Decolorization of industrial azo dye in an anoxic reactor by PUF immobilized *Pseudomonas oleovorans*

E. Silveira, P. P. Marques, A. C. Macedo, P. G. Mazzola, A. L. F. Porto and E. B. Tambourgi

ABSTRACT

This work reported the full degradation of an azo dye in a synthetic effluent by *Pseudomonas oleovorans* immobilized in polyurethane foam (PUF). For each fed-batch experiment, a screw-top vessel containing 160 mL of nutrient broth was inoculated with 0.16 g L^{-1} of fresh culture, incubated at 32 °C and supplemented with 50 mg L^{-1} of dye every 24 hours. Afterwards, the *P. oleovorans* were immobilized in PUF and inoculated in an anoxic reactor. The results showed that at fed-batch conditions, *P. oleovorans* was capable of removing 50 mg of dye in 192 hours. However, when the decolorization was performed in an anoxic reactor, it was capable of fully degrading 25 mg of dye in only 24 hours.

Key words | anoxic reactor, azo dye, immobilization, polyurethane foam, *Pseudomonas*

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INTRODUCTION

There are more than 100,000 commercially available dyes with more than 7×10^7 tons of dyestuff produced annually worldwide (Robinson *et al.* 2001; Akhtar *et al.* 2005). Its production in Brazil reaches 26,500 tons per year (Ulson de Souza *et al.* 2007; Silveira *et al.* 2009b). These dyes are widely used in a number of industries, such as textiles, food, cosmetics and paper printing, with the textile industry being the largest consumer of dyes (Pandey *et al.* 2007).

Considerable attention has been given to issues associated with the presence of coloured compounds in aqueous wastewater generated from textile industries, since water is the only efficient carrier for dyes and other compounds that are used in the dyeing and finishing processes. An average textile finishing company uses 100,000–150,000 litres of water per ton of textile material treated (Li & Guthrie 2010).

Colour is the first contaminant to be recognized in wastewater; however, in addition to the aesthetic problem, dyes obstruct light penetration and oxygen transfer in water bodies. Without an adequate treatment, dyes are stable and can remain in the environment for an extended period of time (Silveira *et al.* 2009b).

Currently, the most popular methods of colour removal from wastewater involve physical and chemical processes that can be costly and usually include the formation of a concentrated sludge that creates a secondary, highly significant disposal problem (Chang & Kuo 2000). Although integrated chemical methods seem to be feasible for the treatment of such wastewater, biological methods should be used preferably considering cost and technical advantages (Yu *et al.* 2010).

Environmental biotechnology is constantly expanding efforts in the biological treatment of dye-contaminated wastewater. Although numerous microorganisms are capable of decolorizing dyes, only a few are able to mineralize these compounds into CO_2 and H_2O (Junghanns *et al.* 2008). Under aerobic conditions, azo dyes are not easily metabolized by bacteria (Robinson *et al.* 2001); however, under anaerobic conditions, several bacterial strains,

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including *Pseudomonas oleovorans*, can enzymatically reduce the azo bonds in the dye molecule to produce colourless by-products (Silveira *et al*. 2009b).

Several research papers have reported the results of combined anaerobic–aerobic bioreactor treatment of azo dye-containing wastewater (O'Neill *et al*. 1999; Sponza & Isik 2002; Kapdan *et al*. 2003; Khehra *et al*. 2006; Isik & Sponza 2008). However, the colour removal is mainly associated with the anaerobic stage, whereas further decolorization in the aerobic stage is usually limited to a few extra percent (van der Zee & Villaverde 2005). According to Minke & Rott (2002), a two-stage anaerobic–aerobic colour removal process achieved 70% higher decolorization than that of a one-stage aerobic treatment process. Therefore, it is reasonable to assume that anaerobic colour removal is mainly due to azo dye reduction.

This work reports the decolorization of an industrial azo dye by PUF immobilized *P. oleovorans* using an anoxic reactor.

EXPERIMENTAL

Microorganisms

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

The microorganisms were 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 (Silveira *et al*. 2009b).

Dyes and reagents

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-sterilized with 0.2 μm filter (Millipore, USA), prior to addition to the sterile culture medium. All other reagents were of analytical grade.

Pre-culture conditions

For each experiment, an Erlenmeyer flask containing 20 mL of nutrient broth (3 g L^{-1} meat extract and 5 g L^{-1} peptone) was inoculated with a single glass bead from the same cryotube and incubated at 28 °C for 24 hours, when an early stationary or a final exponential phase was reached.

Dye decolorizing cultures

Decolorization was performed, under static conditions, using screw-top bottles (500 mL) containing 125 mg of nutrient broth (adjusted pH = 8.5), supplemented with 50 mg L^{-1} of dye, and inoculated with 10 mL of fresh 24-hour-old cultures (approximately 0.16 g 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 (Silveira *et al*. 2009a).

Decolorization in fed-batch cultures

After the decolorization, the cultures were fed with a concentrated dye solution, aiming to regain the initial dye concentration. The feed was performed every time the dye concentration reached values below 3.6 mg L^{-1}.

Determination of cell growth and decolorization

The samples from decolorization cultures were collected and analysed according to the methodology described previously (Silveira *et al*. 2009b). As all samples contained biomass and dye, biomass concentration (first and second steps) and dye (third step) were evaluated as follows:

1. $OD_{600\,nm}$ of sample mixtures without centrifugation: $OD^{X+dye}_{600\,nm} = OD^{dye}_{600\,nm} + OD^{X}_{600\,nm}$;
2. $OD_{600\,nm}$ of sample supernatant (sup) after centrifugation for 10 min at 10,000 *g*: $OD^{sup}_{600\,nm} = OD^{dye}_{600\,nm}$; and
3. $OD_{609\,nm}$ of sample supernatant after centrifugation: $OD^{sup}_{609\,nm} = OD^{dye}_{609\,nm}$.

The biomass produced was determined by subtracting the value obtained in the first step from the value obtained in the second. Color removal efficiency was determined by

the following equation:

$$\text{Decolorization} = \frac{A_{\lambda\text{initial}} - A_{\lambda\text{final}}}{A_{\lambda\text{initial}}} \quad (1)$$

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

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

Cell immobilization on PUF

P. oleovorans cells from an early stationary or a final exponential phase were centrifuged at 10,000 *g* for 10 min and then immobilized in polyurethane foam (PUF). The PUF was cut in 1-cm^3 cubes, washed in distilled water, autoclaved twice and oven-dried at 37 °C. Approximately 9×10^9 cells in culture medium were added to screw-top bottles containing PUF cubes.

The content of the bottles was mixed on a magnetic stirrer for two hours. The bottles were incubated on an orbital shaker for one hour at 140 RPM, and then incubated under static conditions for another two hours. Finally, the dye was added to the culture medium and then incubated at the conditions described elsewhere (Silveira *et al.* 2009a).

Anoxic reactor

A jacketed glass column with an effective volume of 140 mL, with an internal diameter of 3 cm and a height of 20 cm was used as the reactor (Figure 1). The column was autoclaved and dried at 37 °C before each run.

Rubber corks were perforated for silicon tubing passage used for the column's inlet and outlet. Temperature was controlled using a water bath with water cycle. The column was packed with a PUF cylinder with a main diameter of 3 and 19 cm height.

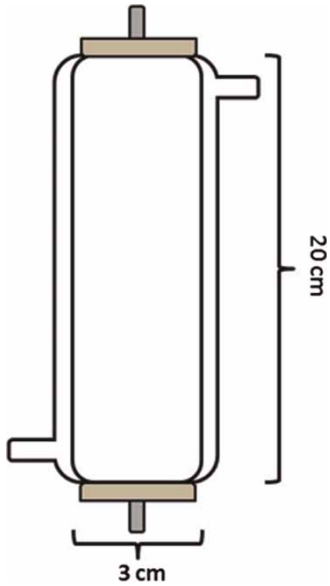

Figure 1 | A schematic of the column used for industrial dye decolorization.

Reactor operation

The experiments were conducted after the immobilization of *P. oleovorans* on PUF. The immobilization within the column was performed after 20 mL inoculation of fresh culture (approximately 9×10^9 cells) diluted in 100 mL of nutrient broth at 200 cm h^{-1} and sealed overnight at 32 °C.

The reactor was fed with synthetic effluent at a linear velocity of 15 cm h^{-1}, and at this rate no cell washout was observed. At given linear velocity, the hydraulic residence time (HRT) was approximately 2 hours. The reactor outlet was connected with the feed vessel to provide the dilution of the feed along the process time. The feed was stocked in a 1 litre bottle.

Synthetic effluent

The composition of the synthetic effluent was B15 industrial dye 25 mg L^{-1}; soluble starch 1,000 mg L^{-1}; acetic acid 150 mg L^{-1}; $(NH_4)_2SO_4$ 280 mg L^{-1}; NH_4Cl 230 mg L^{-1}; KH_2PO_4 67 mg L^{-1}; $MgSO_4{\cdot}7H_2O$ 40 mg L^{-1}; $CaCl_2{\cdot}2H_2O$ 22 mg L^{-1}; $FeCl_3{\cdot}6H_2O$ 5 mg L^{-1}; yeast extract 200 mg L^{-1}; NaCl 150 mg L^{-1}; $NaHCO_3$ 1,000 mg L^{-1}; and 1 mL L^{-1} of trace elements solution ($ZnSO_4{\cdot}7H_2O$ 10 mg L^{-1}; $MnCl_2{\cdot}4H_2O$ 100 mg L^{-1}; $CuSO_4{\cdot}5H_2O$ 392 mg L^{-1};

$CoCl_2 \cdot 6H_2O$ 248 mg L^{-1}; $NaB_4O_7 \cdot 10H_2O$ 177 mg L^{-1}; and $NiCl \cdot 6H_2O$ 20 mg L^{-1}).

Scanning electron microscopy

Samples of PUF before and after *P. oleovorans* immobilization were fixed in 2% glutaraldehyde (v/v) and 4% formaldehyde (v/v) at room temperature. The samples were then postfixed with 1% OsO_4 plus 0.8% $K_4[Fe(CN)_6]$ and 1% tannic acid, dehydrated in graded ethanol, critical point-dried with CO_2. The samples were then dehydrated in an ethanol series, transferred to propylene oxide, air-dried, mounted on aluminium stubs with double-sided tape (Scotch™), coated with gold in sputter system in a high vacuum chamber and examined in a Jeol JSM 5600LV at an acceleration voltage of 8 kV and a working distance of 8 mm.

RESULTS

Time-dependent and fed-batch cultures

The fed-batch decolorization was carried out for 216 hours, and the result is shown in Figure 2, which describes the decolorization of B15 dye in fed-batch. It was also observed that the biomass reached equilibrium after 42 hours. After 192 hours, *P. oleovorans* was able to decolorize up to 50 mg of dye; during this time, bacterial fatigue was not observed.

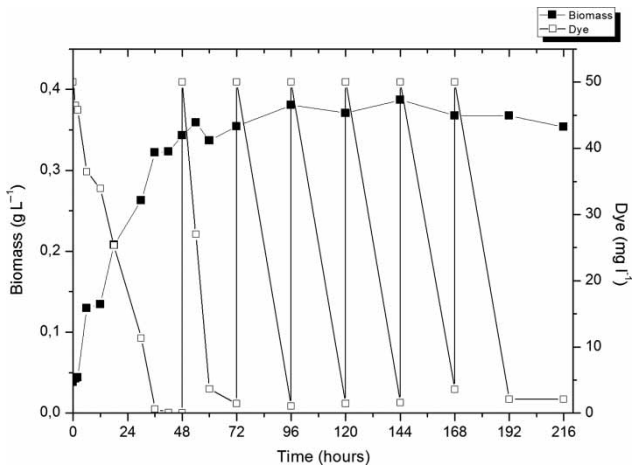

Figure 2 | Decolorization of B15 dye in fed-batch.

It was also observed a total decolorization of B15 dye, performed in only 36 hours in the first run, and substantial cell growth occurred along this first run. However, the cells did not seem to absorb the dye, since the centrifuged biomass showed no characteristics of absorption, remaining creamy white during the entire process.

It was also observed that there were two distinct behaviour patterns onto the fed-batch decolorization process of *P. oleovorans*. The first one was related to the cell growth, with a low decolorization rate (approximately 1.39 mg L^{-1} h^{-1}). The second one was presented along with the reactor feeding, with a raised decolorization rate (approximately 2.08 mg L^{-1} h^{-1}).

Immobilization of *P. oleovorans* on PUF

P. oleovorans was firstly immobilized on PUF cubes of 1 cm^3 and, after the immobilization process, the bacterial decolorization capacity was measured. Figure 3 shows the decolorization pattern of non-immobilized and immobilized cells with recycles.

Along with the decolorization process, and also at the beginning of each decolorization cycle, it was possible to observe some colour absorption by the foam. However, at the end of each run the support regained its original colour. It was also observed that after the first run, the decolorization process time was reduced to 30 hours on the following runs.

Figure 4 shows the scanning electron microscopy (SEM) of polyurethane foam (PUF) containing *P. oleovorans* cells.

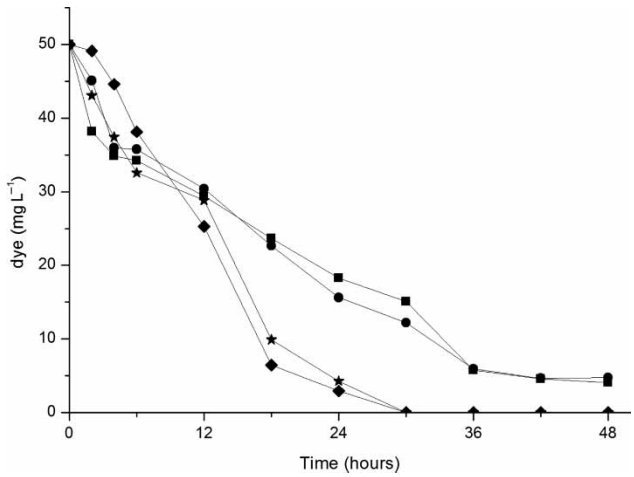

Figure 3 | Decolorization of B15 dye by: ■ – Non-Immobilized cells; and Immobilized cells ● – 1st run; ◆ – 2nd run; and ★ – 3rd run.

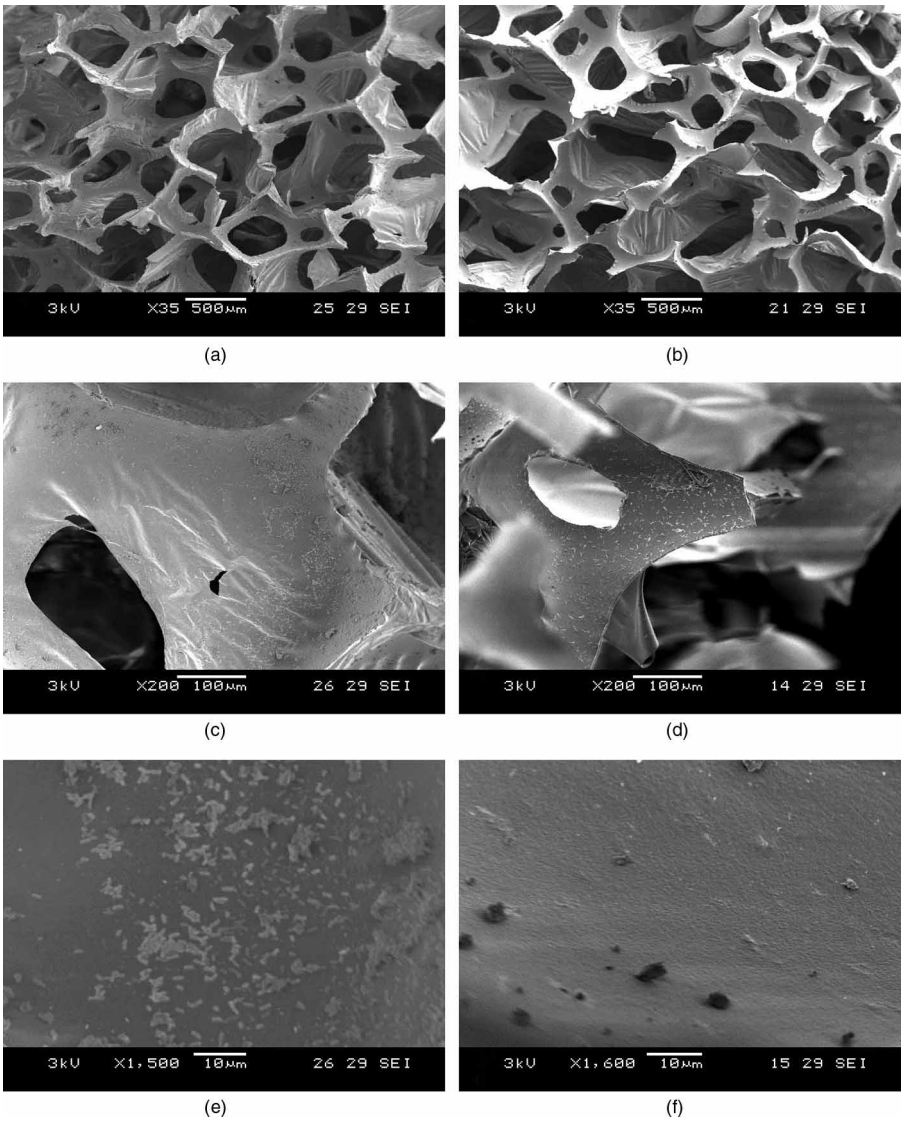

Figure 4 | Scanning electronic microscopy of immobilized cells in polyurethane foam (PUF). (a) PUF cross section after immobilization; (b) PUF cross section before immobilization; (c) PUF surface after immobilization; (d) PUF surface before immobilization; (e) PUF cross section surface containing immobilized with *P. oleovorans*; (f) PUF cross section surface before immobilization with *P. oleovorans*.

PUF was chosen as support due to its high porosity, inert nature, easy access and low cost. The PUF polyhedral structure can be observed in Figures 4(a) and (b), which provide a considerable large surface area to *P. oleovorans* biofilm development (Figure 4(e)).

Reactor decolorization process

Column packing with PUF cubes gave a non-ideal behaviour, in which was observed formation of channelling and stagnant zones within the reactor. Based on that, the reactor was packed with a PUF cylinder, which could fill all its inner space, thus giving it an ideal behaviour. Figure 5 shows the reactor before and after the decolorization process, and Figure 6 shows the decolorization pattern along the process time.

It was observed that in addition to using a lower concentration than the ones used in batch and fed-batch reactors, 25 and 50 mg L^{-1}, respectively, the reactor was able to decolorize a higher mass of dye per cycle. Therefore, the reactor could process 1 litre of effluent and the process time was reduced 6-fold.

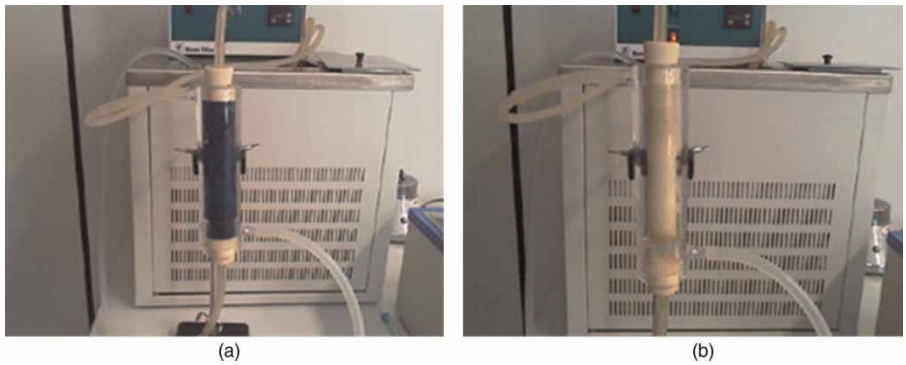

Figure 5 | Anoxic reactor packed with PUF cylinder. (a) Before and (b) after the decolorization.

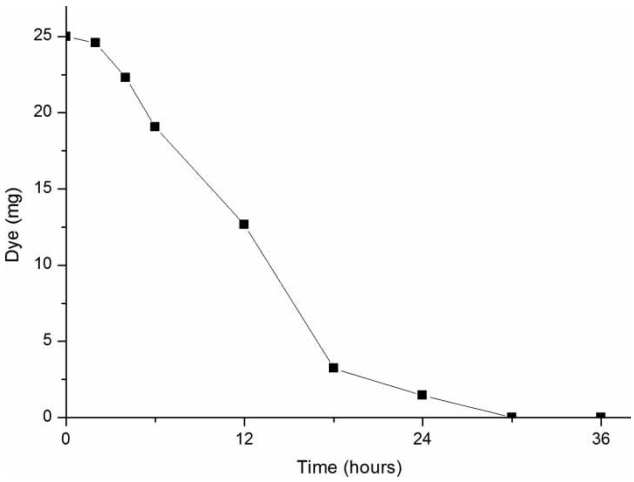

Figure 6 | B15 dye degradation pattern by *P. oleovorans* immobilized in PUF in an anoxic reactor.

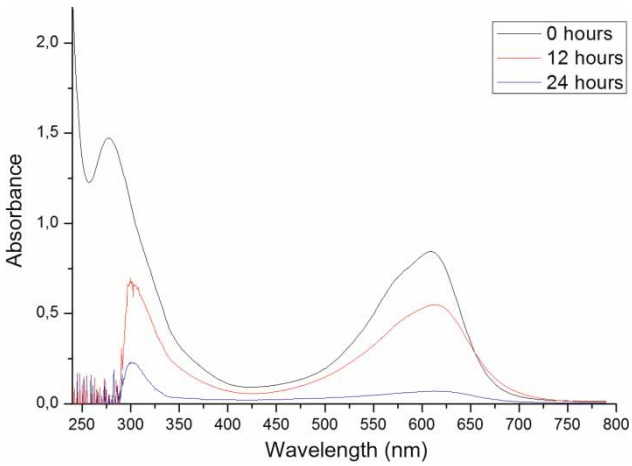

Figure 7 | Synthetic effluent wavescan along the decolorization process in the anoxic reactor.

Figure 7 shows the wavescan in several times along the decolorization of the effluent treated by the anoxic reactor packed with *P. oleovorans* immobilized on PUF. It was observed that apart from the colour reduction in the wavelength between 400 and 790 nm, there was a drastic reduction in the range of 200–350 nm.

DISCUSSION

Azo dye decolorization under anaerobic conditions has been described extensively (Carlieel *et al*. 1996; Chinwetkitvanich *et al*. 2000; Guo *et al*. 2006; Kim *et al*. 2008); however, little is described about total dye degradation. The behaviour present with the fed-batch decolorization suggests that cells were adapting during the first cycle, in which the cell machinery was adapting to consume an alternative and more recalcitrant carbon source. From the second cycle onwards, where all the cell machinery is already ready for the process, it was simpler to the cell to consume the dye as sole carbon source.

Zhang *et al*. (1999) described the use of a fed-batch reactor in a fluidized bed containing white-rot fungus, where the fungus was reused during nine cycles of decolorization. In addition, Chang & Lin (2000) described the decolorization of Reactive Red 22 by a *Pseudomonas luteola* strain in fed-batch, in which the dye was fed in the reactor without the total elimination of the initial dye.

This strategy resulted in a higher decolorization rate by *P. luteola*; however, the dye decolorization should undergo substrate inhibition, leading to an incomplete decolorization

at the final process. Nevertheless, the only advantage presented by the processes described previously was the increase of dye concentration in the effluent. The decolorization performed by *P. oleovorans* showed a significant increase in the colour removal rate, making it possible to achieve total colour removal.

Several researches describe the immobilization of microorganisms on polyurethane foam (PUF), but highlighted the immobilization of bacterial consortium (Khehra *et al.* 2006), semi-solid fungal fermentation (Susla *et al.* 2007) and fungi in submersed cultures (Casieri *et al.* 2008). Khehra *et al.* (2006) described an anoxic–aerobic sequential bioreactor using a bacterial consortium immobilized on PUF, where it was capable of reaching complete decolorization of a synthetic effluent containing 100 mg L^{-1} of Acid Red 88 dye.

Decolorization of much more mass of dye diluted in medium by *P. oleovorans* is possible provided the period of process time is increased (Silveira *et al.* 2009b). Furthermore, the microscopy results diverge from the ones described by Kim *et al.* (2003) and by Khehra *et al.* (2006) on the biofilm formation, probably due to the treatment prior to the microscopy.

According to Sharma *et al.* (2004), a plug flow reactor is capable of decolorizing synthetic effluent contaminated with dyes with a HRT of 23 hours. However, the results obtained showed that, with a decreased HRT of approximately 2 hours, it was possible to decolorize dyes, since the reactor is connected with the recycle.

The recycle, in other words the outlet flow return to the feed inlet, should be done for two reasons: (a) to preserve the media while it was not totally converted into the desired product; and/or (b) to increase the process yield (Missen *et al.* 1999). With the recycle, it was possible to reach a decolorization rate higher than 95%, without the need for a sequential reactor.

Khehra *et al.* (2006) described the use of an anoxic-aerobic sequential bioreactor, in which full decolorization of acid red 88 was achieved, however, it used a bacterial consortium (*Bacillus cerus*, *Pseudomonas putida*, *Pseudomonas fluorescens* and *Stenotrophomonas acidaminiphila*) in the process, making it necessary to use a second reactor to reach full dye degradation and its by-products.

The colour removal in several sequential reactors is basically associated with the anaerobic stage, where the further decolorization in the aerobic stage is normally limited to a few percent (van der Zee & Villaverde 2005). Minke & Rott (2002) described that two-stage decolorization (anaerobic and aerobic) was 70% higher than a single aerobic stage. The results showed that a second aerobic stage is not necessary, since *P. oleovorans* was capable of decolonizing the synthetic effluent in only one stage.

According to Pearce *et al.* (2008), the dye reduction capacity consists basically in the cell's ability to reduce azo bonds (–N = N–) or its intermediary ketohydrazone (–N–NH–). The results suggest that *P. oleovorans* is not only capable of reducing azo bonds, but also of degrading aromatic compounds present in the effluent.

According to Brand & Eglinton (1965), the strong absorbance at 200–300 nm of a solution is consistent with aromatic amines. The formation of aromatic amines resulting from decolorization is a common problem originating from biological treatment of dyes (Ulson de Souza *et al.* 2007). Pearce *et al.* (2008) described the decolorization of pigments by *Shewanella* strain J18 143, in which there was a reduction in its μ_{max} and an increase in the UV spectrum, suggesting the formation of high levels of aromatic amines.

Ulson de Souza *et al.* (2007) suggested that biological treatment should be considered as a pre-treatment, requiring a post-treatment to remove organic compounds (i.e. aromatic amines) remaining in the effluent. This should not be a concern with *P. oleovorans* degradation of industrial dyes, since an almost complete removal of UV spectrum was observed.

CONCLUSION

The ever-increasing legislation restrictions regarding effluent disposal combined with their toxicity, carcinogenicity and mutagenicity makes dye contamination both an environmental problem and a public health problem. The immobilization of *P. oleovorans* along with its use in fed-batch reactors, as well as in an anoxic reactor, is a promising green technology to the decolorization of dye-contaminated effluents, since it was possible to achieve full degradation of

industrial dye in a short period of time. Further work should be performed aimed at the scale-up of this process.

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