Journal of Hazardous Materials 181 (2010) 315–323

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Bioremediation of soil contaminated with pentachlorophenol by *Anthracophyllum discolor* and its effect on soil microbial community

M. Cea[a,*], M. Jorquera[a], O. Rubilar[a], H. Langer[b], G. Tortella[a], M.C. Diez[a]

[a] *Scientific and Technological Bioresources Nucleus, Universidad de La Frontera, Av. Francisco Salazar 01145, Casilla 54-D, Temuco, Chile*
[b] *Agrícola Nacional SACEI (ANASAC), Panamericana 5 Sur km 645, Lote 1 Parque Industrial, Lautaro, Chile*

ARTICLE INFO

Article history:
Received 27 January 2010
Received in revised form 3 May 2010
Accepted 4 May 2010
Available online 11 May 2010

Keywords:
Anthracophyllum discolor
DGGE
Pentachlorophenol
Microbial community

ABSTRACT

Bioaugmentation is a promising technology to clean up sites contaminated with recalcitrant chemicals. White-rot fungi have proven to be effective in the degradation of pentachlorophenol. Here, we report the bioremediation of soil contaminated with pentachlorophenol (PCP) by *Anthracophyllum discolor* and its impact on the soil microbial community. In this study three types of microcosms were established: fresh soil (C_0), fresh soil plus wheat straw (WS_0) and, fresh soil plus wheat straw inoculated with *A. discolor* ($WSAD_0$). Additionally, similar treatments and a control of sterile soil spiked with PCP (C_{250}, WS_{250} and $WSAD_{250}$) were used to evaluate the remediation and adsorption of PCP. The PCP removal, total microbial activity, and enzymatic activities were evaluated. This study also investigated the structure of soil microbial community by denaturing gradient gel electrophoresis (DGGE), identifying some of the dominant bacterial and fungal species. The results showed that PCP was effectively degraded in soils by *A. discolor* and by indigenous soil microorganisms. The addition of wheat straw increased the PCP degradation and enzymatic activities. Only laccase activity was negatively affected by PCP contamination. The PCP degradation was associated with changes in microbial communities, mainly stimulation of members of bacterial phylum *Proteobacteria* (*Xanthomonadaceae*, *Burkholderiaceae* and *Enterobacteriaceae*), and fungal phylum *Ascomycota* and *Basidiomycota*. This study shows the ability of *A. discolor* to degrade PCP from contaminated soil, and demonstrates that agricultural residues, such as wheat straw, can be used as growth substrate by microorganisms in PCP-contaminated soil, demonstrating a great potential of autochthonous microorganisms for soil remediation.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The use of microorganisms to detoxify and degrade contaminants has received considerable attention as an effective biotechnological approach to clean up polluted environments. Bioaugmentation may an effective approach in the case of recalcitrant chemicals that tend to resist degradation by native microbial communities. Among the microorganisms capable of degrading pentachlorophenol (PCP) are the white-rot fungi (basidiomycetes). White-rot fungi are able to degrade all major wood polymers and numerous studies have demonstrated their ability to tolerate and remediate PCP-contaminated environments [1].

The degradation system in white-rot fungi is induced by nutrient deprivation and the enzymes involved in the pollutant transformation process are usually expressed under nutrient-deficient conditions that are prevalent in many contaminated soils [2]. Thus, the capacity for transforming a range of pollutants by fungi depends on the nutrient status and the presence of appropriate carbon and nitrogen growth substrates [3]. Currently, inexpensive growth substrates, such as wood chips, sawdust, wheat straw and apple pomace are being used to stimulate white-rot fungi in soil [4]; in addition their effect on pollutant degradation, oxidative enzyme expression and soil microbial community structure is being investigated. Particularly *Anthracophyllum discolor* (*Xerotus discolor* Mont. 1853), a white-rot fungus isolated from the southern forest of Chile, has demonstrated a high capacity to degrade PCP [3,5] due to the production of ligninolytic enzymes, mainly manganese peroxidase (MnP) and to a lesser extent laccase (Lac), lignin peroxidase (LiP), and manganese-independent peroxidase (MiP) [5]. Biodegradation of PCP in soil slurry cultures by *Bjerkandera adusta* and *A. discolor* has been studied by Rubilar et al. [3]. They observed high PCP degradation rates upon addition of lignocellulosic waste increased the ligninolytic enzyme activity, especially when *A. discolor* was used.

Microbial communities within contaminated ecosystems tend to be dominated by those organisms capable of utilizing or surviving toxic contamination. Macnaugton et al. [6] found that the

* Corresponding author. Tel.: +56 45 325494; fax: +56 45 325053.
E-mail address: mcea@ufro.cl (M. Cea).

0304-3894/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2010.05.013

Table 1
Primer sets used in this study.

Microorganism and primer	Genomic target	Sequence (5′–3′)	Reference
Bacteria			
EUBf933-GC[a,d]	16S rRNA	GCA CAA GCG GTG GAG CAT GTG G	Iwamoto et al. [14]
EUBr1387	16S rRNA	GCC CGG GAA CGT ATT CAC CG	
358F-GC[b]	16S rRNA	CCT ACG GGA GGC AGC AG	Muyzer et al. [15]
907R	16S rRNA	CCG TCA ATT CMT TTG AGT TT	
Fungi			
NS1	18S rRNA	GTA GTC ATA TGC TTG TCT C	O'Donnell et al. [16]
NS8	18S rRNA	TCC GCA GGT TCA CCT ACG GA	
NS7-GC[c]	18S rRNA	GAG GCA ATA ACA GGT CTG TGA TGC	de Souza et al. [17]
F1Ra	18S rRNA	CTT TTA CTT CCT CTA AAT GAC C	

[a] GC-clamp, CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG.
[b] GC-clamp, CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GG.
[c] GC-clamp, CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG.
[d] All GC-clamps were attached to the 5′-end of primer.

structure and diversity of the dominant bacterial communities changed substantially when compared to an uncontaminated soil. They also observed competition between inoculated bacteria and the autochthonous microbial community that rendered the inoculum ineffective. Using denaturing gradient gel electrophoresis (DGGE), Mahmood et al. [7] detected changes in relative abundance of microbial communities for PCP-amended soils when compared to controls. As a consequence of contamination, soil microbial activity and enzyme activities can change. However, Kähkönen et al. [8] reported that the total microbial activity measured as fluorescein diacetate (FDA) hydrolysis was not sensitive to added PCP in a contaminated area with a history of chlorophenol contamination. In terms of enzyme activities, Diez et al. [9] demonstrated that Lac activity was negatively affected in soil contaminated with PCP.

In this context, the objective of the present study was to evaluate the degradation of PCP from a contaminated soil by *A. discolor* and its effect on the autochthonous soil microbial community structure.

2. Materials and methods

2.1. Soil

PCP degradation was studied on an Andisol collected from the Temuco Series, located in southern Chile at 38° 42′S, 73° 35′W. The soil sample was collected from 0 to 20 cm depth, passed through a 2 mm sieve and then moistened to 60% of its water holding capacity (WHC) prior to use in this study. Sterile soil was prepared as follows: soil samples (75 g) at 60% of its WHC were put in glass pots ensuring a maximal depth lower than 2.5 cm. The pots were covered with aluminum foil and autoclaved at 121 °C for 40 min. After that, the samples were incubated during 24 h, and autoclaved again for 40 min.

The chemical characteristics of soil were 5.5 + 1.1 mg kg^{-1} of available N, 15 + 0.3 mg kg^{-1} of P_{Olsen}, 12 + 0.1% of organic matter and a pH value of 5.7 + 0.1. The analyses were done according with methodology described in Sadzawka et al. [10].

2.2. Fungal inoculation

The inoculum was prepared as followed: 5 agar disks (6 mm in diameter) of active mycelia of *A. discolor* from 5-day-old cultures on LBM medium [5] were placed 150 mL of culture broth (10 g L^{-1}glucose, 5 g L^{-1} peptone and 2.5 g L^{-1} yeast extract) and incubated at 30 ± 1 °C in darkness for 7 d. After incubation, the flask was homogenized and 4 mL (11 g L^{-1} of mycelium) was added to 1 g of sterile wheat straw of 1 cm length and incubated at 30 °C for 7 d.

2.3. Soil microcosms assays

Experiments to evaluate the PCP removal, total microbial activity, enzyme activities and microbial populations were performed with three microcosm treatments: (1) 75 g of moistened fresh soil (C_0) were placed in closed glass flask, (2) moistened soil plus 1 g of non-inoculated wheat straw (WS_0), and (3) moistened soil plus inoculated wheat straw ($WSAD_0$). The three microcosms described above represent the control treatments. Similar treatment microcosms were prepared using soil previously spiked with a stock solution of PCP (10 g L^{-1}) diluted in KOH (0.1 mol L^{-1}) at a final concentration of 250 mg of PCP kg^{-1} of soil (i.e., C_{250}, WS_{250}, and $WSAD_{250}$). An additional treatment was prepared using sterile soil (75 g) spiked with PCP to evaluate PCP adsorption (i.e., SC_{250}).

2.4. Analysis of PCP in soil

The residual concentration of PCP in the soil was determined by HPLC after extraction with hexane:acetone (1:1 proportion). Briefly, 5 g of soil was mixed with 10 mL of hexane:acetone and incubated under shaking for 2 h at 25° C. Later, the samples were sonicated for 5 min and filtered with PTFS membrane (0.45 μm pore size; Millipore) and then injected in a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with 20 μL loop and a Merck Hitachi L-7455 diode array detector. The detector was set at 215 nm and the column was a reverse phase (Lichrosphere 60RP select B, 5 μm). The mobile phase consisted of acetonitrile:phosphoric acid (1:1 proportion) with a flow rate of 1 mL min^{-1} at 25 °C. Instrument calibrations and quantifications were performed against pure reference standards (0.05–5 mg L^{-1}). The procedure described has been checked for PCP recoveries (95 ± 5%). Detection limit was 0.03 mg L^{-1} for PCP considering the noise-to-signal ratio greater than 2.

2.5. Soil biochemical and microbial community analyses

The biochemical and microbial community analyses were determined on the different microcosms as follows:

(i) *Total microbial activity:* The total microbial activity was measured by monitoring FDA hydrolysis [11].
(ii) *Manganese peroxidase activity:* The MnP activity was measured through monitoring the oxidation of 2,6-dimethoxyphenol (DMP). One MnP activity unit was defined as the amount of enzyme transforming 1 μmol DMP min^{-1} [12].
(iii) *Laccase activity:* Lac activity was determined as described by Bourbonnais and Paice [13] with some modifications. ABTS

(2,2′-azinobis-3-ethyl benzothiozoline-6-sulphonate) oxidation was measured at 420 nm.

(iv) *Microbial community:* The microbial community composition in soil was evaluated at 0, 7 and 28 d by DGGE by using specific primer sets for bacteria and fungi (Table 1). Briefly, soil DNA extraction was carried out by using UltraClean Soil DNA Isolation Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA, USA). For bacterial community analysis, fragments of 16S rRNA gene were amplified by touchdown polymerase chain reaction (PCR) with two different primer sets EUBf933-GC/EUBr1387 (454 bp of variable regions V6–V8) and 358F-GC/907R (549 bp of variable regions V3–V4) [14]. For fungal community analysis, fragments of 18S rRNA gene were amplified by nested PCR. Firstly, fragments were obtained by touchdown PCR using the primer set NS1/NS8, followed by a second PCR with the primer set F1Ra/NS7-GC (400 bp of variable region V9). All PCR amplifications were carried out with reagents supplied with GoTaq® DNA Polymerase (Promega, Co. Madison, WI, USA).

The DGGE analysis was performed using a DCode system (Bio-Rad Laboratories, Inc.). Twenty microliters of PCR product was loaded onto a 9% (w/v) polyacrylamide gel with 20% and 70% gradient (urea and formamide). The electrophoresis was run for 16 h at 100 V. The gel was then stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on an UV transilluminator. Representative bands in DGGE gels were carefully excised, re-amplified then ran through another DGGE gel, to avoid excised multiple bands due to the close proximity between them. The selected bands were then sequenced by Macrogen, Inc. (Korea). More than one clone per band was sequenced, to provide convincing evidence of taxonomic assignment and the consensus nucleotide sequences obtained in this study were deposited and compared with those present in GenBank database from the National Center for Biotechnology Information (NCBI) by using BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Statistical analysis

All experiments and analyses were done in triplicate. The values reported for enzymatic activities are averages of the three determinations expressed on an oven-dried soil basis (105 °C). Analyses of variance followed by a least significant difference (LSD) test at the 0.05 level was used to determine significant differences means between treatments. The Pearson correlation (two-tailed) was used to test between two response variables. All statistics were carried out using SPSS version 13.0 software.

3. Results

3.1. PCP removal from soil microcosms

PCP removal was observed in all microcosms, with the greatest difference in treatments observed during the first week of incubation (Fig. 1). During the first 14 d of incubation, the fastest removal occurred for the $WSAD_{250}$ treatment, with more than 76% of PCP removed. However, at the end of the experiment, we observed a significant ($P<0.05$) increment of PCP removed in WS_{250}, which reached >93%. These results were significantly higher than the microcosms inoculated with *A. discolor*.

The results also suggest that PCP removal can be attributed to processes other than microbial degradation, such as irreversible sorption or abiotic degradation. For example, in the SC_{250} treatment we observed a low retention of PCP during the first 7 d (11%), which increased at day 14 (36%) and remained constant during the rest of incubation time. This may be due to a saturation of the active sites in the soil able to retain PCP.

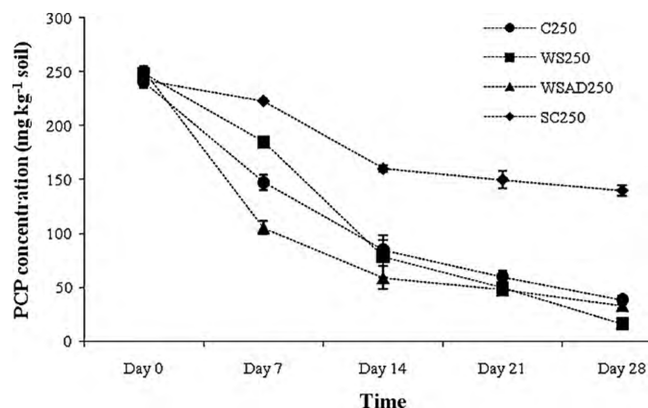

Fig. 1. Residual PCP concentration in soil obtained during incubation.

3.2. Effect of treatments on total microbial activity of soil

FDA hydrolysis showed high variability in the time for all microcosms, mainly in day 21 where the activity diminished significantly in all microcosms (Table 2). Compared with the fresh soil, the addition of wheat straw into the soil microcosms significantly increased the microbial activity when PCP (C_{250} v/s WS_{250}) was present. In general terms, PCP increased the total microbial activity of soil community. Also, we observed different behaviors between the uncontaminated microcosms and those contaminated with PCP. For example, in the microcosms without PCP, the total microbial activity increased from day 7 to 14. However, the total microbial activity remained almost constant in presence of PCP at the same period of time. The increase of microbial activity at the end of experiment could be associated with the presence of dominant bacteria and fungi at day 28 which were not dominant at day 7, as it is observed in DGGE gels (Fig. 3, bands no. 2 and 3; Fig. 4, bands no. 25 and 27).

3.3. MnP activity

From Table 2, it can be observed that MnP activity significantly increased by the addition of wheat straw to the fresh soil without PCP, then diminishing with the time as in the fresh soil. With the PCP incorporation the MnP activity was negatively affected only at the beginning (7–14 d). After 14 d, the activity increased, probably due to an acclimatization of the autochthonous microorganisms. In soil microcosms inoculated with *A. discolor* ($WSAD_0$, $WSAD_{250}$) the MnP activity increased significantly, reaching values of 13.6–45.2 nmol Mn^{3+} min^{-1} g^{-1}, which were higher in $WSAD_{250}$. The higher MnP activity of $WSAD_{250}$ compared with $WSAD_0$ from 21 d can be attributed to a repopulation of the soil microcosm by autochthonous fungi that interact with *A. discolor* in the bioremediation process. High concentrations of MnP have been attributed to PCP degradation in soils by fungi. In this study, we observed a positive correlation ($r=0.353$; $P<0.05$) between MnP activity and PCP removal (Fig. 2). However, when the MnP activity value was above 10 nmol Mn^{3+} min^{-1} g^{-1}, no significant reduction of PCP occurs.

3.4. Lac activity

In fresh soil, the Lac activity increased after 7 d, and then remained constant. After 7 d, a stimulatory effect of wheat straw on Lac activity was observed (Table 2), with significantly ($P\leq0.05$) higher values (3–5 μmol ABTS min^{-1} g^{-1}) obtained in the treat-

Table 2
Enzyme activities and PCP removed in different microcosms during the time.

Microcosm	PCP treatment (mg kg^{-1})	Time (*D*)	Manganese peroxidase (nmol Mn^{3+} min^{-1} g^{-1})	Laccase (μmol ABTS min^{-1} g^{-1})	Fluorescein released (mg g^{-1})	PCP removed (%)
Fresh soil (C)	0	7	10.3 ± 0.91	0.8 ± 0.04	27.8 ± 4.6	–
		14	4.6 ± 1.31	3.0 ± 0.54	41.1 ± 5.7	–
		21	2.8 ± 0.21	2.7 ± 0.00	16.6 ± 0.3	–
		28	2.9 ± 0.10	2.6 ± 0.15	25.2 ± 1.8	–
	250	7	6.4 ± 0.26	2.2 ± 0.06	30.5 ± 0.5	41.2 ± 2.8
		14	1.3 ± 0.26	1.1 ± 0.30	33.6 ± 3.3	66.0 ± 3.6
		21	5.7 ± 0.69	1.9 ± 0.42	19.6 ± 3.7	76.0 ± 2.0
		28	4.3 ± 0.18	1.4 ± 0.06	26.3 ± 6.2	84.4 ± 1.6
Fresh soil + wheat straw (WS)	0	7	13.0 ± 1.85	0.9 ± 0.06	32.5 ± 0.5	–
		14	1.9 ± 0.96	3.8 ± 0.48	42.9 ± 6.8	–
		21	2.3 ± 0.18	5.4 ± 0.60	20.4 ± 1.0	–
		28	2.3 ± 0.13	3.1 ± 0.24	28.1 ± 0.3	–
	250	7	4.8 ± 0.37	1.1 ± 0.03	42.0 ± 3.6	26.0 ± 0.8
		14	7.2 ± 0.78	0.9 ± 0.03	43.4 ± 7.5	68.4 ± 7.8
		21	10.2 ± 0.00	2.5 ± 0.54	30.1 ± 2.2	80.0 ± 2.8
		28	9.0 ± 0.53	1.9 ± 0.66	36.4 ± 1.1	93.6 ± 0.0
Fresh soil + inoculated wheat Straw (WSAD)	0	7	13.6 ± 1.24	1.0 ± 0.06	37.9 ± 7.8	–
		14	44.1 ± 1.83	0.7 ± 0.03	45.9 ± 5.2	–
		21	15.5 ± 0.78	1.0 ± 0.00	24.5 ± 8.0	–
		28	19.6 ± 1.25	1.2 ± 0.21	37.2 ± 9.2	–
	250	7	19.3 ± 0.19	1.7 ± 0.09	47.7 ± 9.0	58.0 ± 2.4
		14	32.2 ± 3.76	2.9 ± 0.06	36.5 ± 6.1	76.4 ± 4.4
		21	26.8 ± 6.53	1.8 ± 0.09	31.2 ± 2.7	80.8 ± 1.6
		28	45.2 ± 0.36	0.9 ± 0.18	39.7 ± 3.9	86.8 ± 0.8
$LSD_{0.05}$ [a]			2.13	0.34	6.08	–
$LSD_{0.05}$ [b]			–	–	–	5.60

[a] Least significance difference ($P < 0.05$) for the microcosm–PCP–time interaction.
[b] Least significance difference ($P < 0.05$) for the microcosm–time interaction.

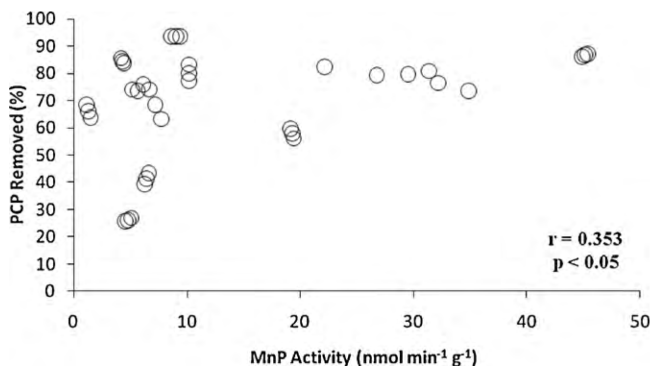

Fig. 2. Pearson correlation observed between Manganese peroxidase activity and PCP removal.

ment WS_0 compared with other treatments (≤3 μmol ABTS min^{-1} g^{-1}). Initially, the addition of PCP produced a significant ($P \leq 0.05$) increase on Lac activity in the C_{250}, WS_{250} and $WSAD_{250}$ treatments when compared with the controls without PCP (C_0, WS_0 and $WSAD_0$). However after 7 d, a decrease on Lac activity was observed for of these treatments. One exception was the soil inoculated with *A. discolor* and contaminated with PCP ($WSAD_{250}$); however, this treatment did not show the higher values of Lac activity during the incubation time.

3.5. Effect of treatments on the microbial community

The effect of wheat straw and PCP amendments on bacterial and fungal communities is shown in Figs. 3 and 4. The dominant band patterns in the treated and control microcosms were different, suggesting that the bacterial and fungal communities were affected by the wheat straw and PCP addition. In particular, in the treatments with PCP-contaminated soil (C_{250}, WS_{250} and $WSAD_{250}$) was stimulated the presence of bands with phylogenetic similarity to β-proteobacteria (bands no. 9, 13 and 14) and γ-proteobacteria (bands no. 1 and 10) groups, especially the families *Xanthomonadaceae* and *Burkholderiaceae*. On the other hand, compared with the treatment C_{250}, the addition of PCP plus wheat straw (WS_{250} and $WSAD_{250}$) showed the stimulation of other bands phylogenetically assigned to *Xanthomonadaceae* (band no. 12) and *Enterobacteriaceae* (bands no. 2 and 3). The appearance of bands also suggests a synergic effect of PCP and wheat straw, stimulating different bacterial populations in soil compared with treatment with addition of PCP or wheat straw only. A similar effect was also observed when fluorescein was released (see Table 2).

The DGGE analysis of the fungal community did not show a specific pattern related to treatments tested with PCP and wheat straw. The dominant bands in the fungal community were mainly assigned to Ascomycota and Basidiomycota (Table 3). It is noteworthy that in the treatment $WSAD_{250}$ at 28 d the band no. 27 (close to *Rhizopus oryzae*, according to phylogenetic tree, Fig. 4) was stronger compared with *A. discolor* band. Despite soil bioaugmentation by *A. discolor* addition, this result suggests that autochthonous fungi contained in soil can actively participate in PCP degradation.

4. Discussions

This study showed PCP removal from Andisol soil by biaugmentation with *A. discolor* grown in wheat straw, and also by the soil indigenous microorganisms. Similar findings have been described by Mahmood et al. [7] for PCP removal: first, like us they reported removal of PCP in two phases and second, they also demonstrate degradation of PCP by indigenous soil bacterial communities.

In this study, we also observed PCP removal in the sterile soil (SC_{250}), which can be attributed to the strong adsorption of PCP in this type of soil as was described by Cea et al. [18,19]. Moreover, Cea et al. [18] demonstrate a high interaction of PCP with soil surfaces.

Such as have been previously observed by Rubilar et al. [3], *A. discolor* presented a high capacity to remove PCP from contaminated soils. However, in our study we observed a significant PCP degradation by autochthonous microorganisms, as revealed the microcosms conducted in non-sterile soil without fungal addition (C_{250}). In this context, Rubilar et al. [3] demonstrated that indigenous microflora exert an inhibitory effect on the growth and degradation capacity of the two white-rot fungi evaluated (*B. adusta and A. discolor*) for the remediation of soil contaminated with PCP in a slurry reactor.

Both *A. discolor* and autochthonous microorganisms presented a good response to PCP in terms of total microbial activity as measured by FDA hydrolysis. Similar findings have been reported in other studies for the indigenous microflora. For example, Kähkönen et al. [8] found that FDA hydrolysis was not sensitive to PCP in a contaminated area with aged chlorophenols. However, other microbial activities are more sensitive, such as butyrate-esterase, acetate esterase and endogenous carbon dioxide evolution, which were inhibited in the sites with higher PCP content. Here the researchers indicate that the most sensitive microbial species were probably replaced with more tolerant ones in these sites.

According to our results, only MnP presented a positive correlation with PCP removal. Several reports have attributed to the enzymatic ligninolytic activity in the degradation capacity of white-rot fungi [1,3] and it has also been demonstrated that soil microorganisms produce this type of extracellular oxidoreductases (MnP and Lac) involved in the oxidative coupling processes of chlorophenols [20]. In this study, an increase in MnP activity resulted in a higher percentage of PCP removal (Fig. 2). However, this occurred when the MnP activity was <10 nmol min^{-1} g^{-1}. For the higher values of MnP activity, no relation with PCP removal was found. In fact, according to the results obtained by Rubilar et al. [3], the degradation of these compounds may be attributed to the action of other cellular and extracellular fungal enzymes. These enzymes can be phenoloxidases and cellobiase dehydrogenases, which may participate concomitantly with ligninolytic enzymes in the degradation process [21,22].

When PCP was added to the natural soil (C250) the behavior of MnP was quite similar to the result obtained by Diez et al. [9] who observed an inhibitory effect on this enzyme by PCP and, considering the extracellular nature of the enzyme, attributed this phenomenon to a stabilization period due to interaction with organic and inorganic soil colloids. However, we observed changes in microbial communities (see Figs. 3 and 4) that suggest an adaptation of the soil microflora to the contaminant, during this time.

As reported by Diez et al. [9], when PCP was initially added to the natural soil the Lac activity was higher than the control (C_0), then (after 7 d for us) Lac activity decreased significantly (Fig. 2c). In fact, Gianfreda et al. [23] found that the higher phenol concentrations pronounced the loss of Lac activity, results explained a progressive entrapment and/or adsorption of active enzyme molecules on phenol polymeric aggregates formed. On the other hand, the low quantity of Lac produced in this study for all treatments, is similar with other studies, where it is indicated that white-rot fungi produce constitutively low concentrations of laccase [2,15].

Agricultural crop wastes have been reported as a growth substrate to cultivate white-rot fungi in soil [4]; supporting the idea that inducible oxidative enzymes may prevail in several species of fungi (native or inoculated) given appropriate growth conditions [1]. Our results showed an increment of MnP and Lac activities in soil amended with wheat straw. In fact, Mancera-López et al. [24] found a stimulatory effect of sugarcane bagasse over native fungi in

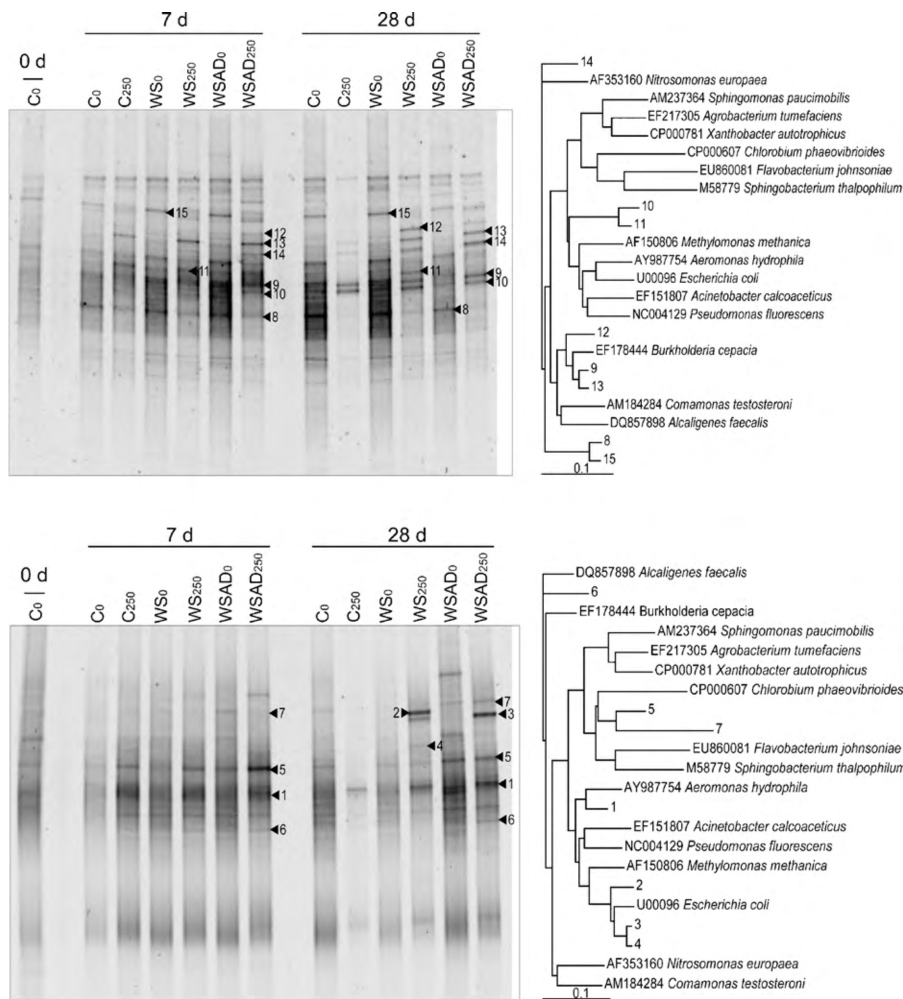

Fig. 3. The DGGE banding patterns and neighbor-joining phylogenetic trees of the bacterial 16S rRNA genes amplified with the primer sets: (a) EUBf933-GC/EUBr1387 and (b) 358F-GC/907R. The neighbor-joining trees were constructed based on some sequences of control strains taken from the NCBI database and by using ClustalX software. Scale of bar indicated 10% sequence divergence and a bootstrap analysis was performed with 1000 trials.

a combined system of biostimulation–bioaugmentation with filamentous fungi, where the native fungi are able to grow in a complex mixture of hydrocarbons and to removes the contaminants but to a lesser degree than inoculated fungi.

Several authors show that chlorinated phenols and their derivatives are degraded by diverse soil bacteria, such as *Sphingomonas*, *Pseudomonas* and *Acinetobacter* [7,25]. Similarly to our results, analysis by DGGE has revealed the presence of *Burkholderia* spp.

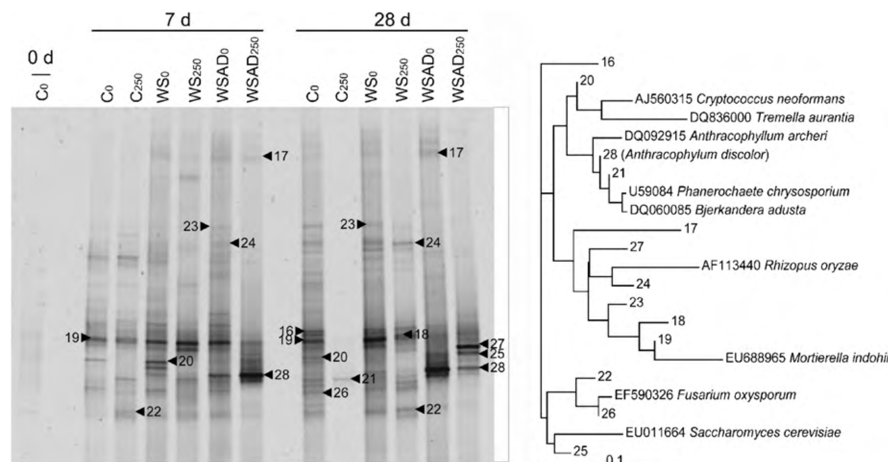

Fig. 4. The DGGE banding pattern and phylogenetic tree of the fungal 18S rRNA genes amplified with the primer set F1Ra/NS7-GC. The neighbor-joining tree was constructed based on some sequences of control strains taken from the NCBI database and by using ClustalX software. Scale of bar indicated 10% sequence divergence and a bootstrap analysis was performed with 1000 trials.

Table 3
Phylogenetic assignment of DGGE bands after 28 d of incubation.

Band[a]	Taxonomic group[b]	Closest relatives or cloned sequences (accession no.)	Similarity (%)[c]	Accession no.
Bacteria				
1	*Proteobacteria*, *Gammaproteobacteria*, *Xanthomonadaceae*	*Luteibacter rhizovicinus* from root nodule of legume growing in sand loam soil (FJ561269)	99	FJ357540
2	*Proteobacteria*, *Gammaproteobacteria*, *Enterobacteriaceae*	*Rahnella* sp. from soil of Great Wall Station in China (FJ546061)	99	FJ357541
3	*Proteobacteria*, *Gammaproteobacteria*, *Enterobacteriaceae*	*Pantoea* sp. from rhizhosphere of plant growing on a lateritic soil (GQ478260)	98	FJ357542
4	*Proteobacteria*, *Gammaproteobacteria*, *Enterobacteriaceae*	*Enterobacter* sp. from soil (FN555398)	98	FJ357543
5	*Proteobacteria*, *Alphaproteobacteria*, *Methylobacteriaceae*	Uncultured bacterium from soil (FJ433811)	93	FJ357544
6	*Proteobacteria*, *Betaproteobacteria*, *Burkholderiaceae*	*Burkholderia* sp. from forest soil (FJ685990)	96	FJ357545
7	*Bacteroidetes*, *Sphingobacteria*, *Chitinophagaceae*	Uncultured bacterium from soil (GQ127925)	92	FJ357546
8	*Proteobacteria*, *Betaproteobacteria*, *Burkholderiaceae*	Uncultured bacterium from rice paddy soil (AB487113)	98	FJ357547
9	*Proteobacteria*, *Betaproteobacteria*, *Burkholderiaceae*	Uncultured *Burkholderia* sp. from soil (AM930510)	98	FJ357548
10	*Proteobacteria*, *Gammaproteobacteria*, *Xanthomonadaceae*	Uncultured *Luteibacter* sp. from soil (AM930508)	99	FJ357549
11	*Proteobacteria*, *Gammaproteobacteria*, *Xanthomonadaceae*	*Dyella* sp. from rhizosphere soil (GQ181136)	99	FJ357550
12	*Proteobacteria*, *Gammaproteobacteria*, *Xanthomonadaceae*	*Ralstonia* sp. from soil contaminated with 2,4-D (AY238507)	99	FJ357551
13	*Proteobacteria*, *Betaproteobacteria*, *Burkholderiaceae*	*Burkholderia* sp. from rhizhosphere of plant growing on a lateritic soil (GQ478268)	100	FJ357552
14	*Proteobacteria*, *Betaproteobacteria*, *Methylophilaceae*	Uncultured Methylophilaceae from agricultural soil (EF072076)	98	FJ357553
15	*Proteobacteria*, *Betaproteobacteria*, *Burkholderiaceae*	Uncultured beta proteobacterium from agricultural soil (EF664963)	98	FJ357554
Fungi				
16	*Ascomycota*, *Saccharomycetes*, *Saccharomycetaceae*	*Pichia guilliermondii* from soil (EU784644)	99	FJ357555
17	*Chytridiomycota*, *Monoblepharidomycetes*, *Oedogoniomycetaceae*	Uncultured fungus from soil (EF628894)	90	FJ357556
18	*Fungi incertae sedis*, *Basal fungal lineales*, *Mortierellaceae*	Uncultured fungus from black soil (GU568171)	97	FJ357557
19	*Fungi incertae sedis*, *Basal fungal lineales*, *Mortierellaceae*	Uncultured fungus from soil (EF628725)	99	FJ357558
20	*Basidiomycota*, *Tremellomycetes*, *Tremellaceae*	Uncultured Tremellaceae from rhizosphere soil (EF023436)	100	FJ357559
21	*Basidiomycota*, *Agaricomycetes*, *Coriolaceae*	Uncultured Agaricomycetes from pig stable (GU306000)	99	FJ357560
22	*Ascomycota*, *Sordariomycetes*, *Clavicipitaceae*	Uncultured fungus from soil (DQ865807)	100	FJ357561
23	*Glomeromycota*, *Glomeromycetes*, *Archaeosporaceae*	Uncultured Archaeospora from root (AM384929)	94	FJ357562
24	*Chytridiomycota*	Uncultured fungus from non-fertilized agricultural soil (AM114806)	97	FJ357563
25	*Ascomycota*, *mitosporic Ascomycota*, *Scytalidium*	*Scytalidium lignicola* associated with root (AY762623)	100	FJ357564
26	*Ascomycota*, *Sordariomycetes*, *Fusarium*	*Fusarium oxysporum* from potato field soil (AB521041)	100	FJ357565
27	*Ascomycota*, *Pezizomycetes*, *Rhizinaceae*	Uncultured fungus from soil (EF628645)	94	FJ357566
28	*Basidiomycota*, *Agaricomycetes*, *Tricholomataceae*	*Anthracophyllum discolor* (*Xerotus discolor*; FJ357568)	100	FJ357567
	Anthracophyllum discolor			FJ357568[d]

[a] Corresponding DGGE bands shown in Figs. 2 and 3.

[b] The phylogenetic assignment is based on sequence analysis by the RDP classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) or GenBank database from NCBI (http://www.ncbi.nlm.nih.gov). It is given the phylum as well as the lowest predictable phylogenetic rank.

[c] Based on partial sequencing of 16S and 18S rRNA gene and comparison with those present in GenBank by using Blastn.

[d] Sequence obtained from pure culture and used to validate the presence of DGGE band of *A. discolor* (FJ357567).

during degradation of chlorinated aromatic compounds [26,27]. Based on information available in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) and MetaCyc Encyclopedia of Metabolic Pathways (http://metacyc.org/), some *Burkholderia* species (strains: 383, ATCC 17616, E264 and NCTC 10247) are able to produce PCP hydroxylases. A xanthomonad with capacity to degrade chlorinated compounds has also been recently reported [28]. In Vietnam, a recent DGGE analyses showed diverse bacteria in soils, including bacteria such as *Ralstonia* during biodegradation of phenoxy herbicides [26]. Despite these findings, to our knowledgement there were no studies reporting *Ranella* as chlorinated aromatic compounds-degrading bacteria. In addition, analysis of PCP-degrading bacterial consortia from a paper mill pulp revealed the presence of the enterobacterium *Escherichia coli* [29].

In relation to fungal populations, studies have revealed that diverse members of Ascomycetes (*Rhodococcus* and *Paecilomyces*) and Basidiomycetes (*Phanaerochaete*, *Trametes* and *Bjerkandera*) are high producers of phenoloxidases and efficient degraders of chlorophenols [1,30]. In addition, *Rhizopus oryzae* was identified as one of the more dominant fungi present in $WSAD_{250}$, which was more dominant than *A. discolor,* and has also been previously reported as a zygomycete capable of tolerating and removing efficiently PCP from contaminated soil [31].

It is noteworthy that the differences between stimulated bacterial populations, obtained by DGGE with the two different primer sets assayed, could be related to mismatches between PCR primers and DNA template, reducing the amplification efficiency [32,33]. To solve this limitation, it is recommended to use more diverse primer sets to analyze the same bacterial community.

5. Conclusions

This study demonstrates the great potential of *A. discolor* and autochthonous soil microorganisms to degrade PCP from contaminated soil. This study also shows that agricultural crop residues, such as wheat straw, can be used as an efficient growth substrate by microorganisms in PCP-contaminated soils, increasing the PCP degradation, total microbial activity and enzymatic activity. Moreover, the DGGE analysis allowed identify members of phylum *Proteobacteria* (*Xanthomonadaceae*, *Burkholderiaceae* and *Enterobacteriacea*) which were dominant during PCP remediation. This result suggest that *Proteobacteria* can actively participate in the remediation of chlorophenols in soils together with *A. discolor*.

Acknowledgments

This study was financed by Fondecyt Nos. 3080039, 3080013, 1050614, DIUFRO DI07-3001. The authors thank J.P. Baquiran from Department of Environmental Science of The University of California-Riverside for his assistance in English writing. M. Cea thank Dr. C. Demergasso (Universidad Católica del Norte) for her assistance in the DGGE technique. M. Jorquera thanks the Bicentenary Program in Science and Technology PSD26, Conicyt.

References

[1] O. Rubilar, M.C. Diez, L. Gianfreda, Transformation of chlorinated phenolic compounds by white rot fungi, Crit. Rev. Environ. Sci. Technol. 38 (2008) 227–268.
[2] A. Dhouib, M. Hamza, H. Zouari, T. Mechini, R. Hmidi, M. Labat, M.J. Martinez, S. Sayadi, Screening of ligninolytic enzyme production by diverse fungi from Tunisia, World J. Microb. Biot. 21 (2005) 1514–1523.
[3] O. Rubilar, G. Feijoo, C. Diez, T.A. Lu-Chau, M.T. Moreira, J.M. Lema, Biodegradation of pentachlorophenol in soil slurry cultures by *Bjerkandera adusta* and *Anthracophyllum discolor*, Ind. Eng. Chem. Res. 46 (2007) 6744–6751.
[4] M. Walter, L. Boul, R. Chong, C. Ford, Growth substrate selection and biodegradation of PCP by New Zealand white-rot fungi, J. Environ. Manage. 71 (2004) 361–369.
[5] G.R. Tortella, O. Rubilar, L. Gianfreda, E. Valenzuela, M.C. Diez, Enzymatic characterization of Chilean native wood-rotting fungi for potential use in the bioremediation of polluted environments with chlorophenols, World J. Microb. Biot. 24 (2008) 2805–2818.
[6] S.J. Macnaugton, J.R. Stephen, A.D. Venosa, G.A. Davis, Y.-J. Chang, D.C. White, Microbial population changes during bioremediation of an experimental oil spill, Appl. Environ. Microb. 65 (1999) 3566–3574.
[7] Sh. Mahmood, G.I. Paton, J.I. Prosser, Cultivation-independent *in situ* molecular analysis of bacteria involved in degradation of pentachlorophenol in soil, Environ. Microbiol. 7 (2005) 1349–1360.
[8] M. Kähkönen, M. Tuomela, A. Hatakka, Microbial activities in soils of a former sawmill area, Chemosphere 67 (2007) 521–526.
[9] M.C. Diez, F. Gallardo, G. Saavedra, M. Cea, L. Gianfreda, M. Alvear, Effect of pentachlorophenol on selected soil enzyme activities in a chilean andisol, R.C. Suelo Nutur. Veg. 6 (2006) 40–51.
[10] A. Sadzawka, M.A. Carrasco, R. Grez, M.L. Mora, H. Flores, A. Neaman, Métodos de Análisis Recomendados Para Los Suelos Chilenos, Serie Acta INIA No. 34, Instituto de Investigaciones Agrarias, Ministerio de Agricultura, Santiago, Chile, 2006.
[11] G. Adam, H. Duncan, Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils, Soil Biol. Biochem. 33 (2001) 943–951.
[12] M.T. Moreira, G. Feijoo, R. Sierra-Alvarez, J. Lema, J.A. Field, Biobleaching of oxygen delignified kraft pulp by several White rot fungal strains, J. Biotechnol. 53 (1997) 237–251.
[13] R. Bourbonnais, M.G. Paice, Oxidation of non-phenolic substrates: an expanded role of lacasse in lignin biodegradation, FEBS Lett. 267 (1990) 99–102.
[14] T. Iwamoto, K. Tani, K. Nakamura, Y. Suzuki, M. Kitagawa, M. Eguchi, M. Nasu, Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE, FEMS Microbiol. Ecol. 32 (2000) 129–141.
[15] G. Muyzer, T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, C. Wawer, Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, in: A.D.L. Akkermans, J.D. van Elsas, F.J. de Brijn (Eds.), Molecular Microbial Ecology Manual, vol. 3.4.4., Kluwer Academic Publishers, Dordrecht, The Netherlands, 1998, pp. 1–27.
[16] K. O'Donnell, Fusarium and its near relatives, in: D.R. Reynolds, J.W. Taylor (Eds.), The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics, CAB International, Wallingford, UK, 1993, pp. 225–233.
[17] F.A. de Souza, G.A. Kowalchuk, P. Leeflang, J.A. van Veen, E. Smit, PCR-denaturing gradient gel electrophoresis profiling of inter- and intraspecies 18S rRNA gene sequence heterogeneity is an accurate and sensitive method to assess species diversity of Arbuscular Mycorrhizal fungi of the genus *Gigaspora*, Appl. Environ. Microb. 70 (2004) 1413–1424.
[18] M. Cea, J.C. Seaman, A.A. Jara, M.L. Mora, M.C. Diez, Describing chlorophenol sorption on variable-charge soil using the triple layer model, J. Colloid Interface Sci. 292 (2005) 171–178.
[19] M. Cea, J.C. Seaman, A.A. Jara, B. Fuentes, M.L. Mora, M.C. Diez, Adsorption behavior of 2,4-dichlorophenol and pentachlorophenol in an allophonic soil, Chemosphere 67 (2007) 1354–1360.
[20] L. Gianfreda, J.-M. Bollag, Isolated enzymes for the transformation and detoxification of organic pollutants, in: R.G. Burns, R. Dick (Eds.), Enzymes in the Environment: Activity, Ecology and Applications, Marcel Dekker, NY, 2002, pp. 495–538.
[21] B.M. Halberg, G. Henriksson, G. Pettersson, A. Vasella, C. Divne, Mechanism of the reductive half-reaction in cellobiose dehydrogenase, J. Biol. Chem. 278 (2003) 7160–7166.
[22] A.M. Montiel, F.J. Fernández, J. Marcial, J. Soriano, J. Barrios-González, A. Tomasini, A fungal phenoloxidase (tyrosinase) involved in pentachlorophenol degradation, Biotechnol. Lett. 26 (2004) 1353–1357.
[23] L. Gianfreda, F. Sannino, M.T. Filazzola, A. Leonowicz, Catalytic behaviour and detoxifying ability of laccase from the fungal strain *Cerrena unicolor*, J. Mol. Catal. B: Enzyme 4 (1998) 13–23.
[24] M.E. Mancera-López, F. Esparza-García, B. Chávez-Gómez, R. Rodríguez-Vázquez, G. Saucedo-Castañeda, J. Barrera-Cortés, Bioremediation of an aged hydrocarbon-contaminated soil by a combined system of biostimulation–bioaugmentation with filamentous fungi, Int. Biodeter. Biodeg. 61 (2008) 151–160.
[25] R.K. Jain, M. Kapur, S. Labana, B. Lal, P.M. Sarma, D. Bhattacharya, I.S. Thakur, Microbial diversity: application of microorganisms for degradation of xenobiotics, Curr. Sci. 89 (2005) 101–112.
[26] N.L. Huong, K. Itoh, K. Suyama, 2,4-dichlorophenoxyacetic acid (2,4-D)- and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) – degrading bacteria community in soil-water suspension during the enrichment process, Microb. Environ. 23 (2008) 142–148.
[27] S. Morimoto, K. Togami, N. Ogawa, A. Hasebe, T. Fujii, Analysis of a bacterial community in a 3-chlorobenzoate-contaminated soil by PCR-DGGE targeting the 16S rRNA gene and benzoate 1,2-dioxygenase gene (benA), Microb. Environ. 20 (2005) 151–159.
[28] N. Manickam, R. Misra, S. Mayilraj, A novel pathway for the biodegradation γ-hexanochlorocyclohexane by a *Xanthomonas* sp. Strain ICH12, J. Appl. Microb. 102 (2007) 1468–1478.
[29] I.S. Thakur, Biodegradation of chlorinated benzoic acid by a *Pseudomonas fluorescens* from a chemostat, Indian J. Microb. 41 (2001) 27–32.

[30] R. Steiman, J.-L. Benoit-Guyod, F. Seigle-Murandi, L. Sage, A. Toe, Biodegradation of pentachlorophenol by micromycetes. II. Ascomycetes, basidiomycetes, and yeast, Environ. Toxic. Water 9 (1994) 1–6.
[31] H. León-Santiesban, R. Bernal, F.J. Fernández, A. Tomasini, Tyrosinase and peroxidase production by *Rhizopus oryzae* strain ENHE obtained from pentachlorophenol contaminated soil, J. Chem. Technol. Biotech. 83 (2008) 1394–1400.
[32] G.C. Baker, J.J. Smith, D.A. Cowan, Review and re-analysis of domain-specific 16S primers, J. Microbiol. Methods 55 (2003) 541–555.
[33] M.N. Marshall, L. Cocolin, D.A. Mills, J.S. VanderGheynst, Evaluation of PCR primers for denaturating gradient gel electrophoresis analysis of fungal community of compost, J. Appl. Microbiol. 95 (2003) 934–948.