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Phenol and color removal in hydrous ethanol vinasse in an air-pulsed bioreactor using *Trametes versicolor*

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Abstract

Vinasse is a problem in worldwide and one that is predicted to increase over the years to come. Total phenol and color removal from this type of vinasse was evaluated using an air-pulsed bioreactor inoculated with *Trametes versicolor*. Batch operation of the bioreactor removed 71% of total phenol, 18% of color and 40% of chemical oxygen demand (COD). Maximum laccase activity achieved was 428 U/L. The air-pulsed bioreactor was subsequently operated in continuous mode for a period of 25 days, removing 80% of total phenol, 17% of color and 60% of COD. Laccase activity ranged from 956 to 1630 U/L. The results indicate that continuous operation of an air-pulsed bioreactor under the conditions proposed in this study favored biodegradation of vinasse.

Keywords: Vinasse, air pulsed bioreactor, *Trametes versicolor*, total phenol, color.

Introduction

A traditional ethanol plant generates between 9 and 15 liters of vinasse per liter of ethanol. Vinasse has a low pH (3.5 – 5) and presents significant coloration, the latter mainly due to the presence of phenolics compounds from the feedstock and melanoidins from Maillard reaction of sugars with proteins (España-Gamboa et al. 2011; Rajasundari and Murugesan 2011). COD is found within the range of 50 – 150 g/L and the total phenol content is between 8,000 – 10,000 mg/L (Jiménez et al. 2006). Parnaudeau et al. (2008) stated that vinasse has been used as a fertilizer in crop irrigation due to the presence of nutrients like potassium. Nevertheless, applying it to sugarcane crops has been observed to negatively affect crop growth without reducing fertilizer requirements (Quiroz-Guerrero 2010). The literature suggests that the negative effects on crops through irrigation are caused by compounds such as melanoidins, phenols and polyphenols (Parnaudeau et al. 2008).

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To date, a wide range of microorganisms have been studied in terms of their extracellular ligninolytic enzyme system. This system essentially consists of peroxidases and/or laccases capable of degrading complex compounds such as melanoidins and phenols that cannot be removed by other microorganisms (Chairattanamanokorn et al. 2005; Ferreira et al. 2010; Potentini and Rodríguez-Malaver 2006; Seyis and Subasioglu 2009; Strong 2010). The majority of these studies were performed in Erlenmeyer flasks and little is known about vinasse degradation using an aerobic reactor.

The white-rot fungus *Trametes versicolor* has been reported to be an excellent laccase producer and is used in the degradation of a wide variety of pollutants, such as textile dyes, phenols, chlorophenols and polycyclic aromatic hydrocarbons (Ryan et al. 2007). *Trametes versicolor* has been found to efficiently degrade textile dyes when treating them in an air-pulsed bioreactor. Furthermore, a strategy for partial biomass renovation has previously been established during a continuous decolorization process using this type of reactor (Blánquez et al. 2006; Blánquez et al. 2008). In this study, we therefore sought to evaluate the capacity of *Trametes versicolor* to degrade total phenol and color in an air-pulsed bioreactor operated in batch and continuous mode in order to treat hydrous ethanol vinasse.

In the present paper we report results of experiments which were designed to provide information on the photoautotrophic growth phase dependent hydrogen production in GT strain under the dark anaerobic nitrate-free condition. The hydrogen production kinetics is related to endogenous glucose degradation, cell death, lactate production and acetate production.

Materials and Methods

Microorganism

Trametes versicolor was obtained from the American Type Culture Collection (ATCC #42530). The fungus was stored on malt extract agar plates at 25ºC until use. A mycelial suspension of *Trametes versicolor* was obtained by inoculating

four 1 cm2 plugs from the growing zone of fungi on malt agar (2%) into a 500 mL Erlenmeyer flask containing 150 mL of malt extract medium (2%). Flasks were placed in an orbital shaker (135 rpm, r = 25mm) at 25°C. After 4-5 days, a thick mycelial mass was formed, which was ground with an X10/20 (Ystral GmbH) homogenizer. The resulting mycelial suspension was stored in sterilized saline solution (0.85% NaCl) at 4°C. This suspension was used to obtain pellets by inoculating 1 mL of the suspension into 250 mL of malt extract medium (2%) (adjusted to pH 4.5) in a 1 L Erlenmeyer flask. The flask was incubated in an orbital shaker (135 rpm, r = 25 mm) at 25°C for 5 - 6 days. The pellets had a diameter of approximately 3 mm and were stored in sterilized saline solution (0.85% NaCl) at 4°C, where they remained active for up to 2 months without losing their morphology.

Sugarcane molasses vinasse

The vinasse was collected from "La Gloria" sugar refinery, located in the municipality of Úrsulo de Galván, Veracruz, Mexico. This sugar refinery produces approximately 1 million liters of vinasse per day and its physicochemical characteristics are shown in Table 1. The vinasse was diluted at a 1:10 ratio with distilled water, given that crops are currently irrigated in the country at this concentration (Quiroz-Guerrero 2010). To prevent foam formation in the air-pulsed bioreactor, 4 drops of antifoam (Braun Biotech DF7960) were added to each liter of diluted vinasse. The diluted vinasse was sterilized in an autoclave at 121ºC for 20 min and stored at 5ºC for subsequent use.

Equipment and operating conditions

Reactor: An air-pulsed bioreactor with a working volume of 500 mL was used for the removal of phenol and color from hydrous ethanol vinasse (See Fig 1). Biomass fluidization and homogenization conditions of the liquid-biomass phase were maintained by means of air pulses generated by an electrovalve with a frequency of 0.20 s-1 (Blánquez et al. 2008).

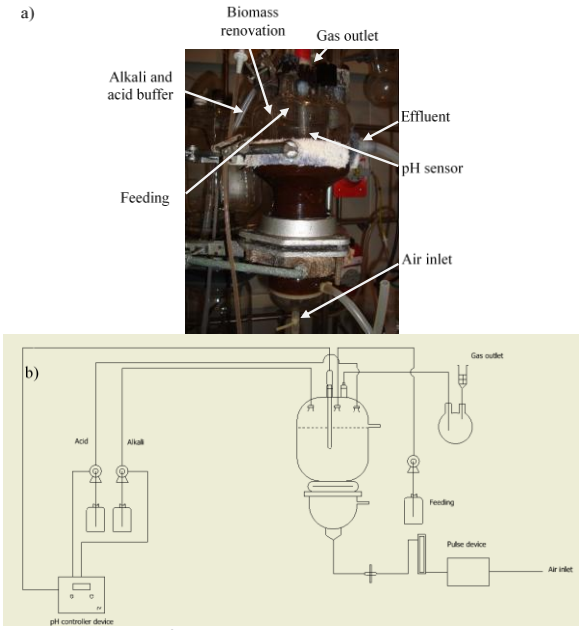

Figure 1: Scheme of the air-pulsed bioreactor. a) Side view; b) Schematic of laboratory setup (Font et al. 2003).

Table 1: Hydrous ethanol vinasse characterization

Parameter	Value[a]
pH	4.39 ± 0.006
Total phenol*	10834 ± 1476
COD	110065 ± 11486
SO_4^{2-}	5300 ± 1416
S^-	241 ± 114
N_{Total}	1720 ± 217
$N\text{-}NH_3$	68 ± 9
N_{org}	1652 ± 219
PO_4^{3-}	415 ± 67

[a] All values except pH are expressed in mg/L
* Expressed in gallic acid

Operating conditions: Two experiments were carried out: batch and continuous. The system was initially operated in batch mode for 7 days, during which time the pH was not controlled. The system was subsequently operated in continuous mode for 25 days and the air-pulsed bioreactor was equipped with an automatic controller (InPro 325X probe, M300 controller, Mettler Toledo, Urdof, Switzerland) to maintain the pH at 4.5. Hydraulic retention time (HRT) was established based on the result obtained in the batch experiment and partial biomass renovation was carried out every 1/3 of the cellular retention time (CRT) according the following methodology. Firstly, 1/3 of the bioreactor content was removed. The liquid medium was then separated from the biomass with a metal strainer. Finally, the same amount of fresh biomass as had been removed previously was resuspended in the separated bioreactor liquid medium and introduced into the bioreactor (Blánquez et al. 2006).

In both experiments, the air-pulsed bioreactor was operated at a temperature of 25ºC and inoculated with a quantity of *Trametes versicolor* pellets equivalent to 2.42 g of dry mass per liter. The biomass, in pellet form, was retained in the air-pulsed bioreactor throughout the experiment with no loss in the effluent.

Analytical methods

The parameters described below were measured on days 1, 5 and 7 when the bioreactor was operated in batch mode. These parameters were measured on days 4, 6, 8, 11, 13, 15, 16, 19, 21 and 25 when the bioreactor was operated in continuous mode.

Determination of total phenol: This was estimated with the Folin–Ciocalteu reagent. 20 µL of sample and 1.58 mL of distilled water were placed in an amber-colored flask. 300 µL of sodium carbonate solution (20% weight/volume) and 100 µL of the Folin–Ciocalteu reagent were then added in rapid succession, mixed, and left to react for 60 minutes in darkness at room temperature. After the reaction time, absorbance at a wavelength of 765 nm was measured with a Varian Cary 3 UV-vis spectrophotometer. Gallic acid was used as the standard for plotting the calibration curve.

COD determination: The Hach colorimetric method was used (Hach Company DR-890), which is analogous to the Standard Methods procedure (APHA 2005).

Color determination: Samples were centrifuged at 18,660 g for 3 minutes. They were then diluted at a 1:10 ratio with distilled water and measured with a Varian Cary 3 UV-vis

spectrophotometer at a wavelength of 475 nm (Ferreira et al. 2010). Decolorization was calculated according to the following formula:

$$Decolorization\ (\%) = \frac{initial\ absorbance - final\ absorbance}{initial\ absorbance}\ x\ 100 \quad (1)$$

Enzyme activity: Laccase activity was measured using a modified version of the method for the determination of manganese peroxidase (Kaal et al. 1993). The reaction mixture consisted of 200 µL of 250 mM sodium malonate at pH 4.5, 50 µL of 20 mM 2,6-dimetoxiphenol (DMP) and 600 µL of sample. DMP is oxidized by laccase even in the absence of cofactor. Changes in the absorbance at 468 nm were monitored for 2 min with a Varian Cary 3 UV-vis spectrophotometer at 30ºC. One activity unit (U) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient was 24.8 $mM^{-1}\ cm^{-1}$.

Results and Discussion

Batch operation of the air-pulsed bioreactor

One of the principal characteristics of an air-pulsed bioreactor is the use of oxygen saturation in the liquid medium, achieving mixing and fluidization of the *Trametes versicolor* pellets by means of air flow (Blánquez et al. 2008). As can be observed in Figure 2, greatest laccase activity was recorded on day 5 of the batch experiment with a value of 428 U/L, and this decreased on day 7. The pH increased from 4.5 to 6.9, and it is clear that the metabolism of *Trametes versicolor* tends to neutralize the medium over time under these operating conditions. Furthermore, *Trametes versicolor* demonstrated the ability to remove phenols, achieving 55% on the first day after inoculation, followed by a linear increase up to 71% on day 7. Similar behavior was observed for COD removal. On the first day, 21% was removed, and a maximum value of 40% was reached on day 5, which then remained constant until day 7. With respect to color variation, a 7% increase was observed on day 1. However, color removal peaked at 18% on day 5 and remained constant until day 7.

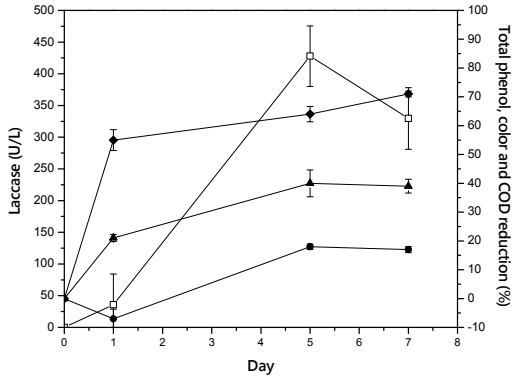

Figure 2: Laccase activity (□), percentage of phenol removal (◆), percentage of COD removal (▲), and percentage of color removal (●) during biodegradation of hydrous ethanol vinasse in a batch operated air-pulsed bioreactor. Error bars represent one standard error.

In general, *Trametes versicolor* was capable of removing phenolic compounds present in hydrous ethanol vinasse, but without producing significant color removal. Seyis and Subasioglu (2009) reported that an acidic pH favors decolorization of vinasse compared to a neutral or alkaline pH, based on their observation that vinasse from molasses inoculated with *Fusarium* species presented an increase in pH without registering decolorization. Conversely, they found that *Trichoderma* species maintained the pH at a value of 4.5 and recorded color removal of between 40% and 55%. Decolorization efficiency can be affected by pH in a variety of ways. As with all enzymes, laccase has an optimum pH value, which is located between 4.2 and 4.5. An increase or decrease in this value could cause inactivation of the enzyme (Koroleva et al. 2002). In wastewaters that possess high levels of phenol compounds, an increase in pH has been reported to increase coloration due to the conversion of these phenols to other compounds known as quinones, which are produced by abstraction of the hydrogen cation from the OH group of the phenol (Strong 2010). Quinone intermediates can spontaneously react with each other to form colored oligomers, depending on substrate and environmental conditions (Majeau et al. 2010). Additionally, pH can affect biosorption potentials of vinasse on the cell walls of microorganisms, given that vinasse contains complex polymeric organic compounds with different aromatic rings and functional groups, and therefore a wide range of ionization potentials at different pH (Kaushik and Thakur 2013). It has also been reported that melanoidins, one of the main compounds that provide vinasse with its color, may precipitate and be removed more easily in an acid medium (Rajasundari and Murugesan 2011). In this study, *Trametes versicolor* was observed to increase the pH inside the air-pulsed bioreactor until it reached a value of 6.9 on day 7, suggesting that this increase may be one of the reasons why we did not observe greater color removal. The rise in pH could have affected biosorption of compounds present in the vinasse on the cell wall of *Trametes versicolor*.

Strong (2010) treated concentrated Amarula vinasse with *Trametes pubescens*, and reported COD and total phenol removal of 70% and 88% respectively 14 days after inoculation, with a final pH of 4.89. Our treatment of hydrous ethanol vinasse with *Trametes versicolor* recorded lower COD removal (40%) and total phenol removal (71%) compared to the results obtained by Strong (2010). This can be attributed to the chemical complexity of hydrous ethanol vinasse, given that it contains 2,041 mg/L of total phenol at a dilution of 10%, which is greater than the 866 mg/L present in the concentrated Amarula vinasse. Increasing the concentration of phenolic compounds has been reported to inhibit the growth rate of *Trametes versicolor*, which is affected at gallic acid concentrations of greater than 10 mM (FitzGibbon et al. 1998).

With regard to color, Strong (2010) found an increase in the first two days of treatment of the vinasse. This was attributed to the production of compounds with greater color than the original ones, which were subsequently either degraded or used during *Trametes pubescens* respiration or growth. The same behavior was observed during the treatment of vinasse from sugarcane molasses at room temperature using *Phanerochaete chrysosporium*. The color increased during the first 8 days of treatment, and removal did not become apparent until day 16, with the maximum value (45%) obtained on day 24. On that same day, COD and phenol removal were 47% and 54% respectively (Potentini and Rodríguez-Malaver 2006). *Trametes versicolor* appears to be a good option for treating hydrous ethanol vinasse to degrade COD and phenol under the conditions reported in this study, in light of the fact that it presents good removal efficiencies for these two parameters over a shorter period of time.

Jiménez et al. (2006) showed that pretreatment of beet molasses vinasse with *Penicillium decumbens*, which removed 67% of total phenols and 71% of COD, improved its

characteristics for subsequent anaerobic digestion. They observed an 80% increase in methane production and a 70% reduction in hydraulic retention time (HRT) at the same organic loading rate (OLR). This was attributed to the removal of complex compounds (including phenols and melanoidins) that inhibited methanogen metabolism. For sequential aerobic-anaerobic vinasse treatment, it is desirable for the anaerobic treatment to remove as much of the organic load as possible in order to produce the greatest quantity of methane. As such, the aerobic treatment should remove as many phenols as possible without eliminating much organic matter. During the batch phase, *Trametes versicolor* recorded high phenol removal (71%), whilst removing just 40% of the COD. This supports the possibility of performing an aerobic-anaerobic sequence for the treatment of hydrous ethanol vinasse from sugarcane molasses using *Trametes versicolor* in the aerobic phase, and subsequently evaluating energy recovery in the form of methane through anaerobic treatment.

Given that greatest laccase production was obtained on day 5 and that color, phenol and COD removal then stabilized up until day 7 in the batch experiment, a HRT of 5 days was chosen for operating the reactor in continuous mode.

Continuous operation of the air-pulsed bioreactor

After successful operation of the air pulsed bioreactor in batch mode, continuous mode was performed. During this experimental stage, a biomass renovation strategy was applied as described by Blánquez et al. (2006), whereby a third of the reactor content is removed every 5 days. The biomass is then separated from the liquid medium and replaced with a third of the initially inoculated biomass, equivalent to 0.78 g/L dry mass. However, excessive *Trametes versicolor* growth was observed on day four of bioreactor operation, causing pellet rupture and preventing adequate fluidization. As a result, 50% of the content of the air-pulsed bioreactor was removed and the biomass present in this volume was replaced with 0.78 g/L of fresh biomass (dry mass). The following renovations were performed on day 10 and 15, as scheduled in accordance with the procedure described by Blánquez et al. (2006). This excessive growth was probably caused by the high levels of organic matter and nitrogen present in the vinasse. Ryan et al. (2007) reported that *Trametes versicolor* grew fastest and achieved the highest biomass accumulation in cultures with the highest concentration of readily available glucose.

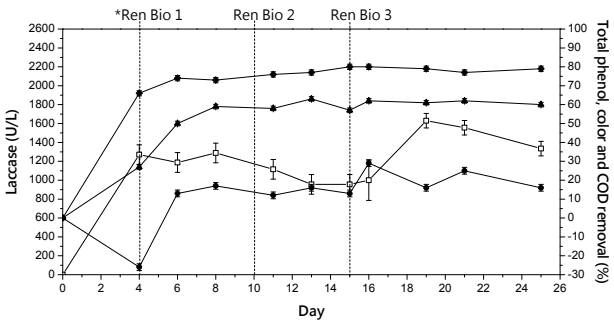

Figure 3: Laccase activity (□), percentage of phenol removal (◆), percentage of COD removal (▲), and percentage of color removal (●) during biodegradation of hydrous ethanol vinasse in a continuously operated air-pulsed bioreactor. *Ren Bio refers to renovation of Trametes versicolor biomass inside the bioreactor. Error bars represent one standard error.

As can be seen in Figure 3, an increase in color (26%) was observed on day 4 compared to the initial value, probably due to the rupture of the *Trametes versicolor* pellets causing the release of biopolymers with greater coloration than were found intracellularly (Strong 2010). Subsequently, removal of 17% was recorded on day 6, and this remained at an average value of 19%. This color removal behavior was similar to that obtained in the air-pulsed reactor operated in batch mode. Figure 4 shows color variation in the air pulsed bioreactor operated in continuous mode. Total phenol removal stabilized at an average value of 80% on day 6, while COD removal remained constant at 60% from day 8 onwards. *Trametes versicolor* was observed to be active throughout the continuous phase, presenting laccase activity with values between 956 U/L and 1,630 U/L. We can therefore infer that it is possible to maintain stable degradation conditions for vinasse over an extended period of time based on adequate biomass renovation inside the reactor.

Ferreira et al. (2010) evaluated color degradation in sugarcane juice vinasse, and found that *Pleurotus sajor-caju* removed 98% of the color by day 9 of incubation. Likewise, Chairattanamanokorn et al. (2005) reported that *Pycnoporus coccineus* removed 45% of the color in vinasse in 13 days. Both studies report color removal percentages that are greater than the 19% obtained by *Trametes versicolor* during the treatment of hydrous ethanol vinasse. This can be attributed to the chemical complexity of hydrous ethanol vinasse compared to the types of vinasse used in the studies by Ferreira et al. (2010) and Chairattanamanokorn et al. (2005).

Laccase activity was not found to be directly related to total phenol and COD degradation in hydrous ethanol vinasse, given that a wide range of enzyme activity was recorded during the continuous experiment without recording changes in the percentages for these two parameters. This suggests that, besides phenol, there are other compounds that could induce laccase activity in *Trametes versicolor*.

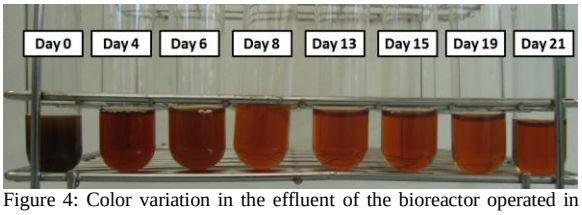

Figure 4: Color variation in the effluent of the bioreactor operated in continuous mode over time.

In general, in this study it was found a phenol, COD and color removal in the vinasse. Nevertheless, it was reported that the combination of different processes, where the first step was an anaerobic treatment followed by an aerobic or physico-chemical process, gives better results in the removal of organic load and color (España-Gamboa et al. 2011; Sangave et al. 2007). After biological treatment, a suitable toxicity/risk assessment test should be done with the approved aquatic and terrestrial test models prior to its discharge from industries to judge whether the treated vinasse is suitable for discharge from industries without any harmful effects in soil and aquatic environment. (Patil and Ghole 2010) reported a similar work, where they evaluated the acute toxicity of anaerobically treated distillery effluent and oxidized effluent on freshwater fish, this study underscores the effectiveness of the two-step process (anaerobic treatment followed by oxidation) for the treatment of distillery effluent in terms of significantly lesser dilution requirements (about 5-fold lesser as compared to the untreated

effluent) before discharging the distillery effluent safely to the ecosystem.

Some of the issues to consider in the implementation of this findings on the industry are the lack of pH and temperature control on the industrial biodegradation processes (Ryznar-Luty et al. 2008). Another problem is culture contamination, common in wastewater treatment, which remains a major challenge in the application of fungi for the color removal especially when large quantities of wastewater are involved and sterilization is not feasible. Further studies must take in account these parameters in order to set a method to scale the process.

Conclusions

The use of *Trametes versicolor* in an air-pulsed bioreactor represents an alternative for the treatment of hydrous ethanol vinasse. Continuous operation degraded total phenol by an average of 80% and removed 60% of COD. Batch operation, meanwhile, removed 71% of total phenol and 40% of COD. There was no significant difference in color degradation between treating vinasse continuously or in batch mode (19% and 18% respectively). For future industrial applications, the degradation capacity of *Trametes versicolor* should be evaluated on higher concentrations of vinasse and vinasse that has not previously been sterilized.

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