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ORIGINAL PAPER

Application of the white rot fungus *Phanerochaete chrysosporium* in biotreatment of bagasse effluent

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Abstract Biotreatment of bagasse effluent using *Phanerochaete chrysosporium* (white rot fungus) is investigated. This study confirmed that lignin is the major pollutant component in this effluent followed by different carbohydrates. The treatment conditions must be very proper, especially in terms of biomass culture to achieve a successful treatment. The best conditions of temperature, biomass concentration, pH and duration for biotreatment of this effluent were 35°C, 552 mg l^{-1}, 6 and 5 to 9 days, respectively. Under these conditions, a 9 days long treatment reduced by 98.7% the original biochemical oxygen demand (of 2,780 mg l^{-1}) and by 98.5% the dissolved chemical oxygen demand (initial 4,200 mg l^{-1}). Moreover, fungal treatment reduced total dissolved solids from 3,950 to 575 mg l^{-1} and color from 560 mg l^{-1} PtCo to 111 mg l^{-1} PtCo.

Keywords *Phanerochaete chrysosporium* · Bagasse effluent · BOD_5 · COD · TDS · Color

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Introduction

Paper industry in fiber deficient countries, to be able to develop national capacity and fulfill the needs, ought to depend on non-wood fiber resources. In Iran, active sugarcane plantations are presently cultivated in southern areas on large industrial scales, producing almost three million tons of wet bagasse (50% moisture) annually, a suitable raw material for paper production. Actually, industrial experience shows that under appropriate bagasse pulping, approximately 400 thousand tons of bleached pulp per year can be produced, corresponding to a bagasse/pulp ratio of 7:1 (www.sugarcane.ir).

Bagasse pulping offers many advantages but has to face the limitation of collecting and storage within a short period of time and utilization during the rest of the year. Therefore, in order to fulfill this requirement, wet storage system has been developed. This system consumes huge volume of water and generates a highly polluted effluent, so-called storage effluent, characterized by a large variety of impurities leading to a very difficult treatment (Sharari et al. 2010). Moreover, the bagasse preparation before pulping implies several washing steps leading to a so-called washing effluent. These two effluents of similar composition are mixed together in a wastewater collection tank giving the "bagasse effluent".

Besides, the world ever increasing population and the progressive adoption of an industrial based lifestyle has inevitably led to an increased anthropogenic impact on our biosphere. Therefore, awareness of environmental problems and potential hazards caused by industrial effluents has prompted many countries to limit the discharge of polluting effluents in receiving waters (Merwe 2002; Anjaneyulu et al. 2005; Asamudo et al. 2005).

Till today, small capacities and the limitation on return on investment as well as cost ineffectiveness had forced non wood pulp mills to escape environmental measures and consequently such mills are now polluting the environment. Recent environmental concerns at global and national levels push these mills to observe pollution regulation and implement appropriate treatment procedures. This obligation involves the development of new alternatives and cost effective treatment processes compared to conventional methods. This is the final aim of this study.

Actually, traditional methods for the treatment of pollutants (sedimentation, flotation…) may not provide adequate treatment in the case of bagasse effluent. Moreover, such treatment processes are often expensive, considering the volume of wastes released during the industrial production process. Consequently, the past two decades have seen tremendous upsurge in research activities for cost effective and environmentally sound alternatives to the conventional methods (Asamudo et al. 2005). Among all the technologies that have been investigated, biotreatment has emerged as the most appropriate and desirable approach for treatment of many pollutants in effluents (Meysami 2001; Shim and Kawamoto 2002; Asamudo et al. 2005).

Biotreatment employs living systems especially microorganisms to catalyze the degradation of pollutants without disruption of the environment (Bumpus et al. 1985; Bumpus and Aust 1986). Enhanced biotreatment technologies increase biodegradation rates by supplying nutrients, electron acceptors, or other factors that are rate limiting (Csuros and Csuros 1999; Emtiazi 2000; Meysami 2001; Sabzali et al. 2005).

Among these microorganisms, white rot fungi are able to degrade and detoxify xenobiotic compounds through the production of extracellular lignin-degrading enzymes (for review, see Haritash and Kaushik 2009; Kasai et al. 2010). Fungi have known a growing interest for the biotreatment (removal or destruction) of effluent pollutants such as heavy metals, inorganic nutrients and organic compounds (Freitas et al. 2009). Fungal systems in addition to be able to decompose substances, appear to be most appropriate for treatment of colored effluents and in the world of fungi, *Phanerochaete chrysosporium* has emerged as a model system in textile, polycyclic aromatic hydrocarbon (PAH) and pulp and paper mill effluent treatment (Lacina et al. 2003; Wang et al. 2009; Sedighi et al. 2009; Enayatzamir et al. 2010). *P. chrysosporium* is a basidiomycetous fungus able to degrade complex compounds such as starch, cellulose, pectin, lignin, hemicelluloses, etc. (Asamudo et al. 2005) which gives many advantages for the treatment of bagasse effluent. Actually, biodegradation of the lignin contained in pulp effluent is not possible by classical techniques (e.g. activated sludge treatment), so many efforts have been made to investigate the use of fungi to degrade lignin in the pulping and bleaching process effluents (Merwe 2002).

This study points out the main results obtained during the laboratory scale experiments on the development of biotreatment using *Phanerochaete chrysosporium* as a cost effective alternative process for pollutant degradation of effluent coming from bagasse storage and washing.

Materials and methods

Bagasse effluent

Bagasse effluent used in this study was collected from the wastewater collection tank that received both bagasse storage effluent and bagasse washing effluent,

at Pars pulp and paper mill in IRAN and was transferred to Grenoble INP-Pagora, Laboratoire de Génie des Procédés Papetiers (LGP2). It was stored in small closed containers at low temperature (−18°C) to prevent natural degradation until it was used (Colombo et al. 2006).

This middle size pulp mill resorts to about 320,000 tons/year bagasse and generates almost 112 $m^3\ h^{-1}$ effluent during period of sugarcane harvesting and 198 $m^3\ h^{-1}$ for the remaining of the year.

Fungal strain, growth

Strain and growth

Pure culture of *P. chrysosporium* strain 1198, was obtained from the culture collection of the Laboratoire d'Ecologie Alpine (CMPG: Collection Mycology Pharmacy Grenoble). Cultures of the strain were transferred to sterile Petri plate of malt extract agar (MEA) media (15 g malt extract, 15 g agar, and 0.1–0.2 chloramphenicol per liter of distilled water) and were incubated at 30°C for 10 days. To produce sufficient mycelium, some Erlenmeyer flasks containing 50 ml sterile broth media were inoculated by 0.5 cm^2 agar plugs. The composition of this broth media was per liter of NH_4Cl 0.1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, KH_2PO_4 2 g, K_2HPO_4 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.05 g, $CaCl_2{\cdot}2H_2O$ 0.1 g, veratryl alcohol 0.4 mM, casein peptone yeast extract 5 g, malt extract 10 g, thiamine hydrochloride 0.01 g. The culture was grown in static flask at 30°C for 5 days until the mycelium formed a mat on surface of the liquid. The mycelial mat was collected, washed with distilled water twice, homogenized in a Warring Blender for approximately 15 s (Molskness et al. 1986), and then was used for the effluent treatment.

Biomass concentration measurement

For the preparation of biomass, fixed volume of suspension containing biomass was centrifuged at 4,000 rpm for 20 min. The pellet was removed, washed, filtered through a pre-dried, pre-weighed glass micro filter. The filter was dried to a constant weight and the dry weight of the biomass was determined as milligram per liter (Hela et al. 2002).

Fungal treatment condition

The study was performed in batch conditions, i.e. in Erlenmeyer flasks of 150 ml that were dispatched on an orbital plate shaker with a rotating speed of 180 rpm to ensure a good mixing. So in each treatment experiment, 100 ml of bagasse effluent (see characteristics in section "Results and discussion") were used and, for providing nutrient for fungus to start biotreatment at early stages, 1% (w/w) of above mentioned broth medium was added to promote the fungal biomass growth, as recommended by many authors (Driessel and Christov 2001; Kanaly and Hur 2005; Cho et al. 2006). The experiments were then realized with different initial biomass concentrations (0, 184, 368 and 552 mg l^{-1}), and under three temperature (25, 30 and 35°C) that was controlled by putting the Erlenmeyer flasks into a bacteriological incubator. Moreover, the pH influence was studied thanks to two sets of experiments at pH 5 and 6. Effluent pH was adjusted with very small additions of hydrochloric acid or soda 1 M solutions at the beginning of the biotreatment. Besides, to estimate reproducibility, each experiment was replicated three times and the values presented in the text or on graphs correspond to the mean. Relative errors have also been calculated and are directly given in the results part to argue the discussion.

Analytical procedures

Chemical oxygen demand measurement

Chemical Oxygen Demand (COD) was determined according to the closed reflux colorimetric method: 2 ml of effluent were oxidized with chemical reactants for 2 h at 148°C (CR/2200-WTW), cooled to room temperature and then the COD was determined at 620 nm using Hach DR/2500 spectrophotometer. Total COD was obtained from 2 ml of raw effluent, while, for dissolved COD, suspended solids contained into bagasse effluent were first removed (method in paragraph related to TDS), and the analysis done on 2 ml of the filtrate. Except for bagasse effluent characterization, COD values presented in the text and figures are related to dissolved COD and were indicated as COD to simplify the reading. The experimental relative error of this method is estimated around 5%.

Color determination

Samples for color determination were filtered through a 0.45 μm Millipore membrane filter after the pH was adjusted to 7.6 with either 1.0 N HCl or NaOH. The color was detected at 465 nm using Hach DR/2500 spectrophotometer according APHA (American Public Health Association) Platinum-Cobalt standard with a range of 0–500 color units (PCU). One color unit equals 1 mg l^{-1} platinum as chloroplatinate ion (Hongve and Akesson 1996). The precision of this measure is about 10%, according to the Hach protocol.

Biochemical oxygen demand determination

Biochemical Oxygen Demand after 5 days of incubation (BOD_5) was done with the OxiTop® measuring system based on pressure measurement via electronic pressure sensors. In each measurement, 20% seed and 80% in ratio of 4 to 1 blank and filtrated effluent were used. BOD_5 was calculated using the following formula:

$$B_E = \frac{(B_D \cdot F) - (0.2 \cdot B_S + 0.64 \cdot B_B)}{0.16}$$

where, B_D is the Digits BOD_5, F is the Volume correction factor (cf. OxiTop® user manual), B_S is the BOD_5 of Seed, B_E is the Effluent BOD_5, and B_B is the BOD_5 of Blank.

The reproducibility of the measure was controlled in parallel and the precision set at ±15%.

Total dissolved solids determination

Total Dissolved Solids (TDS) is measured after filtration of the effluent through a 1.5 μm glass microfibre filter. A certain volume of the filtrat was evaporated to dryness at 105 ± 2°C and then TDS was calculated through mass balance (Clesceri et al. 1998). Experimental error linked to the experimental protocol is 10%.

Carbohydrate determination

High performance anion exchange chromatography coupled with pulsed amperometry, a simple and non-time consuming method, was used to detect and quantify the sugars. A Dionex DX 500 high performance liquid chromatograph with pulse amperometric detection (Dionex Corporation, Sunnyvale, CA) was used for carbohydrate analysis. The Dionex was equipped with a CarboPac PA-10 column (Dionex Corporation) and the flow rate of eluent (98% milliQ H_2O, 2% NaOH 200 mM) was set at 1 ml min^{-1}. The cycle of analysis lasts for 45 min and includes a phase of detection of peaks (25 min) and a phase for washing of the column (20 min). The system was operated at 30°C. After dilution, samples were filtered through 0.22 μm filter and were injected, without further treatment. To measure oligo and polymer saccharides in bagasse effluent, samples (20 ml) were first treated with 1 ml concentrated sulfuric acid in a boiling water bath for 2 h. Samples were then cooled on ice, neutralized with 1 M NaOH solution and filtered through Whatman paper filter. Fucose solution, an internal standard, was added.

Lignin determination

Standard methods were used for lignin determination: TAPPI T 222 om-88 for acid-insoluble lignin (also known as "Klason lignin" and defined as constituent insoluble in 72% sulfuric acid) and TAPPI Useful Methods (1991) for acid-soluble lignin determined by UV spectrophotometry on a filtrate obtained after isolation of the acid-insoluble lignin. The sum of the acid-insoluble and acid-soluble lignin should represent the total lignin content in the bagasse effluent.

Results and discussion

Bagasse effluent characteristics

The average pollution load of the studied bagasse effluent has been determined as 5,860 mg l^{-1} total COD, 4,200 mg l^{-1} dissolved COD, 2,780 mg l^{-1} BOD_5, 3,950 mg l^{-1} TDS, 560 mg l^{-1} PtCo color and pH between 5.1 and 5.3. Furthermore, the bagasse effluent is mainly composed of mono- and oligosaccharides as well as lignin that is classically observed for such effluents (Table 1).

This effluent is highly concentrated in terms of BOD_5 and COD and presents a BOD_5/dissolved COD ratio over 66%, meaning that biological techniques must be efficient to remove the pollutants. Moreover, biological processes do not only transfer the

Table 1 Time evolution of component concentration (mg l^{-1}) in bagasse effluent during fungal treatment ($T = 35°C$, pH 6 and biomass concentration = 552 mg l^{-1})

Component	Treatment period (days)				
	0	3	5	7	9
Lignin	995	749	302	40	8
Glucose	341	164	8.5	5.5	2.9
Arabinose	101	94.5	4.5	4.3	3.3
Galactose	75.2	73	9.3	5.3	4.8
Xylose	78.9	20.3	2.8	2.8	1.7
Mannose	20.8	19.1	3	1.9	1.8

pollution, from water to sludge, they lead to a real consumption of half the pollution (express through COD or BOD_5) thanks to the biological growth activity. The high lignin concentration in this effluent, that represents around 62% of the main components, has motivated the choice of the fungus technique instead of a classical bacterial one. Actually, *Phanerochaete chrysosporium* is well known for its capacity to degrade lignin thanks to specific enzymes (Asamudo et al. 2005).

Biological oxygen demand and chemical oxygen demand

Biomass concentration and temperature influence

The influence of treatment duration, temperature and biomass concentration on the performance of white rot fungi to reduce the content of BOD_5 and COD is shown in Fig. 1a–d respectively. For these experiments the pH was set at 6. Since mesophilic fungi have optimum temperature activity between 20 and 40°C, this temperature range was applied to obtain optimum biotreatment of bagasse effluent (Meysami 2001). During the early stages of treatment, the influence of both temperature and biomass concentration is not significant, but as treatment proceeds, a decrease of COD and BOD_5 is observed whatever the experimental conditions. The most efficient treatment in term of kinetics operates from a biomass concentration corresponding to 368 mg l^{-1} and a temperature of 35°C. After 5 days of fungal treatment, the measured concentrations were 177 ± 17% mg BOD_5 l^{-1} and 265 ± 22% mg COD l^{-1} corresponding to a removal efficiency of 93.6 and 93.7% respectively. After 9 days, the concentrations fall bellow 100 ± 15% mg l^{-1} leading to removal efficiencies over 97% and corresponding to acceptable level satisfying the environmental protection regulation. Besides, during the fungal treatment, both lignin and carbohydrate concentrations decreased meaning that COD and BOD_5 removal results from the degradation and the assimilation of all the components by white rot fungi (Table 1). The runs at 35°C and with a higher biomass concentration of 552 mg l^{-1} give best results after 9 days of biotreatment with efficiencies over 98.5% both for BOD_5 and COD removal. Consequently these conditions were considered as best conditions in our batch experiments on the base of lowest final COD and BOD_5 values obtained and also of the results on TDS removal presented below.

pH influence

The pH of the water used in bagasse pulping process is usually adjusted between 4.5 and 5.5 (because of the dissociation of acetyl group from hemicelluloses, acetic acid is formed which reduces the pH) to help preventing bagasse deterioration during storage. Consequently, the pH of bagasse effluent is in the same region and the samples pH was measured at 5.1–5.3. Therefore, after withholding for temperature and biomass concentration values of 35°C and 552 mg l^{-1} under pH 6, biotreatment performances of white rot fungi was investigated at pH 5 (Fig. 2a and b). After 5 days of treatment the COD concentration drops to 395 ± 10% mg l^{-1} at pH 5 and 576 ± 12% at pH 6. After 9 days these concentration are 95 ± 8% and 64 ± 7% mg COD l^{-1} respectively. Since the difference between these results is not very important if the precision is taken into account (BOD_5 results are also equivalent for the two pH values, Fig. 2a), it can be concluded that the treatment can be performed on both pH (5 and 6) with similar efficiency. Even if previous investigations indicate that any changes in pH would influence the culture medium and consequently the fungi growth rate (Zokayi 1996), our results show the applicability of the proposed biotreatment on the existing effluent without any need for pH adjustment. If the pH of effluent occasionally drops below 5, it is possible to increase the pH above 5 by adding lime which is available in any pulp mill using soda process.

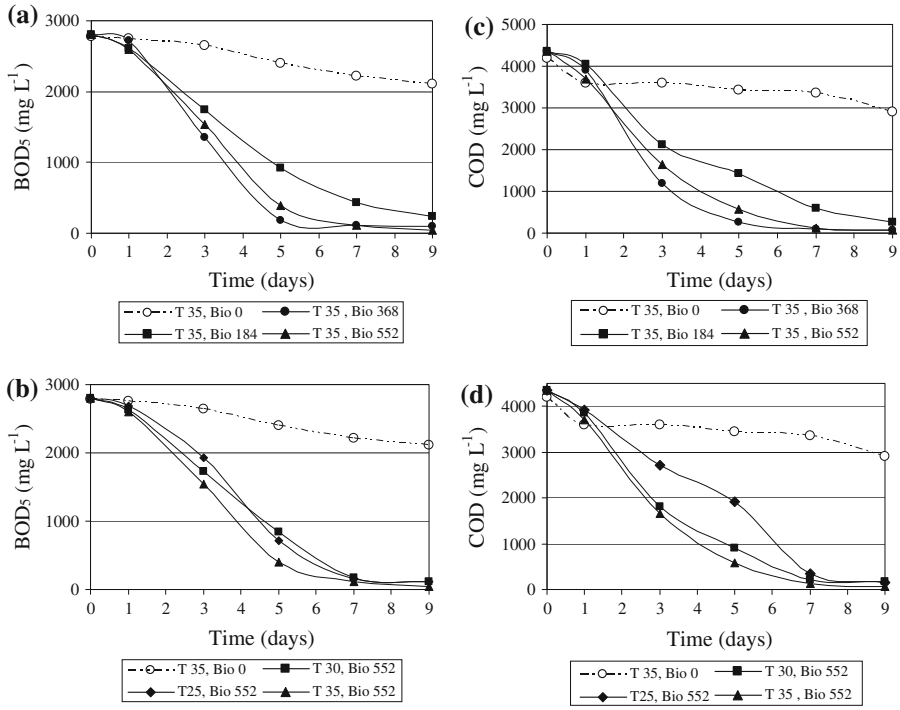

Fig. 1 The influence of biomass concentration and temperature on BOD_5 and COD removal (pH 6)

Total dissolved solids removal

The capability of white rot fungi treatment to remove dissolved solids was also investigated in order to determine the best treatment conditions. As for COD and BOD_5, temperature, biomass concentration and pH were studied. The results show that there was an effective reduction of TDS of bagasse effluent (Fig. 3). It was observed that reduction of TDS in the early periods between 1 and 3 days was fast and then a consistent and continuous decrease followed. Moreover, both temperature and biomass concentration showed great influence on the TDS removal efficiency, with the best conditions for 35°C and 552 mg l^{-1} of biomass (Fig. 3a and b). TDS concentration drops from about 3,950 mg l^{-1} to 575 ± 10% mg l^{-1} which gives a 85.4% efficiency after 9 days at pH 6.

The influence of effluent pH on the performance of biotreatment by fungi was also observed (Fig. 4a). TDS concentration at pH 5 after 9 days is slightly higher than that at pH 6 with a final concentration at 950 ± 10% mg TDS l^{-1}. Otherwise this difference only appears after 8 days of treatment. This suggests that the fungal treatment, once more, can be

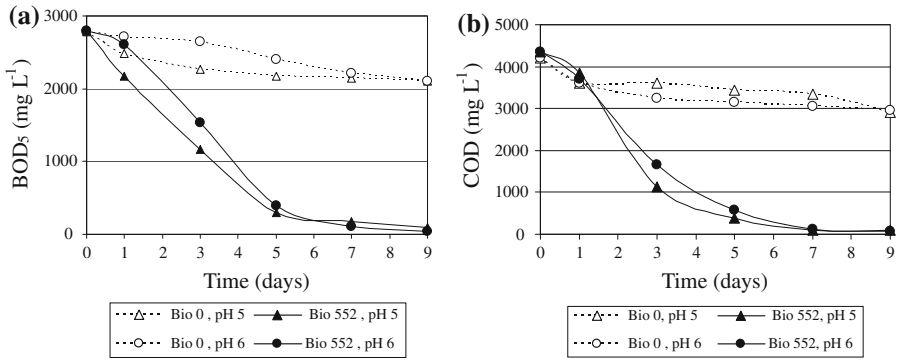

Fig. 2 The influence of effluent pH on BOD_5 (**a**) and COD (**b**) reduction (T = 35°C and biomass concentration = 552 mg l^{-1})

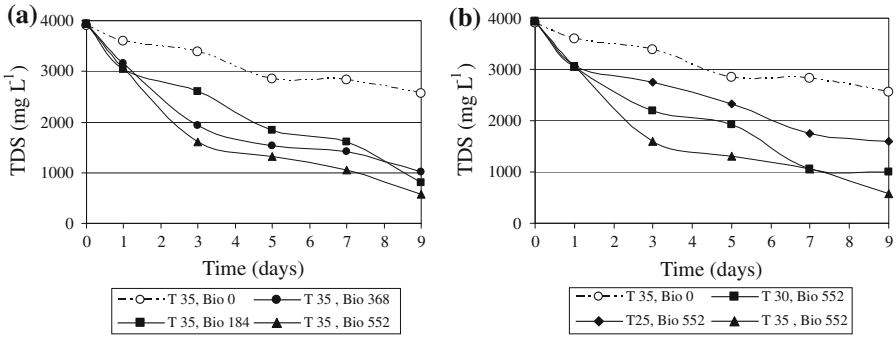

Fig. 3 The influence of biomass concentration (**a**) and temperature (**b**) on TDS reduction of bagasse effluent (pH 6)

performed at both pH 5 or 6, for similar results in the major part of TDS removal.

Color removal

Effluent color influences the first judgment about the pollution load made by a common person even if, for scientists, the real toxicity of the effluent should be the main concern. Consequently, color removal should be of prime interest to any treatment, and this parameter was studied in the experiments. The results (Fig. 5a and b) show that *P. chrysosporium* was able to reduce the color of bagasse effluent from 560 mg l^{-1} PtCo to final value of 111 ± 10 mg l^{-1} PtCo (80.2% removal efficiency) after 9 days of treatment and under 35°C and 552 mg biomass l^{-1}. However, the color evolution during 9 days of treatment is interesting: a fast color drop was observed after 1 day and then a color increase appeared, reaching its maximum after 3 days, before decreasing again. This increase depends on temperature and on biomass concentration: if it is not very pronounced at 25, 30 and 35°C with 552 mg biomass l^{-1}, it is significant for both 184 and 368 mg biomass l^{-1} at 35°C. This phenomenon can be explained by the melanoidin pigment production from interaction of carbohydrate and amine compounds upon heating (Dsouza et al. 2005; Mane et al. 2006; Pant and Adholey 2007). Consequently, increasing the temperature during the biotreatment is a factor for increasing the color of effluent at early stage of biotreatment. In our experimental conditions, this phenomenon seems to be attenuated by the use of a large amount of biomass (552 mg l^{-1}), which explain the better results for these conditions at 35°C. Moreover, all along the treatment, two processes of biosorption and biodegradation of colored segments can occur: (i) In the early stage of fungal treatment, the chromophor groups start to biosorb on mycelium (Driessel and Christov 2001) and, gradually, these compounds will be released from the surface and the degradation process will start with production of smaller molecules. Under mild treatment conditions such as 184 mg biomass l^{-1} (Fig. 5a), system is not strong enough to remove the generated fractions; (ii) As the treatment proceeds, these chromophors will then be decomposed and the color of the effluent reduced. In the case of untreated effluent, during the first 3 days, there is not any chromophor generation and, after this period, effluent color increases. This is probably due to fermentation of carbohydrates in the first stage, followed by the decomposition of lignin which leads to color increase.

The color removal seems to be as efficient under pH 5 or 6 (Fig. 4b). Even if the influence of pH after 3 and 5 days is somehow different, after 7 or 9 days of treatment, the curves approach each other. This difference in the middle of treatment should be explained by the melanoidin pigments present in this effluent. Actually, these pigments are sensitive to pH variations and the increase of pH leads to an increase in the effluent color due to a higher solubilization of melanoidin (Dsouza et al. 2005; Mane et al. 2006; Pant and Adholey 2007). The results obtained for the untreated effluent (Fig. 4b) seem to confirm this hypothesis: color is higher at pH 6 than pH 5.

Lignin and carbohydrate deterioration

Table 1 presents the evolution of lignin and carbohydrate concentrations in bagasse effluent during the fungal treatment. Measurements indicate that the treatment reduced both lignin and carbohydrates. Degradation of hemicelluloses and polysaccharides is the main process in the first phase metabolism of

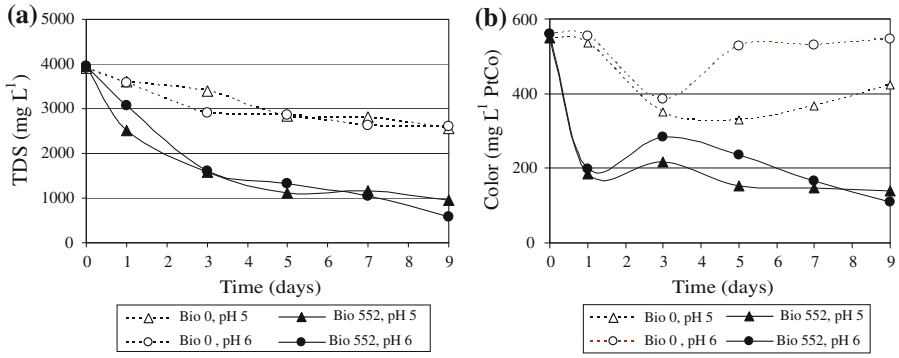

Fig. 4 The influence of pH on TDS reduction (**a**) and on color removal (**b**) of bagasse effluent ($T = 35°C$ and biomass concentration $= 552$ mg l^{-1})

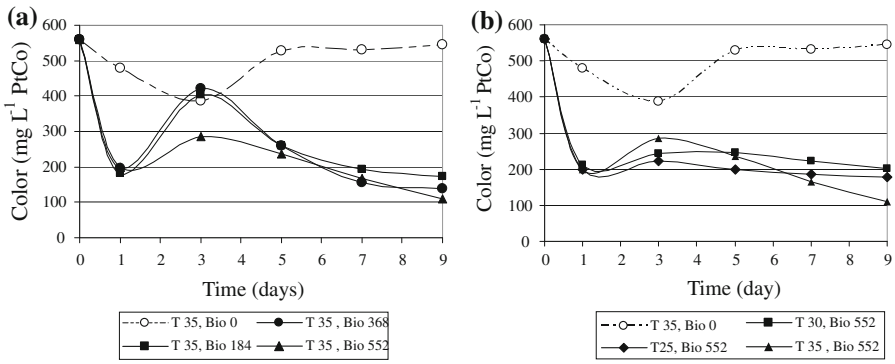

Fig. 5 The influence of biomass concentration (**a**) and temperature (**b**) on color removal of bagasse effluent (pH 6)

white rot fungi. These compounds are decomposed by biodegradation to simple sugars and acetic acid (Abdel-Satera and El-Said 2001; Merwe 2002; Bocchini et al. 2005) which are then directly assimilated by the fungus. During the initial 3 days of treatment, only lignin, glucose and xylose are degraded in great proportions, respectively 24.7, 52 and 74% removal efficiency. After this period, sharp decrease was observed for all the carbohydrates: after 5 days the efficiency of degradation is higher than 85% and reaches more than 92% at the end of treatment. For lignin, removal efficiency is only 70% after 5 days. However, a sharp reduction of lignin concentration occurs between the 5th and 7th days of biotreatment leading to a 96% removal efficiency in accordance with the fact that the biodegradation of this compound starts in the second phase of fungus metabolism process (El-Gammal et al. 1998; Kluczek-Turpeinen et al. 2007).

Since the amounts of carbohydrates were less important than the amounts of lignin and they were deteriorated much more easily than lignin, these components decrease at early stages of treatment and then decomposition of lignin will follow (Table 1). In fact, white rot fungi are able to use carbohydrates as the required substrate metabolism, which reduces the need for addition nutrients. This observation lead to conclude that in industrial conditions, the fungi could be used without external nutrient supply, the bagasse effluent being rich enough in sugar rapidly degradable.

Conclusion

Bagasse becomes a non-wood resource in pulp and paper industry, especially when wooden fibers are lacking. The specificity of bagasse pulping is the need to provide a wet system to prevent bagasse from deterioration during storage. The developed and implemented system requires large quantity of fresh water and generates considerable volume of highly polluted effluent. Considering the very good BOD_5/COD ratio and the high lignin content in bagasse

effluents, a new biological treatment was studied based on the use of *Phanerochaete chrysosporium*, a white rot fungus. Laboratory tests were performed in small closed bioreactors in order to estimate the relevance of the fungal treatment. The results lead to set the best experimental conditions at 552 mg biomass l^{-1} and 35°C for a 9 day-treatment to obtain the higher treatment efficiency: 98.7, 98.5, 85.4 and 80% respectively for BOD_5, COD, TDS and color.

These promising results have been reached in laboratory tests considering growth medium optimization (addition of co-substrate, nutrients, mediator's molecules, and physical parameters optimization) and a good handling of biomasses. It also appeared that the amount of rapidly biodegradable carbohydrates contained in the bagasse effluent, and easily assimilated by the fungi as a carbon sources, offers hope not using this nutrient addition in an industrial treatment process. The next step, continuous process implementation, is still under progress, in particular to confirm the operating conditions. Once more, this work shows the advantages there are to introduce a mycoreactor in effluent treatment lines (Lacina et al. 2003): the enzymatic richness of the fungal biomass permit to be more efficient in specific compound degradation and assimilation than with bacterial biomass.

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