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Azo Dye Degradation by *Phanerochaete chrysosporium* in the Medium Enriched with Nitrogen in the Presence of Primary Co-Substrate

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ABSTRACT

This work sought to evaluate the ability of fungi Phanerochaete chryosporium to degrade congo red azo dye in batch reactor, evaluate the influence of glucose and wheat bran as co-substrates on the removal of the dye in the medium and investigate the influence of ammonium chloride, ammonium nitrate and ammonium sulfate as the inorganic nitrogen source for the process. Wheat bran was not effective satisfactorily for the removal of dye and organic matter had no desired effect for the removal of color and organic matter and showed the lowest values of k_2, 0.008 $M^{-1}.d^{-1}$ and 0.0004 $M^{-1}.d^{-1}$, respectively. Glucose presented the best response with the highest final percentage of dye removal (97%) and rate of dye removal (0.017 $M^{-1}.d^{-1}$), without adding an external source of nitrogen.

Key words: Wheat bran, glucose, congo red, fungi, nitrogen

INTRODUCTION

Nowadays, environmental contamination by organic and inorganic constituents has become a major problem. Many compounds from industrial activities are discharged without treatment, especially in water bodies, making the environment unsuitable for habitation due to their toxic and recalcitrant characteristics (Dellamatrice 2005). Textile industries show high pollutant potential and their wastewaters are considered to be the most hazardous effluents to treat (Fu and Viraraghavan 2001). According to Dellamatrice (2005), there is also a great concern about the human contamination caused by the inadequate discharge of textile wastewaters in water bodies, mainly to the food chain as these compounds are bioaccumulated by the living organisms. Many compounds discharged with these effluents are mutagenic or carcinogenic and their effects in the organism are not completely known.

Azo dyes are considered the most toxic compound to the aquatic biota and carcinogenic to the human being (Binupriya et al. 2008) due to their breakdown products that form intermediates products such as aromatic amines (Chen et al. 2009). Thus, it is essential to remove these dyes before their release into natural water streams that can modify the ecosystem and/or cause damage to the population health (Zanoni and Alves 2001). Various physical–chemical and biological technologies have been applied to remove the dyes

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from the wastewaters but each one has its technological and economical limitations. Biological treatment has received wide attention as a potential alternative for textile wastewater treatment due to its low costs compared to the majority of physical–chemical methods (Van Der Zee and Villaverde 2005).
Since 1980, white-rot fungi have been used as an alternative to remove the colour of effluents and degrade xenobiotic compounds. These fungi are capable of producing enzymatic complexes that degrade a great variety of compounds (Rodrigues et al. 2003). The mechanism of recalcitrant compounds degradation by white-rot fungi involves enzymes of phenol oxidases group, such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases. White-rot fungi are the best-known dye-decolourizing microorganisms. These organisms also synthesize a number of enzymes, which contribute to the decolorization process through extracellular or intracellular enzymatic pathways (Kamida et al. 2005).The enzymes produced by white-rot fungi are regulated by the depletion of nutrients, mainly nitrogen that has stronger effect to this process. Nitrogen is an essential component to the cells as it is present in their composition and constituents (Ben Hamman 1997; Bitton 2005). *Phanerochaete chrysosporium* is the most common fungus associated with microbial degradation (Gomma et al. 2008). This fungus has been used, with promising results, in bioremediation processes of textile effluents (Alam et al. 2009), contaminated areas with polycyclic aromatic hydrocarbons (Ding et al. 2008), bleaching Kraft paper effluents (Freitas et al. 2009) and anthracene-contaminated waters (Mohammadia and Nasernejad 2009).
This work sought to evaluate the ability of *P. chryosporium* to degrade congo red azo dye and the effect of co-substrates such as glucose and wheat bran on the removal of this dye and organic matter in batch assays. Also, the effect of the use of sucrose and glucose as co-substrates on the removal of congo red in the presence of different nitrogen sources (ammonium chloride, ammonium sulphate and ammonium nitrate) was evaluated

MATERIAL AND METHODS

Cultivation of *Phanerochaete chrysosporium*

Phanerochaete chrysosporium was cultivated in a growth medium containing ($g.L^{-1}$) yeast extract (2.0), glucose 20.0, K_2HPO_4 1.0, KH_2PO_4 0.6, $MgSO_4.7H_2O$ 0.5 and peptone 2.0 at 30ºC for 5 d. The spores were removed with Tween 80/isotonic saline solution (1:5) and stored in a sterilized glass flask. The inoculum was transferred to the reactors in the concentration of 2 x 10^6 $spores.mL^{-1}$.

Dye

Congo red ($C_{32}H_{22}N_6Na_2O_6S_2$) (Fig. 1), an azoic and direct dye, with a molecular weight of 696,66, colour index no. 22120 and spectrum of absorbance of 500 nm (Binupriya et al. 2008) was used in this study.

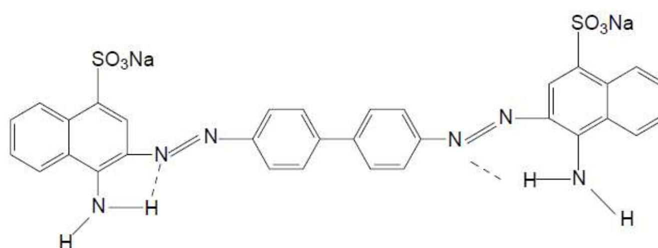

Figure 1 - Chemical structure of Congo red dye.

Influence of the usage of the wheat bran and glucose as co-substrates (Phase I)

Batch studies were carried out in 250 mL Erlenmeyer's flasks containing 150 mL of stock basal medium and 2 x 10^6 $spores.mL^{-1}$ of fungal inoculum. Wheat bran and glucose were added in the concentration of 1% (w/v), and $1g.L^{-1}$ respectively, and tested together and separately. The stock basal medium (SBM) consisted of ($g.L^{-1}$): KH_2PO_4 2.0, $MgSO_4.7H_2O$ 0.5, $CaCl_2.2H_2O$ 0.1 and $(NH_4)_2C_4H_4O_6$ 0.2. The medium was supplemented with 30 $mg.L^{-1}$ of dye and 10 $mL.L^{-1}$ of mineral solution. Tap water was used to prepare the medium. The mineral solution ($g.L^{-1}$) was composed by $MgSO_4.7H_2O$ 3.0, NaCl 1.0, $MnSO_4.H_2O$ 0.5, $FeSO_4.7H_2O$ 0.1,

$ZnSO_4.H_2O$ 0.1, $CoCl_2.6H_2O$ 0.1, $CaCl_2.2H_2O$ 0.082, H_3BO_3 0.01 and $CuSO_4.5H_2O$ 0.01. The pH of the medium was adjusted to pH 5.0 before being sterilized at 121ºC for 15 min.
Reactors were operated under different conditions in duplicate: control reactors (CR) – containing only basal medium; reactors with fungi (FR) – containing stock basal medium and inoculum; reactors with fungi and glucose (FGR) – containing stock basal medium, inoculum and 1 $g.L^{-1}$ of glucose; reactors with fungi and wheat bran (FBR) – containing stock basal medium, inoculum, 1% (w/v) of wheat bran; reactor with fungi, wheat bran and glucose (FBGR) – containing stock basal medium, inoculum, glucose (1 $g.L^{-1}$) and 1% (w/v) of wheat bran. Reactors were agitated on rotary shaker at 30ºC and 150 rpm for 15 d. Aliquots of 2.0 mL were withdrawn after 1, 3, 5, 7, 10, 13 and 15 d to determine the efficiency of dye and organic matter removal.

Influence of the inorganic nitrogen source (Phase II)

Congo red (30 $mg.L^{-1}$) and the same basal medium of the Phase I was used in duplicate in 250 mL Erlenmeyer's flasks, with modified nitrogen source, viz. 0.042 $g.L^{-1}$ ammonia chloride (RFN-I), ammonia sulphate (RFN-II) and ammonia nitrate (RFNIII) with 5 $g.L^{-1}$ of glucose (Phase I) or saccharose (Phase II). RFSN reactors did not receive nitrogen source. Aliquots of 2.0 mL were withdrawn after1, 5, 10, 15, 20, 25 and 30 d.

Determined Variables

Organic matter concentration (COD), pH and dye concentration were determined in the Phase I; dye concentration, pH and manganese peroxidase (MnP) enzyme were determined in the Phase II. COD and pH were determined according to standard APHA procedures (APHA 2005). Dye concentration was measured at the wavelength (λ) of 500 nm by a UV–VIS spectrophotometer (Biospectro - SP 220 - Brazil). A standard curve was obtained for Congo red known concentrations ranging from 0 to 50 mg/L. Before the absorbance measurement, the samples were centrifuged at 3500 rpm for 15 min to separate out the remnants of fungal biomass and the supernatant was analyzed for COD, pH, dye concentration and enzyme assays. All the experiments as well as the controls were run in duplicate.
The manganese peroxidase (MnP) activity was determined using the method described by Aguiar Filho (2008). For this, 50 µL of $MnSO_4$, 50 µL of H_2O_2 in succinate buffer, 100 µL of sodium lactate, 200 µL of bovine albumin and 600 µL of supernatant (sample) were taken in each assay tube. An assay tube was sterilized for 10 min (control) and cooled. The initial time (zero) was determined as the measurement of the absorbance with 100 µL of phenol red. After 10 min, the final time was determined with the measurement of the absorbance after the addition of 40 µL of NaOH to the reaction. The samples and controls were measured against zero cross spectrum at 610 nm. The enzymatic activity was obtained from Equation 1.

$$UI/L = \frac{\Delta abs}{\varepsilon \times R \times t} \times 10^6$$

Where Δabs is the absorbance variation, ε is the molar absorption coefficient (ε_{610nm} = 44600 L/M.cm), R is the quantity of the sample solution (mL) and t is the time of the reaction (min). UI/L is the international unity (µmol/min). Data were analyzed by one-way analysis of variance (ANOVA) with Tukey test to compare results and obtain the statistical parameter. Readings were considered significant when *P* was ≤ 0.05.

RESULTS AND DISCUSSION

Phase I

The reactors with fungi and wheat bran (FBR) and the reactors with fungi (FR) showed maximum efficiencies of dye removal (88 and 77%, respectively) at the 5th day of operation. At the end of the experiment, however, the addition of 1% (w/v) wheat bran as co-substrate did not demonstrate significant differences in the removal efficiencies of the dye by the FBR (76%) and FR (73%) reactors. Significance level of (5.3%) the experimental data was obtained by Tukey test, indicating that FR and FBR reactors presented removal efficiencies statistically similar ($P > 0.05$). This corroborated the fact that the addition of wheat bran as co-substrate did not increase the removal of dye. Dellamatrice et al. (2005) evaluated the removal of colour of industrial effluent by two species of *Pleurotus ostreatus*, using wheat bran (0.05 $g.mL^{-1}$) and sugar cane bagasse (0.12 $g.mL^{-1}$). After 15 days of operation, the authors observed the complete removal of the dye of the industrial effluent. However, *P. chrysosporium* was able to remove 88% of the dye

in a shorter period of time in this study, even with the application of the same co-substrate at the concentration of 1% (w/v).

Figure 2 shows the removal of Congo red dye by control reactors (CR), fungi reactors (FR), fungi and wheat bran reactors (FBR), fungi and glucose (FGR) reactors, fungi, wheat bran and glucose reactors (FBGR).

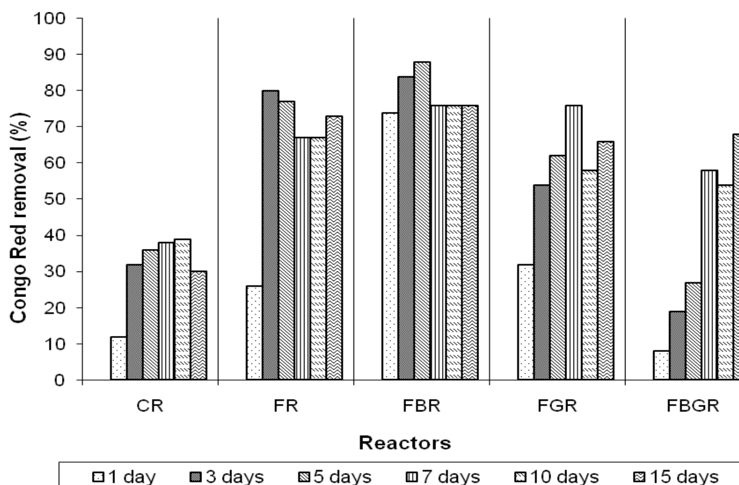

Figure 2 - Performance of the batch reactors in the removal of congo red dye in Phase I.

The removal of the dye by the control reactors (CR) was lower than that observed in the other reactors and equal to 30% at the 15th day of operation. Reactors with fungi and glucose (FGR) reactors achieved maximum removal of Congo red (76%) at the 6th day and 66% at the 15th day. The same behaviour was noted in the reactors containing fungi, wheat bran and glucose (FBGR) with removal of 68%. Although FGR and FBGR presented similar removal efficiencies of Congo red at the 15th of operation, these values were statistically different ($P < 0.05$). Chen et al. (2008) reported 98% removal of Orange G dye in basal medium by *P. sordida* in the simultaneous presence of 0.95 $g.L^{-1}$ of glucose and 0.5 $g.L^{-1}$ of ethanol as co-substrates in a period of 72 h. In the present work, removal of 58% of Congo red dye was obtained in the presence of 1.0 $g.L^{-1}$ of glucose as co-substrate. This removal efficiency was lower than that reported by Chen et al. (2008). This could be due to the fact that Congo red had a more complex molecular structure than Orange G with double azoic linkage between the aromatic rings.

Equation 2 shows the velocity of Congo red dye removal adjusted by the kinetic equation of second order.

$$\frac{1}{C} = \frac{1}{C_0} - k_2 \, x \, t$$

Where C is the dye concentration at time t, Co is the initial dye concentration, k_2 is the velocity constant and Δt is the variation of time. Velocity constant (k_2) and correlation coefficient (r^2) estimated to CR, FR, FBR, FGR and FBGR reactors are tabulated in Table 1.

The values of the velocity constant (k_2) indicated that even with the higher removals of Congo red dye achieved by FR (73%) and FBR (76%) reactors, glucose increased 1.8 times this velocity when compared to the reactors with fungi and wheat bran (FBR). The velocity of Congo red dye removal was 1.6 times higher in the reactors in the absence of co-substrate than that obtained in FBR reactors and similar to that obtained in the FGR reactors. The removal efficiencies achieved by FR and FBR reactors were not different statistically ($P > 0.05$), corroborating the similarity observed between the velocity constants (k_2) of 12 and 14 $L.g^{-1}.d^{-1}$ of these reactors, respectively. The maximum removal of the organic matter in the medium occurred at the last day of operation and was 99, 80, 93, and 87% in FR, FBR, FGR and FBGR, with the exception of CR reactors where it was 25% only (Fig. 3).

Table 1 - Removals of dye in function of the velocity constant (k_2) and correlation coefficient (r^2) to CR, FR, FBR, FGR and FBGR reactors.

Reactor	k_2 (L.g^{-1}.d^{-1})	r^2
CR	1.0	0.843
FR	12.0	0.896
FBR	7.7	0.704
FGR	14	0.956
FBGR	7.0	0.916

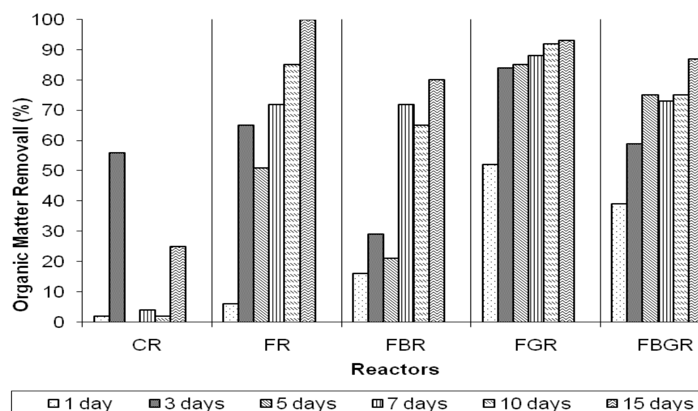

Figure 3 - Performance of the batch reactors in the removal of COD in Phase I.

Guimarães et al. (2005) reported lower COD removal (48%) in a batch rotational contact reactor inoculated with *P. chrysosporium*, containing glucose as co-substrate (5 g.L^{-1}), treating refined sugar industry effluents. No significant variations of pH were observed in the experiments. The variations of pH were of 5.1 to 5.3 (CR), 4.8 to 5.5 (FR), 5.1 to 6.5 (FBR), 5.1 to 6.2 (FGR), 5.1 to 5.6 (FBGR). The slight increase in pH could be related with the consumption of organic acids produced from high degradation of organic matter (Rodrigues et al. 2007).

Phase II

At the end of Phase I, with glucose available to the fungi, removal efficiencies of dye were 97, 83, 76 and 89% were achieved by the reactors containing no nitrogen source, ammonium chloride (FNIR), ammonium sulphate (FNIIR) and ammonium nitrate (FNIIIR), respectively. These results indicated that the addition of a nitrogen source, in the presence of glucose as co-substrate, was not determinant to achieve good removals of dye, as noted by the velocity constant (k_2) in the reactors with fungi and no nitrogen source (FNNR, Phase I) (Table 2).

Figure 4 gives the results of dye removal during the batch experiments using glucose as co-substrate.

With the addition of saccharose as co-substrate, the reactors with no nitrogen source (FNNR), ammonium chloride (FNIR), ammonium sulphate (FNIIR) and ammonium nitrate (FNIIIR) achieved 84, 90, 86 and 93% removal of the dye, respectively. Velocity constant (k_2) and correlation coefficient (r^2) estimated for CR, FNIR, FNIIR, FNIIIR and FNNR reactors in the Phase II are shown in Table 3.

Figure 5 shows the performance of batch reactors on the removal of Congo red dye.

Table 2 - Removals of dye in function of the velocity constant (k_2) and correlation coefficient (r^2) to CR, FNIR, FNIIR, FNIIIR and FNNR reactors – Phase I.

Reactor	k_2 (L. g^{-1}.d^{-1})	r^2
CR	2.0	0.918
FNIR	6.0	0.911
FNIIR	12.0	0.898
FNIIIR	7.0	0.987
FNNR	17.0	0.946

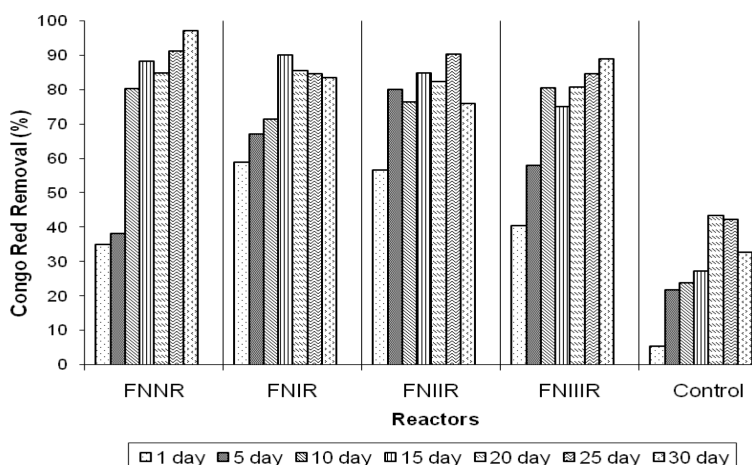

Figure 4 - Performance of the batch reactors with glucose as supplementary carbon source on the removal of congo red dye.

Table 3 - Removals of dye in function of the velocity constant (k_2) and correlation coefficient (r^2) to CR, FNIR, FNIIR, FNIIIR and FNNR reactors – Phase II.

Reactor	k_2 (L. $g^{-1}.d^{-1}$)	r^2
CR	2.0	0.9177
FNIR	7.0	0.9294
FNIIR	3.0	0.8770
FNIIIR	16.0	0.9519
FNNR	3.0	0.9088

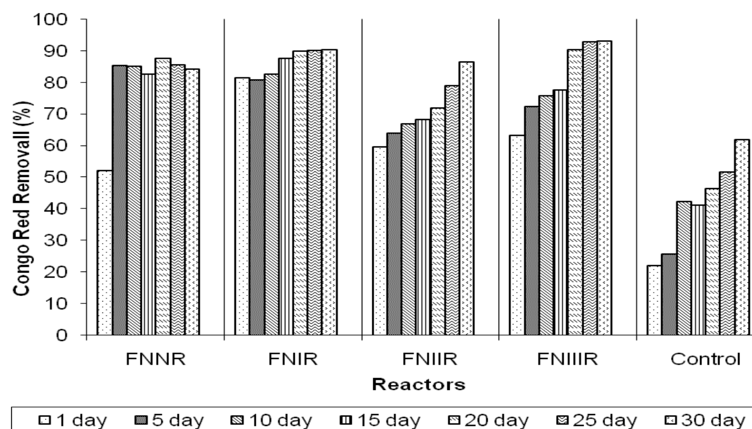

Figure 5 - Performance of the batch reactors with saccharose as a supplementary carbon source on the removal of congo red.

Chen et al. (2008) reported removal of 90 and 97% of Orange G dye using glucose as co-substrate and ammonium sulphate as nitrogen source by *P. sordida* and *Tyromyces lauteus*, respectively. A similar behaviour was noted in this work in the reactors containing ammonium sulphate as nitrogen source (FNIIR) and glucose as co-substrate that achieved velocity constant (k_2) of 12 $L.g^{-1}.d^{-1}$ and maximum removal of dye of 89%.

Comparing the results obtained in Phase I with glucose to saccharose (Phase II), the best response of the reactors to the removal of dye occurred in the reactors containing glucose and with the absence of nitrogen source, followed by the addition of ammonium sulphate (FNIIR). However, when glucose was substituted by saccharose in the Phase II, reactors containing ammonium nitrate as nitrogen source (FNIIIR)

presented the highest efficiency of dye removal (86%). In the reactor without nitrogen source (RFSN), the presence of glucose as co-substrate (Phase I) increased the velocity coefficient (k_2) of dye removal 5.6 times when compared to the velocity coefficient obtained with the addition of saccharose as co-substrate. The consumption of dye in the presence of saccharose (Phase II) the in reactors with ammonia nitrate (FNIIIR) was 2.3 times higher than that with the addition of glucose as co-substrate. Therefore, it was inferred that glucose increased the efficiency of the culture to consume Congo red dye (17 $L.g^{-1}.d^{-1}$). This velocity was drastically reduced to 3 $L.g^{-1}.d^{-1}$ when glucose was substituted by saccharose as co-substrate. In Phase II, the addition of a supplementary nitrogen source was necessary to optimize the removal of Congo red dye by the reactors in the presence of saccharose. Since the reactors containing ammonium chloride (FNIR) did not achieve the best response in any phase, this additional source of nitrogen should not be the ideal for *P. chrysosporium* in the studied conditions.

Shahavali et al. (2000) reported that the presence of a nitrogen source contributed to the colour removal of a textile effluent by *P. chrysosporium* in an air-lift bioreactor containing glucose as co-substrate. However, the application of ammonium chloride as the additional nitrogen source showed minimum effect (60%) in the decolourization process. In the present work, the velocity coefficients (k_2) obtained for the reactors (FNIR) containing ammonium chloride as the supplementary source of nitrogen were low, similar to that observed by Shahavali et al. (2000).

The use of glucose as co-substrate was better for the removal efficiency (97%) and velocity constant (17 $L.g^{-1}.d^{-1}$) of the process, as it resulted the best removal of Congo red dye without any source of nitrogen. In the presence of glucose as co-substrate, pH reduced from 5.0 to 3.8 in FNNR reactors, 5.0 to 3.5 in FNIR and FNIIIR reactors and 5.0 to 4.0 in FNIIR reactors. However, this reduction was more significant in the presence of saccharose, with variations of pH from 5.0 to 2.1 in FNNR, 5.0 to 2.3 in FNIR, 5.0 to 2.2 in FNIIR and 5.0 to 2.5 in FNIIIR. The minor decrease in the pH values in the reactors containing glucose was related to the fact that glucose was used for the synthesis of amino acids in comparison to saccharose that was formed by glucose and fructose. In the synthesis of amino acids, the ammonium ion reacts with an intermediary compound from the metabolization of glucose, the α-ketoglutarate causing the formation of glutamate. Therefore, with the withdrawn of the ammonium ion, the equilibrium equation of ammonia to ammonium ion tends to the formation of ammonium. This fact generates the consumption of ions H^+ of the medium and consequently no decrease in the pH values was observed (Griffin 1994; Motta 2005).

Shahvali et al. (2000) showed the removal of dye by *P. chrysosporium* in an air-lift bioreactor treating textile effluent at pH of 3.0; however, the authors noted decrease in the decolourization at pH higher than 5.0 probably due to the osmotic variations in the medium. In the presence of saccharose, the activity of manganese peroxidase enzyme showed a peak common to the reactors at the last day of operation, probably due to the release of intracellular enzymes from the disruption, or fragmentation of hyphae (Colen 2006).

According to the results, higher enzymatic activity occurred in the reactors containing ammonium nitrate (FNIIIR) in the presence of saccharose, confirming the highest value of velocity constant (k_2) of 0.0105 $\mu mol^{-1}.min$ observed in these reactors at the 5th day of operation. After this, the enzymatic activity reduced during the 10th and 25th days and showed another peak of 0.0613 $\mu mol^{-1}.min$ at the 30th day.

CONCLUSIONS

The results demonstrated the ability of *P. chrysosporium* to remove Congo red dye. The velocity constant (k2) of the dye removal was lower in FBR (8 $L.g^{-1}.d^{-1}$) and FBGR (7 $L.mg^{-1}.d^{-1}$) reactors even with the addition of 1% (m/v) wheat bran. However, FR reactors presented satisfactory removal of dye (77%), with no necessity of co-substrate addition, and removal velocity of 12 $L.g^{-1}.d^{-1}$, similar to 14 $L.g^{-1}.d^{-1}$ obtained in the reactors containing glucose. The highest velocity constant of 17 $L.g^{-1}.d^{-1}$ was obtained in the presence of glucose, with the absence of nitrogen source. The results demonstrated that *P. chrysosporyum* could be used for the treatment of textile effluents, considering the nutritional requirements.

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