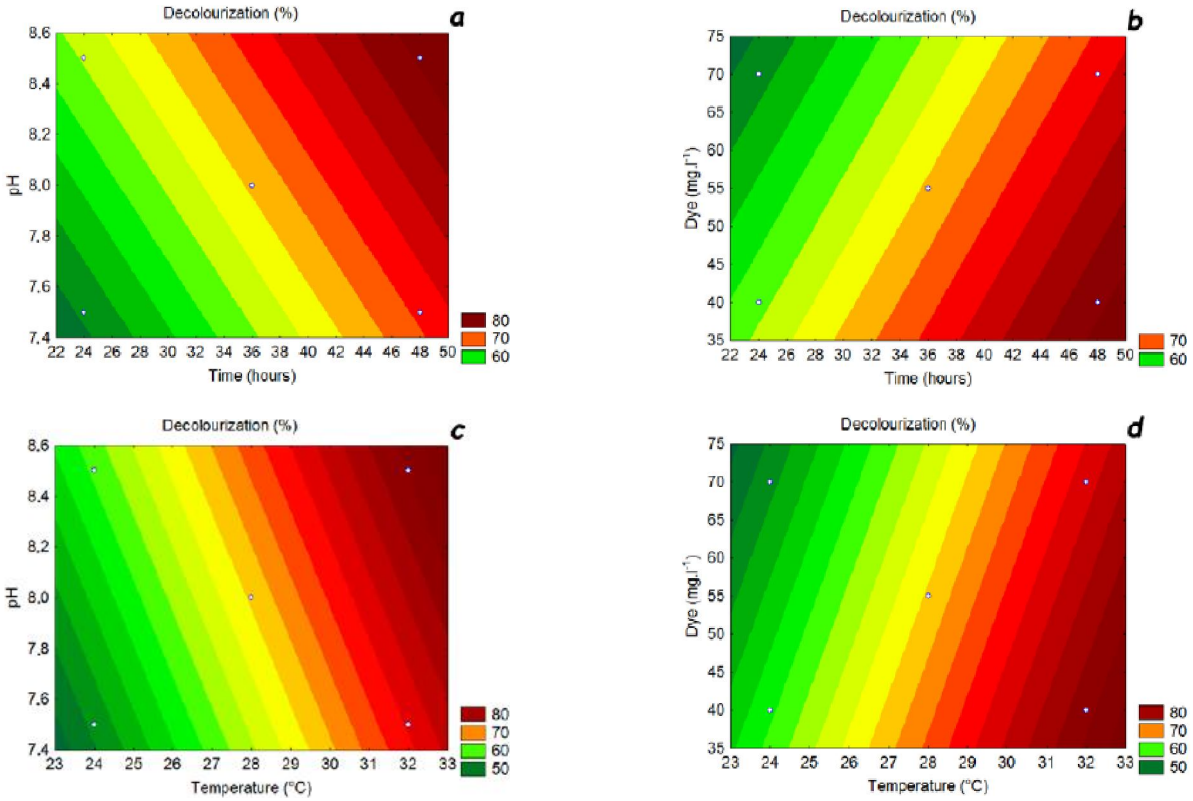

Figure 2 : a) Decolourization as a function of time and pH value; b) Decolourization as a function of time and dye concentration; c) Decolourization as a function of temperature and pH value; and d) Decolourization as a function of temperature and dye concentration.

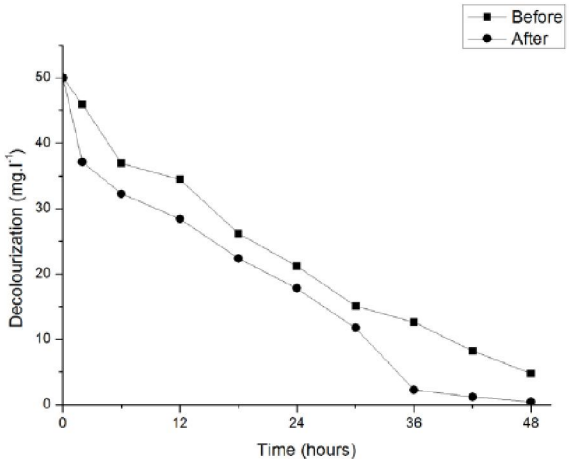

Figure 3 : Decolourization profile before (initial pH 6.5; 37°C; inoculum concentrarion 0.32g·l^{-1}; reactor volume 50%; agitation speed 0 rpm) and after the optimization.

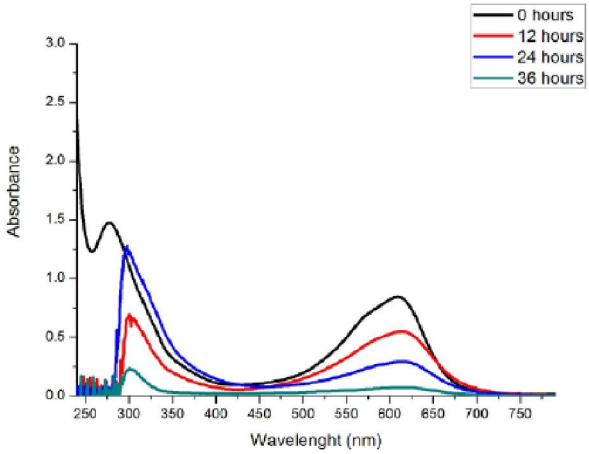

Figure 4 : B15 dye's wavescan along the decolourization process

Response surface methodology

To identify an optimum within the range of variables a Response Surface Methodology (RSM) was established to propose the minimization of the number of experiments[25]. Figure 1 shows the response surface graphic obtained as a function of time and temperature over the decolourization rate. A considerable increase in the decolourization of 1.4-fold was observed with the increase of temperature from 24 to 32°C, as well as an increase of 1.27-fold with the increase of time. The overall increase of the entire process was over 2-fold.

According to Weuster-Botz[25], the success of a statistical experimental design for optimization of a process depends on the randomly correct choice of variable ranges. Figure 2 shows the decolourization pattern as a function of time and pH value (Figure 2a),

as a function of time and dye concentration (Figure 2b), as a function of temperature and pH value (Figure 2c) and as a function of temperature and dye concentration (Figure 2d). There was a slight increase in the decolourization with the increase of pH value, and also an increase of decolourization rate as dye concentration decreased. The main objective of RSM was to determine the optimum conditions for the system or to determine a region that satisfied operating specifications[26].

Decolourization process of optimized culture

Figure 3 shows the *Pseudomonas oleovorans'* decolourization profile before and after optimization of conditions. Before condition optimization the process took over 48 hours to achieve a decolourization of over 95% (colour removal of 45mg·l^{-1}), and after optimization this decolourization was achieved after only 36 hours.

UV-Vis scan (240-790nm) of supernatant at different time intervals is presented in figure 4. The authors observed that the absorbance (609nm) was constantly reduced along the 36 hours of decolourization process; also a reduction in the UV spectrum within the process was also observed.

According to Brand & Eglinton[27], the strong absorbance at 200-300 nm of a solution is consistent for aromatic amines. Pearce et al.[28] described the decolourization of pigments by *Shewanella* strain J18 143, in which there was a reduction of its λ_{max} and an increase in UV spectrum, suggesting the formation of high quantity of aromatic amines.

The formation of aromatic amines resulting from decolourization is a common problem originated from the biological degradation of dyes. Ulson De Souza et al.[3] suggested that biological treatment should be considered as a pre-treatment, requiring a post-treatment for the removal of organic compounds (i.e. aromatic amines) remaining in the effluent. This should not be a concern with *Pseudomonas oleovorans* decolourization of dyes, as an almost complete removal of UV spectrum was observed.

CONCLUSION

P. oleovorans showed to be a good decolourizing tool for treatment of dyes, and the design of experiments along with RSM seems to be an adequate methodology to optimize the colour removal and dye degradation. The use of RSM improved decolourization process efficiency in 2-fold, and not only the dye chromophore group dye was reduced, but the aromatic amines as well. The best conditions for dye decolourization based on RSM and economic factors were pH 8.5, temperature 32°C, 25% of tube volume, an initial culture size of 0.16g·l^{-1}, and dye concentration of 50mg·l^{-1} in static anoxic conditions.

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