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Chemical Engineering Journal 126 (2007) 163–169

Chemical Engineering Journal

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The effect of HRT on the decolourisation of the Grey Lanaset G textile dye by *Trametes versicolor*

Paqui Blánquez [a,*], Glòria Caminal [b], Montserrat Sarrà [a], Teresa Vicent [a]

[a] *Departament d'Enginyeria Química i Unitat d'Enginyeria Bioquímica del CeRBA, Escola Tècnica Superior d'Enginyeria, ETSE, Universitat Autònoma de Barcelona, Campus de Bellaterra 08193, Cerdanyola del Vallès, Barcelona, Spain*

[b] *Unitat de Biocatàlisis Aplicada Asociada al IIQAB (CSIC-UAB), Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain*

Received 12 December 2005; received in revised form 3 August 2006; accepted 12 September 2006

Abstract

The establishment of the operational conditions for the continuous treatment process of the metal complex dye Grey Lanaset G (150 mg l^{-1}), in a fluidised bed bioreactor using air pulses with retained pellets of the white rot fungus *Trametes versicolor* has been carried out. Although the bioreactor operated under non-growth conditions, the fungus activity related to laccase production was maintained. The glucose consumption rate used to maintain the fungus was evaluated in 0.31 ± 0.03 g glucose d^{-1} DCW^{-1}. The effect of the hydraulic retention time (HRT) on the decolourisation yield was also studied. Decolourisation was highly efficient (>80%) for the different HRTs ranging from 18 to 120 h, and the dye removal rates ranged from 6.73 to 1.16 mg l^{-1} h^{-1}. No direct relationship between decolourisation and extracellular enzyme activity was found, and high enzyme activities were not necessary to obtain high decolourisation percentages. The treated effluent fulfils the environmental quality standards in relation to colour, so it could be discharged into a municipal wastewater treatment plant if necessary.

Keywords: Bioreactor; Continuous treatment; Laccase production; White rot fungi

1. Introduction

The ability of ligninolytic fungi to degrade the lignin and a wide range of aromatic compounds is a result of their non-specific extracellular enzyme system [1]. They can oxidise compounds resistant to microbial attacks, such as textile dyes [2]. There are some examples of papers which address treating industrial wastewater with ligninolytic fungi: wastewater from molasses alcoholic fermentation by *Trametes versicolor* [3], *Aspergillus niger* [4] or *Phanerochaete chrysosporium* [5]; paper mill wastewater by *P. chrysosporium* [6], *Aspergillus foetidus* [7], and *T. versicolor* [8,9]; and olive oil mill wastewater by *Phanerochaete flavido-alba* [10]. Different synthetic dye decolourisation, such as azo, anthraquinone, heterocyclic, triphenylmethane, and polymeric triphenilmethane, and partial mineralisation of azo dyes with this fungi have been reported at laboratory scale in batch mode [11–14]. However, before applying it to industry, the bioreactors and culture strategies need to be developed for the long-term activity of the microorganisms in a continuous operation. There are few papers that address continuous or semi-continuous decolourisation by ligninolytic fungi. The systems most commonly used are: stirred tank reactors [15,16], bubble columns [17], fixed beds [18] and fluidised beds [18,19]. In general the worst results are obtained with the continuous packed-bed reactor, probably due to the poor mass transfer caused by the reactor being blocked by excessive mycelial growth. The freely suspended pellets growing in the fluidised bed reactor clearly have much better mass transfer properties [18]. A pulsed packed-bed bioreactor was used to biologically degrade the dyes Orange II and Poly R-478 with *P. chrysosporium* and it showed high stability in long-term operations with a HRT between 24 and 48 h [20–22].

All the configurations mentioned above provide high enzymatic activities, which sooner or later decrease due to the culture ageing and the presence of proteases in the medium [23]. Repeated-batch decolourisation tests using immobilised *P. chrysosporium* cells showed an efficient cycle to cycle decolourisation decrease, as well as biomass ageing [24]. Using a biofilm continuous reactor made it possible to decolourise the Red 533 diazo dye with a HRT between 48 and 144 h [25].

* Corresponding author. Tel.: +34 93 581 2141; fax: +34 93 581 2013.
E-mail address: Paqui.Blanquez@uab.es (P. Blánquez).

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doi:10.1016/j.cej.2006.09.007

Immobilised *Trametes hirsuta* was also used and high degrading efficiency was obtained for different dyes in successive batches [26].

The ability of *T. versicolor* to produce enzymes during growth was exploited for research into the long-term decolourisation capacity of *T. versicolor* in sequencing batch reactors [27]. High, stable degrees of decolourisation of reactive textile dyes were repeatedly achieved without any decrease in activity over time; however, bacterial contamination occurred easily, causing a decrease in decolourisation efficiency.

The aim of the present work is to study the main operational parameters for the continuous treatment of the dye Grey Lanaset G in a pulsed fluidised bioreactor with the fungus *T. versicolor* in pellet form, which was retained in the bioreactor to evaluate the effect of the HRT in the decolourisation process. The dye Grey Lanaset G was chosen because it was the main dye present in the wastewater of a nearby textile industry. The maximum concentration of the dye that could be present in the wastewater was 150 mg l^{-1}, so we used this concentration in the experiments carried out. The results obtained are interesting for future industrial applications, since continuous treatment with an HRT of less than 1 day was successfully achieved.

2. Materials and methods

2.1. Microorganism

Trametes versicolor was obtained from the American Type Culture Collection (ATCC #42530). The fungus was maintained on 2% malt agar slants at 25 °C until use. Subcultures were routinely prepared as required from the mother culture.

2.2. Media and cultures condition

Pellets of *T. versicolor* were obtained as described previously [28].

2.3. Chemicals

Grey Lanaset G, which is a commercial mixture of several metal complex dyes, was complimentarily supplied by Ciba (ref. 080173.5). All other chemicals were reagent grade.

2.4. Equipment and operating conditions

2.4.1. Reactor

A glass fluidised bioreactor with a useful volume of 1500 ml, was used to carry out the decolourisation experiments. The scheme of it and the equipment is shown in Fig. 1. Fluidised conditions were maintained by air pulses generated by an electrovalve [29]. The electrovalve is controlled by a cyclic timer, and the pulsing frequency is defined as the inverse of the sum of opening and shutting times of the electrovalve: $F = 1/(t_0 + t_s)$, where F is the frequency, t_0 the opening time and t_s is the shutting time. In this study t_0 was 1 s, t_s was 5 s and the air flow was 12 l h^{-1}. The bioreactor was furnished with a pH controller in order to maintain pH at 4.5 and the temperature was maintained at 25 °C.

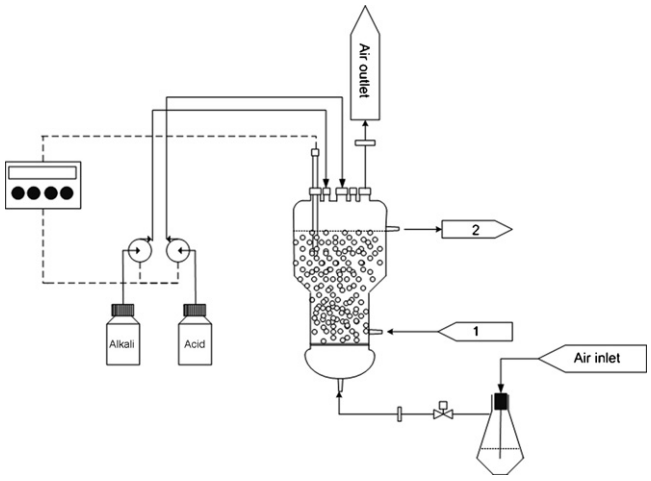

Fig. 1. Scheme of the air pulsed fluidised bioreactor. (1) Inlet and (2) outlet in continuous operation mode.

2.4.2. Reactor medium composition for the starting operation

The batch reactor medium contained per liter: 8 g glucose, 1.9 g NH_4Cl, 11 ml of a supplemented medium [30] and 0.15 g Grey Lanaset G dye. The pH was adjusted to 4.5 with 0.5 M NaOH and the solution was sterilised at 120 °C for 30 min.

2.4.3. Feeding solution

The continuous simulated wastewater consisted of dye (0.15 g l^{-1}), glucose (variable concentration depending on the experiment, g l^{-1}) and supplemented medium (11 ml l^{-1}).

2.4.4. Operating conditions

The start-up of the bioreactor was carried out loading the bioreactor with the batch medium. The culture medium was inoculated with an amount of pellets equivalent to 3.2 g DCW l^{-1}. Once the glucose concentration in the reactor was 2 g l^{-1} approximately the continuous stage was switched on with a hydraulic retention time ranging from 18 to 120 h, depending on the experiment.

The biomass, in pellet form, was retained in the bioreactor throughout the experiment by means of a metal mesh in the outlet, avoiding loss in the effluent.

2.5. Colour determination

Spectrophotometric measurements were carried out at the visible maximum absorbance, 590 nm on a PV 8620 Philips spectrophotometer.

2.6. Glucose determination

Glucose concentrations were measured with an YSI 2000 enzymatic analyzer from Yellow Springs Instruments and Co.

2.7. Enzymatic activity test

Laccase and MnP activity were measured using a modified version [31] of the method for the determination of manganese peroxidase [32]. Where 2,6-dimetoxiphenol (DMP) is oxidised by laccase even in the absence of a cofactor. Conversely, oxidation by manganese peroxidase (MnP) requires the presence of an H_2O_2 as a cofactor and catalytically active Mn^{2+}. One activity unit (AU) was defined as the number of micromoles of DMP oxidised per minute. The DMP extinction coefficient was 10,000 M^{-1} cm^{-1}.

3. Results

3.1. Effect of the glucose feeding rate on the continuous process

Several previous studies have demonstrated the ability of *T. versicolor* to produce laccase under nitrogen limiting conditions. The lack of nitrogen strongly stimulates the ligninolytic enzyme system [33–36]. Moreover, maintaining nitrogen limiting conditions biomass is not growing, avoiding operational problems related to fungal growth on the walls and instrumentation of the bioreactor.

During the continuous process the biomass did not grow due to the lack of nitrogen, however, glucose was fed into it to maintain activity. Experiments were carried out under laccase production conditions, and manganese peroxidase activity was not detected in any experiment. An experiment with unlimited glucose was carried out at a hydraulic residence time of 48 h (run 1). The glucose concentration in the continuous feeding medium was 5 g l^{-1}.

As Fig. 2 shows starting on day 8 the glucose concentration started to increase gradually up to 1 g l^{-1} at the end of the run because the microorganisms were not able to metabolise all the glucose in the system. The glucose profile was decreasing during the first 8 days and then it began to increase. During the continuous process not growing of the biomass was supposed to take place, and in that experiment, as will be shown later, glucose was fed over the glucose consumption rate for the maintenance of the fungus, so it should increase from the moment that the continuous stage was switched on, but it started to increase 4 days later. This behaviour is due to the presence of nutrients in the system from the starting operation reactor medium, until complete depletion of nutrients, the biomass was growing, and the glucose consumption rate was higher than the one calculated for the fungus maintenance.

The presence of glucose in the effluent could be an environmental problem for a dye wastewater treatment, due to the increase in COD in the reactor effluent. The accumulation also implies that a nutrient is wasted and the decolourisation yield decreases slightly. Therefore, the glucose concentration in the effluent must be zero or close to zero.

The glucose specific consumption rate was determined based on the previous results. In general, glucose is consumed for growth and for other energetic or maintenance requirements. The rate of substrate utilisation is described by Eq. (1). If it is assumed that there is no growth, $\mu = 0$, the rate of glucose utilisation is $-r_s = m_s \cdot X$, where m_s is the specific maintenance rate (g d^{-1} g^{-1} DCW) and X is the biomass concentration (g DCW l^{-1}).

$$-r_s = \frac{1}{Y_{X/s}} \cdot X + m_s \cdot X \tag{1}$$

The glucose balance in a continuous system without growth is defined by Eq. (2), where τ is the hydraulic retention time (d), C_e the glucose feeding concentration (g l^{-1}) and C is the outlet glucose concentration (g l^{-1}).

$$\frac{dC}{(1/\tau) \cdot (C_e - C) - (m_s \cdot X)} = dt \tag{2}$$

From the results obtained the specific glucose consumption rate needed to maintain fungus activity was 0.31 ± 0.03 g glucose d^{-1} g^{-1} DCW, corresponding to a glucose consumption rate of 1.5 ± 0.05 g glucose d^{-1}. So, if the previous experiment was longer, the maximum concentration achieved in the stationary state should have been 1.5 g l^{-1}.

Once the glucose consumption rate was determined a second experiment (run 2) was performed. The HRT was maintained at 48 h and the glucose concentration in the continuous feeding medium was 2 g l^{-1}, which corresponds to the glucose consumption rate. The results are shown in Fig. 3. After a 5-day batch

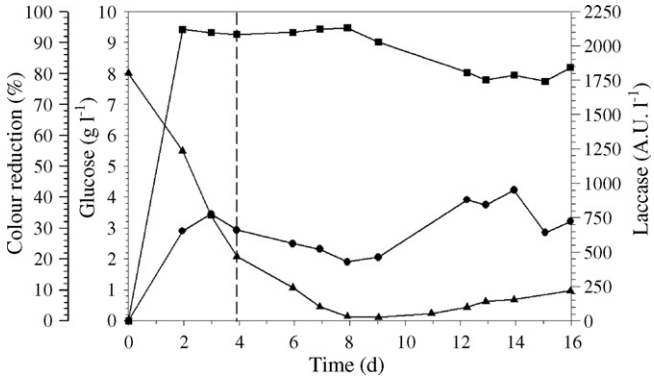

Fig. 2. Time course of glucose concentration (▲), laccase activity (●) and percentage of colour reduction (■) (run 1).

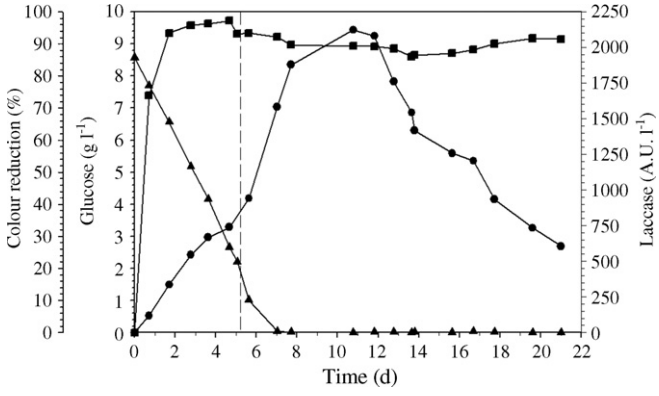

Fig. 3. Time course of glucose concentration (▲), laccase activity (●) and percentage of colour reduction (■) (run 2).

stage, continuous operation was maintained for 16 days, during which the glucose was completely depleted and no accumulation was detected. The system reached decolourisation levels higher than 85%, at the hydraulic stationary state. The maximum enzyme activity, 2123 AU l^{-1}, was reached after 11 days and then the extracellular enzyme activity quickly decreased to 605 AU l^{-1} at the end of the run. However, that enzymatic activity concentration was enough to maintain good decolourisation levels.

In order to check the response of the system to a possible disturbance, such as a deficit in the carbon source feeding, another experiment (run 3) was carried out with a glucose feeding concentration that was 15% lower than the corresponding consumption rate (data not shown). Even though the glucose was limited, the decolourisation percentage was about 80%. The maximum enzyme activity was 2000 AU l^{-1}, obtained after 3 days without glucose in the medium. Activity decreased dramatically to 500 AU l^{-1}, then continued to decrease slowly, however, decolourisation stayed at 80%.

3.2. Effect of the hydraulic retention time

With the aim of analysing the effect of the hydraulic retention time on decolourisation, different experiments were carried out. In all of the experiments the glucose feeding concentration was equivalent to the consumption rate. The results were analysed in the hydraulic stationary state. In the first experiment (run 4) the HRT was increased to 120 h. The volumetric loading rate was 30 mg dye d^{-1} l^{-1}, and the biomass loading rate was 9.4 mg dye d^{-1} g^{-1} DCW. Fig. 4 shows the results obtained in this experiment. Decolourisation remained virtually constant at 90%. The maximum enzyme activity (2028 AU l^{-1}) was reached after 21 days and remained high for a further 10 days. Then, the extracellular enzyme activity decreased through to the end of the run. In the next experiment the HRT was decreased to 24 h (run 5) (data not shown). The volumetric loading rate was 150 mg dye d^{-1} l^{-1} and the biomass loading rate was 46.8 mg dye d^{-1} g^{-1} DCW. The maximum laccase activity (1705 AU l^{-1}) appeared on the fourth treatment day and then the activity quickly decreased to 100 AU l^{-1} at the end of the run. However, the decolourisation values were still higher than

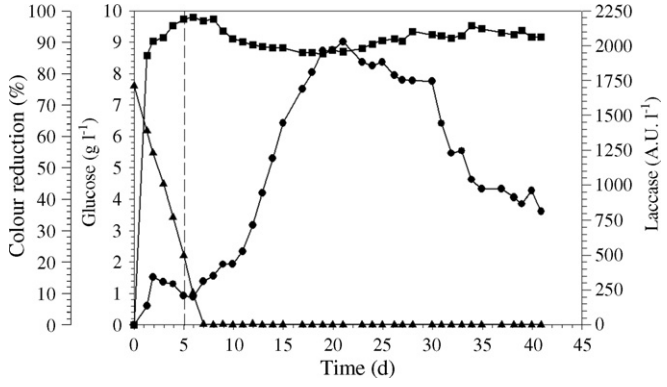

Fig. 4. Time course of glucose concentration (▲), laccase activity (●) and percentage of colour reduction (■) (run 4).

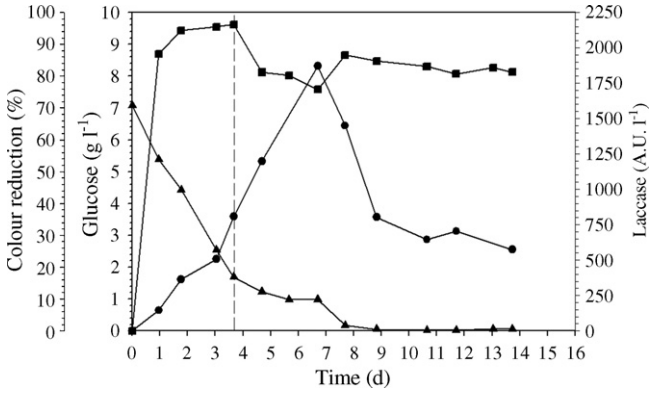

Fig. 5. Time course of glucose concentration (▲), laccase activity (●) and percentage of colour reduction (■) (run 6).

Table 1
Summary of the condition operational and the hydraulic retention time experimental results

	Run			
	4	2	5	6
HRT (h)	120	48	24	18
V (l)	1.5	1.5	1.5	1.5
B_v (mg dye d^{-1} l^{-1})	30	75	150	200
B_x (mg dye d^{-1} g^{-1} DCW)	9.4	23.4	46.8	62.5
Colour reduction (%)[a]	96	90	86	83
Dye removed (mg l^{-1} h^{-1})[a]	1.16	2.93	5.95	6.73
Extracellular laccase production rate (AU d^{-1})[b]	404	1204	1800	2300

[a] Average for the continuous stage.
[b] Average for 6 HRT.

80% even though the enzyme activity was so low. These results encouraged us to decrease the HRT even more to 18 h (run 6). The volumetric loading rate was 200 mg dye d^{-1} l^{-1} and the biomass loading rate was 62.5 mg dye d^{-1} g^{-1} DCW. The experimental results are shown in Fig. 5. Even with the increased load, a colour reduction higher than 80% was obtained. The maximum enzymatic activity (1870 AU l^{-1}) was reached on the seventh day, and then it decreased gradually to 574 AU l^{-1} at the end of the run. Table 1 shows the summary of the operational conditions and the most interesting results of the experiments carried out.

4. Discussion

4.1. Effect of the glucose feeding rate on the continuous process

In many biotechnological processes operating in a continuous mode with immobilised or free cells, the biomass level increases over time and periodic purges are required to avoid operational problems. The strategy followed in the present study clearly separates the growth phase and the dye biodegradation phase. No nitrogen source was added to the feed medium during continuous treatment in order to avoid biomass growth. However, the conditions allowed the fungus to continuously produce the enzyme laccase which is involved in the biodegradation process.

The ligninolytic fungi require an easily accessible carbon source such as glucose or glycerol to produce ligninolytic enzymes [37]. Consequently, to maintain enzymatic production during the continuous process glucose was chosen as a key component. The consumption rate needed to maintain the process was calculated from the results obtained in run 1, in which the glucose was not limited.

We evaluated the effect on the enzymatic production and decolourisation yield from the results obtained in several runs carried out with different glucose feeding rates. When the glucose was not fully depleted (run 1) the enzymatic level reached was lower than 1000 AU l^{-1}, however, the decolourisation percentage only decreased from 90% to 80% during the continuous stage and glucose accumulated in the bioreactor. The proposed hypothesis to explain this behaviour is that glucose is an easily and accessible carbon source, so when there is glucose in the culture medium the microorganisms do not need to degrade the dye, and therefore the enzyme production is lower, but enough to maintain high decolourisation percentages.

On the other hand, if we compare run 2 with run 3 it is observed that after 21 days of treatment, feeding glucose at a consumption rate concentration (run 2), the decolourisation was 86%, and feeding glucose at a concentration that was 15% lower than the consumption rate (run 3), the decolourisation level was 82%. Therefore, a slightly higher colour reduction was obtained by feeding glucose at the consumption rate concentration. However, in both experiments the decolourisation results were highly satisfactory. Dosoretz et al. [38] stated that in glucose limited cultures the fungus produces proteases that reduce the enzyme concentration, which could affect decolourisation. However, in the glucose limited experiment carried out (run 3) a significantly different decrease in the enzymatic activity was not detected compared with the experiment carried out with a glucose consumption feeding rate (run 2). To clearly observe the effect of the proteases on enzyme production it is probably necessary to reduce the glucose concentration in the feed simulated wastewater even more.

The decolourisation percentages obtained over all the continuous stages were almost constant even when the extracellular enzymatic level changed considerably. Therefore, no direct relationship between laccase activity and decolourisation rate exits, as it has been demonstrated in previous works [28]. Though the laccase is involved in the biodegradation process, it is probable that only low enzyme activity is necessary to catalyse the decolourisation of the dye solution.

We observed that there were no negative effects on the decolourisation process when the glucose feeding rate was slightly lower than the glucose consumption rate during a time period of 18 days. However, excessive glucose feeding could have negative effects on the process, such as an increase in COD in the effluent, which as we mentioned previously is an environmental problem.

4.2. Effect of the hydraulic retention time

The HRT is one of the most important parameters of a continuous process. The lower the HRT the more profitable the decolourisation treatment will be, on the condition that the decolourisation level achieved is satisfactory and no operational problems occur. We chose the extracellular enzymatic profile and the decolourisation level as the parameters for evaluating the HRT effect. Comparing the activity profile of run 2 (HRT 48 h) with that obtained in run 4 (HRT 120 h), it is observed that in both experiments the activity profile is the same. The maximum was reached on day 21 for the 120 h HRT, and on day 11 for the 48 h HRT. For the 48 h HRT the flow rate was higher and consequently the enzyme was removed faster. Therefore, the lower the HRT the higher the laccase production, because the maximum extracellular activity level is similar for both experiments. This could be due to the enzymatic system being stimulated by the faster dye feeding conditions. This fact is clearly reflected in Fig. 6 and Table 1. This figure shows the extracellular enzyme production rate for each HRT over a time period equivalent to 6 HRT. It is shown that when the HRT decreases the enzymatic activity production is strongly stimulated. For instance, when the HRT was 120 h the maximum enzyme production rate was 618 AU d^{-1}, while for an 18 h HRT the maximum enzyme production rate was 3740 AU d^{-1}. After 6 HRTs, operating at an 18 h HRT, the total amount of extracellular laccase produced was 10,347 AU, that is, a mean production of 2300 AU d^{-1}. Operating at a 120 h HRT the total amount of lacase produced in 30 days (6 HRTs) was 12,125 AU, so the mean production was 404 AU d^{-1}.

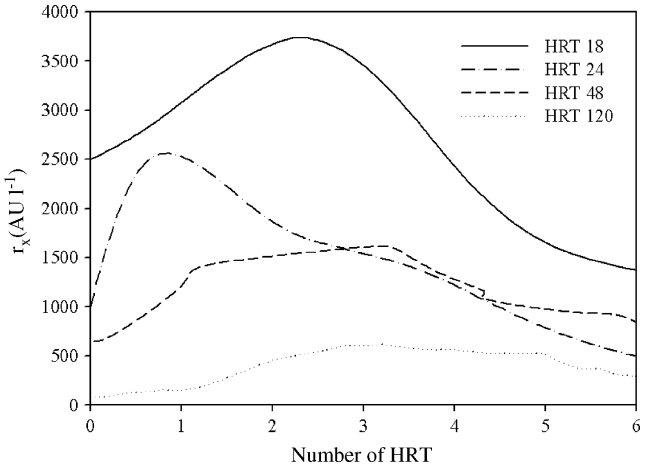

Fig. 6. Extracellular enzyme production rate for each HRT over a time period equivalent to 6 HRT.

Therefore, the lower the HRT the higher the dye volumetric loading rate and the higher the enzyme production. These results suggest that it could be possible to reduce the biomass concentration in the bioreactor, because high enzyme productions are not necessary to obtain good colour reduction yields.

Obviously, the percentage of decolourisation is higher the higher the residence time, however, the decolourisation level obtained with a 48 h HRT was only 13.5% points lower than that obtained with a 120 h HRT. This fact indicates the robustness of the system, since small changes in the residence time do not imply significant changes in the colour reduction.

It is probably not necessary to obtain higher colour reduction percentages if the intension is to reuse the treated wastewater in

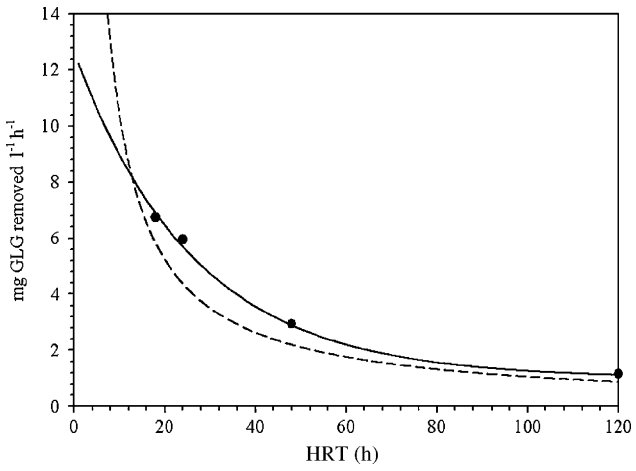

Fig. 7. Relationship between the theoretical required (- - -) and the experimental (—) dye removal rate and the hydraulic retention time.

a stage of the textile plant or to discharge it into a wastewater treatment plant. Therefore, these results show that it is not necessary to operate at an HRT higher than 48 h to obtain very good results. When the HRT was reduced to 24 and 18 h, the colour reduction levels remained over 80% in both cases (Table 1). Colour reduction can be due to the intracellular laccase activity and in previous works the role of the intracellular laccase in the decolourisation process has been demonstrated [28]. Fig. 7 shows the dye removal rate in relation to the HRT. The experimental results (●) are fitted to a decreasing exponential equation (solid line). It can be observed that the lower the residence time, the higher the dye removal rate. Even for low retention times, the decolourisation percentage is high (higher than 80%), so that the amount of dye removed is much higher when the HRT is lower, even though overall decolourisation is higher when operating at higher residence times.

It is possible to compare these experimental data with the necessary theoretical dye removal rates, taking into account that the quality standards established for the effluent need to be fulfilled in order for the effluent to be discharged into a wastewater treatment plant. The limit dye concentration in the reactor effluent is defined so that the solution resulting from the effluent being diluted 30 times must be colourless. Therefore, if the concentration of the dye in the inlet solution is 150 mg l^{-1}, 105 mg l^{-1} must be removed. Fig. 7 shows (in a dashed line) the required theoretical decolourisation rate in relation to the HRT and the experimental curve obtained. The two curves intersect at 13 h HRT, so the minimum HRT at which the system can operate is 13 h, obtaining a 70% decolourisation percentage in relation to the initial solution of 150 mg l^{-1} Grey Lanaset G with an inoculum of 3.2 g DCW l^{-1}.

5. Conclusions

The system is suitable for continuous operation without biomass growth, although the microbial activity is maintained by limiting the nitrogen source and feeding glucose as an accessible carbon source.

Continuous treatment has been carried out at different HRT, 18, 24, 48 and 120 h, achieving decolourisation levels that are higher than 80%. Therefore, the lower the HRT the higher the dye removal rates, ranging from 1.16 mg dye removed l^{-1} h^{-1} with a 120 h HRT to 6.73 mg dye removed l^{-1} h^{-1} with a 18 h HRT.

In working conditions only laccase activity was detected. The enzymatic system is stimulated by the dye volumetric loading rate; therefore, reducing the biomass concentration in the bioreactor when operating at a low HRT could be possible, because to obtain good colour reduction yields high enzyme activity levels are not necessary. The assayed operational conditions show *T. versicolor*'s potential for the continuous production of the enzyme laccase in the presence of an organic pollutant like the textile dye Grey Lanaset G.

Finally, this work demonstrates that continuous operation in a fungal fluidised bed bioreactor permits concentrated dye solutions to be decolourised with an HRT lower than 1 day.

Acknowledgement

This work was funded by the Spanish Commission of Science and Technology (Project PPQ2000-0645-C02-01).

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