Bioresource Technology 102 (2011) 7603–7607

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

Fungal solid state fermentation on agro-industrial wastes for acid wastewater decolorization in a continuous flow packed-bed bioreactor

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ARTICLE INFO

Article history:
Received 28 February 2011
Received in revised form 10 May 2011
Accepted 13 May 2011
Available online 19 May 2011

Keywords:
Fungal solid state fermentation
Apple processing residues
Laccase
Dye
Wastewater

ABSTRACT

This study was aimed at developing a process of solid state fermentation (SSF) with the fungi *Pleurotus ostreatus* and *Trametes versicolor* on apple processing residues for wastewater decolorization. Both fungi were able to colonize apple residues without any addition of nutrients, material support or water. *P. ostreatus* produced the highest levels of laccases (up to 9 U g^{-1} of dry matter) and xylanases (up to 80 U g^{-1} of dry matter). A repeated batch decolorization experiment was set up with apple residues colonized by *P. ostreatus*, achieving 50% decolorization and 100% detoxification after 24 h, and, adding fresh wastewater every 24 h, a constant decolorization of 50% was measured for at least 1 month. A continuous decolorization experiment was set up by a packed-bed reactor based on colonized apple residues achieving a performance of 100 mg dye L^{-1} day^{-1} at a retention time of 50 h.

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1. Introduction

Wastewaters from the textile industry can be considered as the most polluting among all industrial sectors, and their treatment is greatly challenging. The white rot basidiomycetes fungi are, so far, the microorganisms most efficient in degrading synthetic dyes. This property is due to the production of extracellular lignin-modifying enzymes – manganese peroxidases (MnP), lignin peroxidases (LiP), and laccases (Lac) – which are able to degrade a wide range of xenobiotic compounds, including dyes. Laccase is the main enzyme involved in dye decolorization by cultures of *Phlebia tremellosa*, *Pleurotus sajor-caju* and *Pleurotus ostreatus* and several recent studies have been focused on dye degradation by laccases. Most studies dealing with ligninolytic enzymes have been performed in submerged conditions, whereas solid state fermentation (SSF) would be more suited for cultivating basidiomycetes fungi, because it provides conditions very similar to the environment in which they have evolved and it shows several advantages over liquid fermentation systems (Singhania et al., 2009). Many studies reported the exploitation of SSF to give an extra value to agro-industrial residues (Rodriguez Couto, 2008; Sharma and Arora, 2010; Cha et al., 2010) and different reactors for SSF were reported (Singhania et al., 2009).

In this study, a process of SSF of the basidiomycetes fungi *P. ostreatus* and *Trametes versicolor* on apple processing residues was developed for production of ligninolytic and (hemi)cellulolytic enzymes and their use in a packed-bed reactor for decolorization of textile wastewaters.

2. Methods

2.1. Lignocellulosic substrates

Commercially available wheat, employed for fungal pre-culture, was prepared as previously described (Iandolo et al., 2011). Apple residues were collected in a local apple processing farm of Campania (Italy). The residues (peels, seeds and stems) were reduced to small pieces and sieved to have 0.8–2.0 mm dimension particles, and stored by freezing them at −20 °C. Before sterilizing in autoclave at 110 °C for 1 h, 1.5% (w/w) $CaCO_3$ was added to reach a final pH of 6.0. As far as the percentage of humidity is concerned, the starting value of 71% was kept, without further water addition.

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0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.05.029

2.2. Fungal strains

The basidiomycetes fungi used in this study were a strain of the white rot fungus *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC MYA-2306) from ATCC, the Global Bioresource Centre and *T. versicolor* (NBRC4937) from the fungal collection Nite Biological Resource Center (Department of Biotechnology National Institute of Technology and Evaluation, Japan).

2.3. Solid state fermentation

Fungal growth on wet apple residues prepared as above described and samples preparation for analyses were carried out as previously described (Iandolo et al., 2011).

2.4. Analytical determinations

Evaluation of humidity and dry matter content, analysis of soluble reducing sugars content and assays of laccase, manganese peroxidase, lignin peroxidase, filter paper and xylanase activity, and evaluation of total protein content were performed as previously described (Iandolo et al., 2011).

2.5. Non-denaturing PAGE

Non-denaturing PAGE was performed as previously described (Iandolo et al., 2011). The gels were stained to visualize laccase activity by using ABTS as the substrate.

2.6. Decolorization batch experiments

Batch decolorization experiments on previously formulated model wastewaters containing acid industrial dyes (Faraco et al., 2009) were performed with apple wastes after three days of fungal fermentation and crude enzymatic extracts from these samples. An amount of colonized waste (0.3–0.5 or 0.6–1 g) corresponding to a laccase activity of 1 or 2 U or a volume of crude enzymatic extracts corresponding to 1 U of laccase activity were incubated with wastewater models up to a final volume of 4 mL, at room temperature under vigorous agitation by a rotary shaker. Decolorization was evaluated recording UV–VIS absorption spectra at different times (10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 7 h, 9 h, 24 h) of incubation and calculated as the extent of decrease of spectrum area recorded between 380 and 740 nm in comparison with the corresponding area of the spectrum of the untreated wastewater model. To evaluate the extent of color absorption on mycelium and agro-industrial residues, incubation of colored wastewaters with residues after mycelium inactivation by autoclave treatment at 120 °C for 20 min, and measurement of decolorization at different times were performed.

A repeated sequencing batch decolorization experiment was set up by using 0.3–0.5 g of apple residues colonized by *P. ostreatus* at the third day of SSF corresponding to 1 U of laccase activity within a dialysis tube (cut off of 12,000–14,000 KDa) (Delchimica scientific glassware srl, Italy). Each decolorization cycle consisted of addition of 4 ml of the wastewater to the apple residues. A new cycle was initiated after 24 h when no further change in the wastewater decolorization was measured. Decolorization was evaluated as described above. The experiments were performed in three-replicates and reported values are representative of three experiments.

2.7. Continuous packed-bed reactor

For setting up the fixed bed reactor for acid bath decolorization, 12 g of apple processing residues colonized by *P. ostreatus* at the 3rd day of SSF were homogenized in a blender in the presence of 12 mL of 50 mM Na phosphate pH 7 and packed in a dialysis membrane (~480 mm × 4.5 mm, cut off 12,000–14,000 KDa) (Delchimica scientific glassware srl, Italy). The dialysis membrane was placed within a glass column (495 mm × 9 mm) and surrounded by glass beads (500 μm mean diameter) in order to optimize flux distribution around the dialysis membrane (working volume 30 mL) (Fig. 1). The reactor system was operated at room temperature. Absorption at 590 nm (corresponding to the wavelength of maximum absorption of Acid Blue 62) was continuously measured, and at regular time periods of 24 h decolorization % was evaluated as described above (Section 2.6). Reported values are representative of three experiments.

2.8. Detoxification experiments

Detoxification was evaluated on the acid bath (4 mL) after 24 h of treatment with apple processing residues colonized by *P. ostreatus* at the third day of SSF within the dialysis tube (final activity 1 U) and on the acid bath treated in the continuous packed-bed reactor. Toxicity was determined as previously described (Palmieri et al., 2005). The experiments were performed in duplicate and reported values are representative of at least two experiments.

2.9. Protein identification by mass spectrometry

Protein identification by mass spectrometry was performed on slices of interest from the non-denaturing PAGE as previously described (Lettera et al., 2010).

3. Results and discussion

3.1. Development of solid state fermentation process on apple residues

A process of SSF with *P. ostreatus* and *T. versicolor* was developed on apple residues, demonstrating the ability of both fungi to colonize the waste quickly and extensively. It is worth noting that addition of either a material support or water was not necessary to allow fungal colonization of the waste. During SSF, no significant changes in humidity and reducing sugars level were measured (data not shown). A continuous reduction of dry mass was assessed for both fungi (Table 1), possibly due to waste conversion into volatile compounds by fungal metabolism.

3.2. Time courses of ligninolytic and glycosyl hydrolytic enzymes

The production of ligninolytic (Lac, LiP and MnP) and glycosyl hydrolytic (xylanase and cellulase) enzymes was evaluated during *T. versicolor* and *P. ostreatus* SSFs (Table 1). As far as laccase activity levels are concerned, *P. ostreatus* reached similar levels to those produced on tomato pomace, while *T. versicolor* achieved a ten fold lower maximum value with respect to tomato pomace SSF (Iandolo et al., 2011). In comparison with SSF processes previously reported with bith other *P. ostreatus* strains (Elisashvili et al., 2008; Stajic et al., 2006; Kurt and Buyukalaca, 2010) and other fungi (Elisashvili et al., 2008; Moldes et al., 2003; Rosales et al., 2002), SSF of the *P. ostreatus* strain tested in this study allowed the achievement of similar values, although neither optimization of culture conditions nor nutrient/water addition were performed in this study. As far as peroxidase production is concerned, only *T. versicolor* secreted detectable levels of MnP activity, while neither *P. ostreatus* nor *T. versicolor* produced appreciable values of LiP. Among the investigated glycosyl hydrolases, only xylanolytic enzymes were produced at detectable levels by both fungi, measuring lower values than those previously reported in other studies on SSFs with fungi

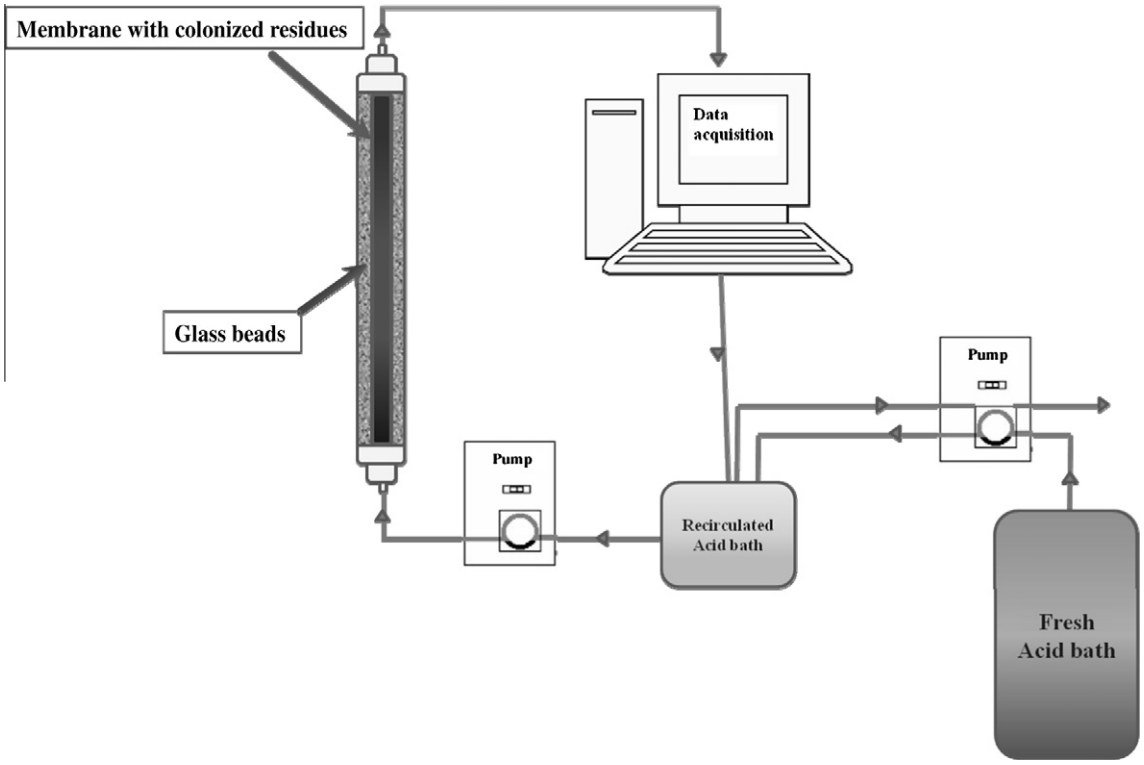

Fig. 1. Schematic diagram of the packed-bed reactor based on apple residues colonized by *P. ostreatus* employed, operating in a continuous mode.

Table 1
Dry mass content and enzyme activity values during SSF. Results of the analytical determinations reported correspond to mean values of three-replicates of two independent experiments.

Time (days)	Dry mass content (g)	MnP activity (U/g d.m.)	Laccase activity (U/g d.m.)	Xylanase activity (U/g d.m.)
Pleurotus ostreatus				
0	17.6 ± 0.3	n.d.	0	0
1	14.6 ± 0.4	n.d.	1.27 ± 0.15	44.08 ± 4.83
2	14.3 ± 1.1	n.d.	2.76 ± 0.14	55.55 ± 2.10
3	12.5 ± 1.1	n.d.	9.05 ± 0.97	45.26 ± 8.12
4	13.6 ± 0.5	n.d.	5.88 ± 0.93	76.94 ± 10.12
5	13.9 ± 0.8	n.d.	6.27 ± 0.31	46.87 ± 3.42
7	11.7 ± 0.9	n.d.	3.32 ± 0.69	36.60 ± 1.99
Trametes versicolar				
0	15.1 ± 0.3	0	0	0
1	15.2 ± 0.7	0	1.11 ± 0.17	36 ± 1
2	15.0 ± 0.4	0.47 ± 0.06	3.82 ± 0.07	31.30 ± 0.35
3	14.6 ± 0.3	0.56 ± 0.03	4.12 ± 0.36	36.85 ± 1.43
4	13.5 ± 0.2	0.60 ± 0.08	1.94 ± 0.00	18.10 ± 1.70
5	14.1 ± 0.4	0.89 ± 0.06	2.70 ± 0.18	30.15 ± 1.00
6	13.7 ± 1.9	0.64 ± 0.04	1.44 ± 0.38	34.15 ± 1.04
7	13.0 ± 0.2	0.65 ± 0.03	1.80 ± 0.11	24.24 ± 3.38

n.d.: not detectable.

A. terreus (Gawande and Kamat, 1999), *P. dryinus* and *P. ostreatus* (Elisashvili et al., 2008), *Penicillium* sp. (Nair et al., 2008). Hence, both fungi were shown to be able to produce hemicellulolytic but not cellulolytic activities under the analysed conditions. Moreover, for both fungi, the correlation between production times for laccase and xylanase activities (Table 1) suggests their synergistic action in waste transformation.

3.3. Wastewater decolorization

Crude enzymatic extracts from apple residues colonized by *P. ostreatus* or *T. versicolor* at the 3rd day (corresponding to the maximum laccase activity production) were tested for decolorization ability on model wastewater containing acid dyes. A maximum decolorization of 40% was achieved after 24 h of incubation with 1 U of laccase activity from *T. versicolor* while just 3 h of incubation were needed to gain the same effect with 1 U of laccase activity from *P. ostreatus*. The latter result is similar to the decolorization level previously achieved with extracellular enzymes containing laccase (2 U mL^{-1}) from *P. ostreatus* liquid culture after 24 h of incubation (Faraco et al., 2009). Experiments of dye decolorization with crude enzymatic extracts from fungi cultivated under solid state conditions have been previously reported for *T. hirsuta* (Couto and Sanroman, 2006), *T. pubescens* (Osma et al., 2007) and *T. versicolor* (Xin and Geng, 2011), producing not better performances than those reported hereby.

Apple residues colonized by *P. ostreatus* and *T. versicolor* at the 3rd day were also used in decolorization experiments. As far as treatment of acid bath with *P. ostreatus* colonized residues is concerned, around 43% of decolorization was achieved in only 10 min of incubation with an amount of colonized waste corresponding to a laccase activity of 0.25 U ml^{-1} of wastewater, and 34% of the decolorization obtained accounts for enzymatic degradation. After 24 h of incubation of wastewater with colonized apple residues, 68% of total decolorization, with a 24% due to enzyme activity, was achieved, showing an increasing contribution of absorption over enzymatic degradation when incubation goes on. Similarly, when apple residues colonized by *T. versicolor* at the 3rd day were tested, 60% and 71% of total decolorization (with 39% and 21% due to enzymatic degradation, respectively) were achieved after 10 min and 24 h of incubation, respectively. When a fresh sample of acid wastewater was added to *P. ostreatus* colonized apple residues that had been recovered after the first 24 h decolorization round, a total decolorization of 58% including an enzymatic contribution of 18% was achieved after a new 24 h cycle. The leaching of about 60% of laccase activity in the acid wastewater sample removed after the first decolorization round can explain the lower enzymatic performance at the second decolorization round. Therefore, a repeated sequencing batch decolorization experiment was set up using the apple residues within a dialysis membrane to avoid the leaching of enzymes during the acid bath incubation. These experiments allowed reaching a 50% decolorization due to enzymatic degradation in 24 h without loss of enzymatic activity in the wastewater. Every 24 h, fresh wastewater was added and a decolorization of 50% due to enzymatic degradation was kept constant for at least 1 month. Complete detoxification of the wastewater was also achieved after 24 h incubation.

3.4. Acid bath decolorization in the continuous packed-bed reactor

In order to carry out decolorization experiment in continuous mode, a fixed-bed reactor was set up by packing an amount of apple processing residues (after three days of fungal colonization) corresponding to a laccase activity of 40 U in a dialysis tube within a glass column surrounded by glass beads. 100 mL of acid bath were recirculated through the column at a flux of 2 mL/minute, corresponding to a passage time of 15 min, up to reaching a stationary decolorization of 70% as reduction of Abs_{590nm} and 38% as reduction of the extent of spectrum area between 380 and 740 nm after 7 days (data not shown). Then, a continuous wastewater feeding was started by adding fresh wastewater to the reservoir at a flux of 0.01 mL/minute corresponding to a retention time of around 2 days (Fig. 1) and the decolorization was then kept constant, with a performance of 100 mg dye L^{-1} day^{-1}. Another packed-bed bioreactor for dye continuous decolorization based on laccases produced by fungal SSF has so far been developed with the fungus *T. hirsuta* (Couto et al., 2006) grown on orange peelings. In this study, a worse performance was achieved for the dyes Methyl Orange and Poly R-47816 (16 and 28 mg L^{-1} day^{-1}, respectively), using a similar retention time (3 days).

The main aspect of our bioreactor is that continuous decolorization was carried out by using a low cost equipment and a simple reactor design, without a strict control of operative conditions that is required for fungal solid state fermenters instead. The absence of living biomass avoids problems associated with requirement of continuous substrate feeding to sustain the growth, biomass wash-out or bioreactor clogging. The only drawback of our bioreactor can be found in the expected decrease of the enzymatic activity due to laccase denaturation. However, our system was shown able to work for at least one month. The high ratio between the retention time of wastewater feeding and the passage time due to the liquid recirculation (about 100) assures the good mixing of the flow packed bed. Consequently, dead zones inside the packing are avoided and mass transfer to the dialysis membrane is enhanced.

3.5. Enzymes identification

When fractionation on native PAGE and staining of bands for laccase activity were carried out on extracts from apple residues that have been colonized by the fungus at the 3rd day, the pattern of laccase isoenzymes was proved similar to that obtained during SSF on tomato pomace (Iandolo et al., 2011). The activity positive protein bands were excised and subjected to protein identification by MS/MS ion search on a MASCOT server against the annotated *P. ostreatus* genome (http://genome.jgi-psf.org/PleosPC15_1). POXA3 and POXC could be unambiguously identified (2 peptides with 6% sequence coverage, and 5 peptides with 16% of sequence coverage, respectively) as well as the Dye-decolorizing peroxidase (6 peptides with 10% of sequence coverage) as already previously identified (Faraco et al., 2007). Hence, POXA3 and POXC laccases and the Dye-decolorizing peroxidase are the main responsible for decolorization by apple residues colonized by *P. ostreatus* after three days of SSF.

4. Conclusions

The developed SSF showed to be an effective system for apple residues upgrading by production of high xylanase activity levels and development of a laccase-based system for continuous dye decolorization. The developed system was very suitable to its application for dye decolorization, since it was able to operate in a continuous mode giving a performance of 100 mg dye L^{-1} day^{-1} with a retention time of 50 h, very low equipment cost and no operational problems. Considering that the tested wastewater simulates a real textile effluent, this work indicates the possibility of implementing the developed techniques for the treatment of textile-dyeing wastewaters.

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