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Effects of soya lecithin on the removal and biodegradation of chlorpyrifos and pentachlorophenol by *Anthracophyllum discolor*

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Effects of soya lecithin on the removal and biodegradation of chlorpyrifos and pentachlorophenol by *Anthracophyllum discolor*

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"A mi mamá,

por su amor, comprensión, entereza y apoyo incondicional"

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Chapter 1

General Introduction

General introduction

1.1 Introduction

Contamination of groundwater, soils and sediments is a consequence of a wide range of toxic organic and inorganic compounds released by natural processes (Gribble, 1994), industrial activities and industrial accidents (Kiem et al., 2003). This fact has resulted in an increase of the contaminated sites in the world. Therefore, it is an important environmental problem word wide.

Several strategies have been developed to remediate contaminated sites. However, some are expensive, sometimes difficult to perform, inefficient and often exchange one problem for another (Bollag and Bolag, 1995). Bioremediation is an economical, versatile, environmentally friendly technology and can be applied on a large number of contaminants (Jain et al., 2005).

Bioremediation is a complex system that depends on many factors, such as adequate microbial community, bioavailability of contaminants and environmental factors (Bollag and Bollag, 1995; Conte et al., 2005). Low water solubility and adsorption to soil are two factors of hydrophobic contaminants that limit their availability to degrading microorganisms in the bioremediation processes. However, several studies have demonstrated that the addition of surfactants can enhance the bioavailability of this type of contaminants, thus increasing their degradation (Abdolhamid et al., 2009; Kang et al, 2010).

Surfactants are organic molecules that present a hydrophobic tail and hydrophilic head in aqueous systems (West and Harwell, 1992). This structure confers properties to surfactants such as detergent, emulsifying, foaming, dispersing, and solubilization leading their applications in several industrial areas, as it has been reviewed by Desai and Banat (1997), Van Hamme et al., (2006) and Banat et al. (2010). The water solubility of the surfactant is due to the reduction of surface tension of the solution and interfacial tension between single molecules at the surface and interface respectively (West and Harwell, 1992).

In general, surfactants are characterized by properties such as critical micelle concentration (CMC), hydrophilic-lipophilic balance (HLB), charge, origin source, and chemical structure (Wets and Harwell, 1992; Van Hamme et al., 2006). According to their hydrophilic or ionic part, surfactants are classified as anionic, cationic, zwitterionic or nonionic (West and Harwell, 1992).

The properties of surfactants have led their use in several environmental applications, such as: increasing desorption (Zhu and Aitken, 2010), enhancing aqueous solubility (Eddouaouda et al., 2011), improving biodegradation processes (Gottfried et al., 2010), enhancing soil washing (Franzetti et al., 2009), and removing inorganic compounds such as heavy metals (Rufino et al., 2011). Chemically produced surfactants are identified as synthetic surfactants whereas that biosurfactants are produced by numerous microorganisms and represent a wide diversity of chemicals and molecular structures (Desai and Banat, 1997). Biosurfactants have been of great interest in the last years, because they present several advantages over synthetic surfactants, such as low toxicity, biodegradability, and ecological acceptability (Desai and Banat, 1997). Another group of biosurfactants is phytogenic surfactants, such as saponins and lecithins. Both have been applied in studies of bioelimination of phenanthrene and fluoranthene in aqueous systems (Soeder et al., 1996). Both quillaya saponin and soya lecithin solubilize polycyclic aromatic hydrocarbons at high concentrations (Fava et al., 2004). Besides, degrading bacteria used these surfactants as substrates for growth (Soeder et al., 1996).

Surfactant can enhance the bioremediation processes by increasing their bioavailability or mobilizing or removing the hydrophobic contaminants in contaminates sites. The application of surfactants of surfactants can exert both positive and negative effects on the biodegrading microorganisms. In general, have been established that the synthetic cationic surfactant are most toxic and have been applied as antimicrobials. Besides, the synthetic nonionic and microbial surfactants are often considered as low or non-toxic (Van Hamme et al., 2006). The effects of surfactants application in biodegradation processes is not predictable, and is dependent of several factors such as surfactant and contaminant concentration, degrader microorganisms, environmental conditions, among others (Van Hamme et al., 2006; Banat et al., 2010).

White rot fungi can degrade a wide range of contaminants, such as polychlorinated phenols, pesticides, synthetic dyes, and polycyclic aromatic hydrocarbons (D'Annibale et al., 2005; Cea et al., 2010; Rubilar et al., 2011). The capability to degrade these compounds is associated with the extracellular ligninolytic system, formed by lignin peroxidase, manganese peroxidase, manganese independent peroxidase, or laccase (Reddy, 1995). *Phanerochaete chrysosporium*, *Trametes versicolor* and *Pleurotus osteratus* have been the fungi most applied for contaminants degradation. However, numerous investigations have been development to find new fungal strain with high ability for the degradation of pollutants. In this context, Tortella et al. (2008) and Rubilar et al. (2011) showed that *Anthracopyllum discolor*, a native fungus isolated from forests of southern Chile, producer of manganese peroxidase with capability to degrade pentachlorophenol. Although the white rot fungi have been widely studied in biodegradation processes, little information is available respect to the effects of surfactant on white rot fungi, the most data are related with bacteria (Soeder et al., 1996; Franzetti et al., 2009; Eddouaouda et al., 2011).

The enhancement of biodegradation processes by using of surfactants is an attractive option. However, this technology depends on complex interaction of the physical, chemical and biological factors within environment.

1.2 Hypothesis and research objectives

1.2.1 Hypothesis

Considering the previously mentioned facts related to hydrophobic toxic compounds with low solubility, the effect of surfactants to solubilize pollutants, and the capacity of the enzymatic system of *Anthracophyllum discolor* to degrade pollutant, it is possible to establish the following working hypothesis:

"The addition of soya lecithin, as natural surfactant, will enhance solubility of chlorpyrifos and pentachlorophenol, increasing their bioavailability for the enzymatic system of *A. discolor*. On the other hand, this surface active agent will increase the ligninolytic activity of *A. discolor*, enhancing the degradation of these compounds".

1.2.2 Research objectives

1.2.2.1 General objective

To study the effect of soya lecithin on the removal and biodegradation of chlorpyrifos and pentachlorophenol by *Anthracophyllum discolor*.

1.2.2.2 Specific objectives

1. To evaluate the effect of soya lecithin addition on production of ligninolytic enzymes by *Anthracophyllum discolor* in liquid medium.
2. To evaluate the effect of soya lecithin addition on degradation of chlorpyrifos and pentachlorophenol by *Anthracophyllum discolor* in liquid medium.
3. To evaluate the effect of soya lecithin addition on degradation of chlorpyrifos and pentachlorophenol by *Anthracophyllum discolor* in contaminated soil.

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Chapter 2

Biosurfactants useful tools for the bioremediation of contaminated soil

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Biosurfactants useful tools for the bioremediation of contaminated soil

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Abstract

Bioremediation processes are negatively affected by the low aqueous solubility of some contaminants; therefore their bioavailability may be enhanced by the addition of surfactants. These compounds are organic molecules that can be chemically and biologically produced. Surfactants contain both hydrophilic and hydrophobic groups, therefore reducing surface and interfacial tensions of immiscible fluids and increasing the solubility and sorption of hydrophobic organic and inorganic compounds. This article provides an overview of characteristics of natural and synthetic surfactants and the effects of biosurfactants on solubility, sorption and biodegradation of hydrophobic organic contaminants; as well as the effects of biosurfactants on degrader microorganisms as white-rot fungi. Finally, some examples of application of natural surfactants for bioremediation of contaminated soils are shown. In general, this overview indicates the great potential of biosurfactants on the remediation of contaminated sites.

Keywords: Biosurfactants, hydrophobic organic compounds, soil.

2.1 Introduction

Soil pollution is a consequence of the accumulation of a wide range of chemical compounds generated either by natural or industrial processes. The existence of contaminated sites is an important environmental problem today.

Several strategies involving biological, physico-chemical, and thermal processes have been developed to remediate contaminated sites (Bollag and Bollag, 1995; Vidali, 2001; Rubilar et al., 2011). Methods such as incineration, excavation, landfilling and storage are expensive, sometimes difficult to execute (Vidali, 2001; Jain et al., 2005), inefficient, and often exchange one problem for another (Bollag and Bollag, 1995). Alternatively, biological processes offer several advantages over conventional technologies, because they are often more environmentally friendly, economic and versatile, and they can reduce the concentration and toxicity of a large number of contaminants (Vidali, 2001; Jain et al., 2005). However, these processes are limited by the low water solubility of the contaminants, limiting their availability to microorganisms (Bollag and Bollag, 1995; Volkering et al., 1998).

Bioavailability of a contaminant is largely controlled by its hydrophobicity and ease of desorption from the solid phase of the soil to the aqueous solution (Semple et al., 2003). The molecular structure, concentration and physico-chemical characteristics of the pollutants limit their bioavailability (Volkering et al., 1998; Alexander, 2000). Low bioavailability is also related to the ageing of the pollutants in the soil (Alexander, 1995; Semple et al., 2003). The addition of a surfactant to a contaminated soil can reduce the interfacial tension thus increasing the mass transfer of the contaminants (Mulligan et al., 2001; Gao et al., 2007; Franzetti et al., 2008). In this context, several researchers have shown that various surfactants can enhance desorption (Aronstein et al., 1991; Mata-Sandoval et al., 2002; Xu et al., 2006), solubilization (Garon et al., 2002; Prak and Pritchard, 2002; Doong and Lei, 2003), biodegradation of organic compounds (Fava and Di Gioia, 2001; Kim et al., 2001), and removal of heavy metals from soil (Dahrazma and Mulligan, 2007; Rufino et al., 2011).

Thus, the aim of this review is to provide an overview of characteristics of natural surfactant (biosurfactant) and synthetic surfactants (surfactant), the effects of biosurfactants on solubility, sorption and biodegradation of hydrophobic organic contaminants (HOCs). The effects

of biosurfactants on degrader microorganisms and white-rot fungi are also presented. Finally, some examples of application of biosurfactants for bioremediation of contaminated soils are shown.

2.2 Characteristics and properties of the surfactants

The surface activity of surfactants derives from their amphiphilic structure, meaning that their molecules contain both water soluble and water insoluble portions (West and Harwell, 1992). The water solubility of the surfactants is due to the hydrophilic portion (polar group), while the hydrophobic portion (nonpolar chain) tends to concentrate at the air-water interfaces or in the center of micelles, reducing the surface tension of the solution (West and Harwell, 1992; Desai and Banat, 1997; Volkering et al., 1998). Surfactants form aggregates or micelles; this ability confers certain properties such as emulsifying, foaming, dispersing, and the capacity to act as a detergent, making surfactants very versatile chemical compounds. They are applied in several industrial sectors such as the cosmetics, pharmaceuticals and food, petroleum, agrochemical and fertilizer industries, as it has been reviewed by Kosaric (1992), Deleu and Paquot (2004) and Banat et al. (2010).

Surfactants are characterized by properties such as critical micelle concentration (CMC), hydrophilic-lipophilic balance (HLB), chemical structure and charge, as well as properties from their origin source (Van Hamme et al., 2006). The surfactants exist as monomers or single molecules at low concentration in aqueous solutions; over the CMC, the surfactant molecules assemble together, forming aggregates. The CMC depends on surfactant structure, composition, temperature, ionic strength, and the presence and types of organic additives in the solutions (Fuget et al., 2005). At the CMC of surfactant solutions, a drastic change occurs in many physicochemical properties (surface tension, conductivity, or turbidity) (Figure 2.1) (Hanna et al., 2005; Zana, 2005). Micelles are capable of dissolving hydrophobic contaminants in their hydrophobic core, which results in an increased apparent aqueous solubility of the pollutants (Edwards et al., 1991; Prak and Pritchard, 2002).

The HLB number is also an important parameter of the surfactants, describing their physical properties and is specific for each surfactant. This number is determined by the relationship of the hydrophilic and the hydrophobic parts of the surfactant molecule (Tiehm,

1994). This indicates the types of oils that can emulsify them and can be also used to determine their suitability for use. Surfactants with a low HLB are lipophilic whereas a high HLB is indicative of better water solubility (West and Harwell, 1992; Tiehm, 1994). In terms of the hydrophilic portion, surfactants are classified as anionic (negative charge), cationic (positive charge), zwitterionic (both negative and positive charges), or nonionic (no charge) (West and Harwell, 1992; Volkering et al., 1998). Table 2.1 shows the CMC and HBL number, as well as charge type, of some surfactants.

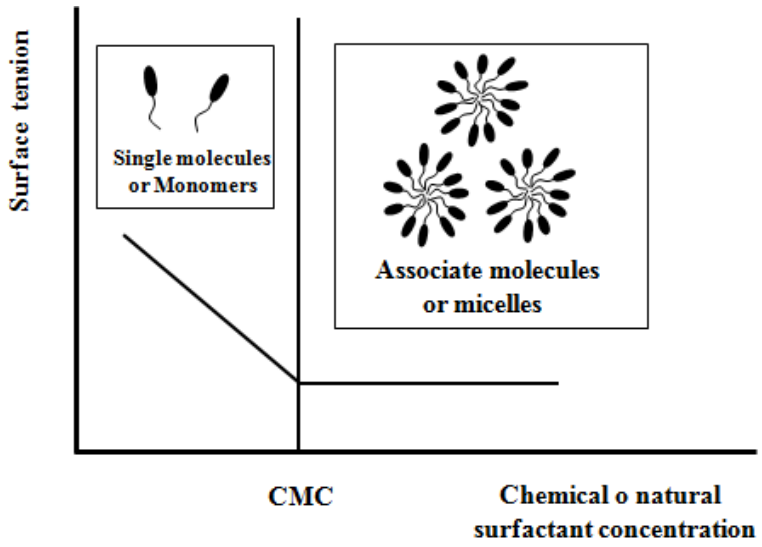

Fig. 2.1 Surface tension as a function of chemical or natural surfactant concentration, CMC represents critical micelle concentration (Patist et al., 2000 and Whang et al., 2008).

2.2.1 Synthetic surfactants

Surfactants that are produced chemically are known as synthetic surfactants. The hydrophobic chain of these surfactants are paraffins, olefins, alkylbenzenes, alkylphenols and alcohols; the polar group is usually either a sulphate group, a sulphonate group, or a carboxylate group for anionic surfactants, or a quaternary ammonium group for cationic surfactants. For nonionic surfactants the polar groups are polyoxyethylenes, sucrose, or polypeptides (Volkering et al., 1998). The most common chemical surfactants are sodium dodecyl sulfate (SDS), Triton X-100 (TX100) and Tween 80 (TW80).

Synthetic surfactants are readily available and of (relatively) low cost, so are extensively utilized in remediation processes of contaminated water or soil. On the other hand, the low yields and high costs that can be incurred in the production of biosurfactants (Deleu and Paquot, 2004; Mukherjee et al., 2006; Banat et al., 2010) have restricted their use. However, considerable attention has been paid to the production and study of biosurfactants, since they offer several advantages over synthetic surfactants: low toxicity, low CMC, biodegradability, ecological acceptability, high selectivity, and specific activity at extreme temperatures, pH, and salinity (West and Harwell, 1992; Desai and Banat, 1997; Kosaric, 2001; Anandaraj and Thivakaran, 2010).

2.2.2 Biosurfactants

Natural surfactants or biosurfactants can be produced extracellularly or as part of the cell membrane by a wide variety of microorganisms such as bacteria, fungi, and yeast. Some examples include *Pseudomonas aeruginosa* (produces rhamnoplids), *Bacillus subtilis* (produces a lipopeptide called surfactin) (Ron and Rosenberg, 2001; Mata-Sandoval et al., 2002; Mulligan, 2005), *Nocardia amarae* (Moussa et al., 2006), and *Saccharomyces lipolytica* CCT-0913 (Lima and Alegre, 2009). Most biosurfactants are either anionic or neutral, only a few are cationic, containing amine groups. The hydrophobic part is based on long chain fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxy fatty acids. The hydrophilic group can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Mulligan et al., 2001).

Biosurfactants are grouped mainly by their chemical composition and their microbial origin. The main classes of these compounds include glycolipids; lipopeptides and lipoproteins; fatty acids, phospholipids, and neutral lipids; and polymeric biosurfactants, as has been reviewed by Desai and Banat (1997), Kosaric (2001), Rahman and Gakpe (2008) and Gautam and Tyagi (2006). Besides, biosurfactants can be classified according to their molecular weight into two main classes, low-molecular-weight molecules called biosurfactants and high-molecular-weight polymers or bioemulsans (Neu, 1996; Rosenberg and Ron, 1999). Biosurfactants lower surface and interfacial tension; this group includes glycolipids, lipopeptides, phospholipids, and proteins. On the other hand, bioemulsans are more effective as emulsion-stabilizing agents, i.e. stabilize oil-in-water; this group includes polymers of polisaccharides, lipoproteins, and particulate

Table 2.1 The charge type, critical micelle concentration (CMC) and hydrophilic-hydrophobic balance number (HLB) of some surfactants, adapted from Doong and Lei (2003).

Surfactant	MW †	Charge type	CMC	HLB	Reference
SDS†	288 g mol^{-1}	Anionic	8.10 mM	40.0	Eriksson et al. (2002)
TX100	628 g mol^{-1}	Nonionic	0.31 mM	13.5	Eriksson et al. (2002)
TW80	1310 g mol^{-1}	Nonionic	0.01 mM	15.0	Eriksson et al. (2002)
Brij 35	1198 g mol^{-1}	Nonionic	0.05 mM	16.9	Yeom et al. (1996), Zhu and Feng. (2003)
Igepal CA-720	735 g mol^{-1}	Nonionic	0.23 mM	14.6	Saichek and Reddy (2004)
Rhamnolipid JBR515	577 g mol^{-1}	Anionic	0.02 mM	22-24	Xie et al. (2005), Nguyen et al. (2008)
Saponin	1800-2000 Da	Nonionic	87.60 mg L^{-1}	-	Soeder et al. (1996), Urum and Pekdemir (2004), Rigano et al. (2009)
Lecithin	773 g mol^{-1}	Zwiterionic	610.00 mg L^{-1}	3-4	Soeder et al. (1996), Bergenstahl and Fontell (1983), Cubero et al. (2002), Aulton (2004)
Tergitol NP-10	683 g mol^{-1}	Nonionic	0.05 mM	14.0	Laha and Luthy (1991), Mulder et al., (1998)

†: MW = Molecular weight

SDS = Sodium dodecyl sulfate

surfactants (Neu, 1996; Rosenberg and Ron, 1999; Perfumo et al., 2010).

In this context, surfactin and rhamnolipids are low-molecular mass biosurfactants with molecular weight of 1036 and 802 Da, respectively (Mulligan and Gibbs, 1990). Besides, emulsan an extracellular lipopolysaccaharide biosurfactant produced by *Acinetobacter calcoaceticus*, is a high-molecular-weight bioemulsifier with an average molecular weight of about 1000 kDa (Kim et al., 1997). Alasan is another bioemulsifier complex produced by *Acinetobacter radioresistens* KA53, with an average molecular weight of 1 MDa (Navon-Venezia et al., 1995).

Biosurfactants can be synthesized using different microorganisms and carbon sources and production is influenced by the composition of the medium and by culture conditions (Desai and Banat, 1997; Franzetti et al., 2009). The carbon sources used for biosurfactant production are hydrocarbons, carbohydrates, vegetable oils and oil wastes, olive oil mill effluent, lactic whey and distiller wastes, starchy substrates, renewable resources, industrial and/or municipal wastewater, under aerobic conditions (Kosaric, 1992; Desai and Banat, 1997; Gautam and Tyagi, 2006). In this context, Franzetti et al. (2008) found three new bacterial strains hydrocarbon-degrading *Gordonia* genus. They were isolated from a site chronically contaminated by diesel. These strains were able to grown using a wide range of straight and branched aliphatic hydrocarbons as carbon and energy sources and to produce at least two classes of surface-active compounds, emulsifying agents and water-soluble substrates. Cell-bound biosurfactants, which reduce surface tension, were produced in hydrocarbons; however their production was lower in water soluble substrates. *Gordonia* sp. BS29 synthesized, and then released extracellularly, bioemulsions during the exponential phase with *n*-hexadecane as carbon and energy source. The production of biosurfactants started in the exponential phase and their concentration increased following linear growth. Calvo et al. (2008) isolated *Ochrobactrum anthropi* strain AD2 from the waste water treatment plant of an oil refinery. This bacterium produced exopolysaccharide AD2 (EPS emulsifiers) in glucose nutrient broth media with various added hydrocarbons; such as n-octane, mineral light and heavy oils and crude oils. In addition, Franzetti et al. (2009) studied the cultural factors that affecting the production of the cell-bound biosurfactants by *Gordonia* sp. BS29. Their research evaluated the type and concentration of the carbon source, the concentration of phosphates and sodium

chloride, and the interactions among these factors. The results showed that with the optimized cultural conditions a 5-fold increase in the biosurfactant concentration, compared to the un-optimized medium, was obtained. The optimization did not change the number and type of the glycolipid biosurfactants produced by *Gordonia* sp. BS29.

The phytogenic surfactant is another group of biosurfactants, such as saponins and lecithins (Soeder et al., 1996), and humic acids (Conte et al., 2005). The phytogenic surfactants are released from decaying roots, and can be found in considerable amounts in the rhizosphere, where phosphatidylcholine (PC), the most important component of lecithin, is the major phospholipid (Soeder et al., 1996).

Cyclodextrins are another group of substances that enhanced the apparent solubility and biodegradation of HOCs in aqueous solutions and contaminated soil (Boyle, 2006). These substances can imitate the function of surfactants since they can form soluble complexes with hydrophobic compounds. Cyclodextrins have a non-polar cavity into which the HOCs partition to form inclusion complexes and a polar exterior that provides the molecule with a relatively high aqueous solubility. Moreover they are of interest in microbial processes because they do not exhibit the toxicity of many synthetic surfactants (Singh et al., 2007).

The most important characteristic of biosurfactants is their environmental acceptability, as they are biodegradable, have lower toxicity than synthetic surfactants, their own specific action, effectiveness at extremes of temperature, pH and salinity, and are ecologically safe, as it has been reviewed by Kosaric (1992) and Desai and Banat (1997). These properties have allowed use of biosurfactants in the remediation of inorganic compounds such as heavy metals (Kosaric, 1992; Zouboulis et al., 2003), and in the remediation of organic compounds such as hydrocarbons (Franzetti et al., 2008). Also, the ability to reduce the interfacial tension of oil in water has allowed applied of biosurfactants for the removal of water from emulsions prior to processing (Mulligan, 2005) and therefore they are applied in oil recovery (Plaza et al., 2008; Abdolhamid et al., 2009). Moreover, natural surfactants have been used in the food processing industry, and the health care and cosmetics industries (Desai and Banat, 1997). The properties of biosurfactants have generated a large number of investigations, which have allowed identification of new microorganism producers of natural surfactants, determination of their structure, finding

new sources of carbon and energy, enhancing the production processes, and generating several patents (Shete et al., 2006).

2.3 Influence of biosurfactants on the bioavailability of hydrophobic organic compounds

The bioavailability of HOCs can be enhanced by biosurfactants through the following mechanisms: emulsification of non-aqueous phase liquid contaminants (Edwards et al., 1991; Volkering et al., 1998; Jiménez Islas et al., 2010), enhancement of the apparent solubility of the pollutants (Edwards et al., 1991; Volkering et al., 1995), and facilitated transport of the pollutants from the solid phase (Yeom et al., 1996; Jiménez Islas et al., 2010). These mechanisms may cause enhanced mass transport and their relative contributions strongly depend on the physical state of the pollutants (Volkering et al., 1998). A fourth possible mechanism has been suggested by Tang et al. (1998) and Poeton et al. (1999): the biosurfactants help microorganisms adsorb to soil particles occupied by the contaminant, thus decreasing the diffusion path length between the sites of adsorption and the site of bio-uptake by the microorganisms.

In the first mechanism, emulsification of non-aqueous phase liquid contaminants, the biosurfactants can decrease the interfacial tension between and aqueous and non-aqueous phase. This may guide the formation of micro and macro emulsions. This results in an increase in the contact area, enabling improved mass transport of the contaminants to the aqueous phase and in mobilization of sorbed liquid-phase contaminants (Edwards et al., 1991, Volkering et al., 1998).

The second mechanism, enhancement of the apparent solubility of the HOCs, is due to presence of micelles that contain high concentrations of HOCs in the hydrophobic center of the micelles (Edwards et al., 1991, Volkering et al., 1995). Brown (2007) explains the apparent aqueous solubility of the contaminant as the sum of the aqueous (C_{aq}) and micellar (C_{mic}) HOC concentrations. Edwards et al. (1991) established that micellar-phase HOC concentration can be modeled using a linear partition relationship of the form:

$$C_{mic} = k_{mic}\, S_{mic}\, C_{aq} \qquad (1)$$

where k_{mic} is the HOC-micelle partition coefficient and S_{mic} is the micelle concentration. The S_{mic} correspond to difference between the total surfactant concentration and their CMC.

Finally, facilitated transport of the contaminants from the solid phase can involve several processes, such as the interaction of contaminants with single biosurfactant molecules, the interaction of surfactants with separate-phase or sorbed hydrocarbons, the mobilization of contaminants by swelling of the organic matrix, and the mobilization of contaminants trapped in soil caused by lowering of the surface tension of the soil particle pore water in soil particles, as it has been reviewed by Volkering et al. (1998).

The use of biosurfactants can improve the bioremediation processes by mobilization, solubilization or emulsification (Urum and Pekdemir, 2004; Nguyen et al., 2008). The mobilization and solubilization mechanisms are promoted low-molar mass biosurfactants, at below and above the CMC, respectively. Whereas, the emulsification processes is promoted by high-molar mass biosurfactant (Urum and Pekdemir, 2004; Pacwa-Plociniczak et al., 2011).

2.3.1 Emulsification and solubilization of hydrophobic organic contaminants by biosurfactants

Whang et al. (2008) studied the capacity of rhamnolipid and surfactin to reduce surface tension. The biosurfactants were produced by *P. aeruginosa* J4 and *B. subtilis* ATCC 21332, respectively. The results showed that the biosurfactants were able to reduce surface tension to less than 30 from 72 dynes cm^{-1} with CMC values of 45 and 50 mg L^{-1} for surfactin and rhamnolipid, respectively. Also, the results of diesel dissolution experiments demonstrated that the diesel solubility was enhanced with increased biosurfactant addition.

Most studies of the effect of surfactants on the solubilization of polycyclic aromatic hydrocarbons (PAHs) have been performed under mesophilic conditions. Related to this, Wong et al. (2004) evaluated the influence of TW80, TX100 and the biosurfactants produced from *P. aeruginosa* strain P-CG3 and *P. aeruginosa* strain ATCC 9027 on the solubilization of phenanthrene under thermophilic conditions. They found that the surfactants enhanced the solubility of phenanthrene at 50ºC. The biosurfactant from P-CG3

was the most effective with a 28-fold increase in apparent solubility of phenanthrene at a concentration of 10 x CMC, compared with the controls (TW80 and TX100).

Franzetti et al. (2009) determined that the BS29 bioemulsans, produced by *Gordania* sp. strain BS29, effectively remove crude oil and PAHs from soil. The crude oil removal by BS29 bioemulsans is comparable with rhamnolipid in the same experimental conditions.

Barkay et al. (1999) evaluated the effect of alasan in the enhancement of solubilization of PAHs. Alasan is a high-molecular-weight bioemulsifier complex of an anionic polysaccharide and proteins. The concentration of solubilized PAHs increased linearly with the addition of the biosurfactant (50 to 500 μg mL^{-1}). The apparent aqueous solubilities of PAHs were increased higher than their solubilities without alasan. The results of physicochemical characterization of the solubilization activity suggest that alasan solubilizes PAHs by physical interaction, likely of the hydrophobic nature, and that this interaction is slowly reversible. Moreover, the increase in apparent aqueous solubility of PAHs does not depend on the conformation of alasan and is not affected by the formation of multimolecular aggregates of alasan above its saturation concentration. Also, alasan enhances the biodegradation of PAHs.

Tecon and van der Meer (2010) evaluated the effects of two types of biosurfactants produced by *Pseudomonas* sp. (cyclic lipopeptides and rhamnolipids) on phenanthrene bioavailability. They measured the bioavailability from growth rates on contaminants and from specific induction of a phenanthrene-responsive green fluorescent protein (GFP) reporter in *Burkholderia sartisoli* strain RP037. Their results showed that the co-culturing of strain RP037 with lipopeptide-producing bacterium *Pseudomonas putida* strain PCL1445 enhanced GFP expression, compared to a single culture, but this effect was not significantly different when strain RP037 was co-cultivated with a non-lipopeptide-producing mutant of *P. putida*. The addition of partially purified supernatant extracts from the *P. putida* lipopeptide producer also did not unequivocally enhance phenanthrene bioavailability for strain RP037, compared to controls. In contrast, a 0.1% rhamnolipid solution strongly augmented RP037 growth rates on contaminants and led to a significantly larger proportion of cells in culture with high GFP expression.

2.3.2 Desorption of hydrophobic organic compounds by biosurfactants

The HOCs in contact with the soil are associated with organic matter by different mechanisms: adsorption and electrostatic and covalent bonding (Alexander, 1995). Adsorption is the most important mode of interaction between soil and HOCs. Adsorption processes of these pollutants in soil occur from complete reversibility to total irreversibility. Prolonged exposure time to the pollutant decreases its bioavailability. The extent of adsorption depends on the properties of the soil (mineral and organic matter content) and of the contaminant (solubility, polarity, molecular structure), as has been reviewed by Semple et al. (2003) and Gevao et al. (2000). Hence, the application of biosurfactant solutions, in soil-water systems, may result in the transfer of HOCs from the soil-sorbed phase to the aqueous phase, allowing mass-transfer processes and biodegradation (Aronstein et al., 1991; Tiehm, 1994; Volkering et al., 1995; Jiménez Islas et al., 2010).

Biosurfactants can be effective in facilitating desorption of the pollutants from soil as a possible integral part of a biodegradation process (Mata-Sandoval et al., 2002) or in an aqueous soil washing method, where a biological or nonbiological process is subsequently applied to remove the contaminants from the recovered aqueous washing (Singh et al., 2007). Bioemulsifiers from bacterias were able to emulsify n-octane, toluene, xylene, mineral oil and crude oil and they looked promising for remediation application (Toledo et al., 2008). Urum and Pekdemir (2004) evaluated the ability of aqueous biosurfactant solutions for possible applications in washing crude oil contaminated soil. The results showed that the biosurfactants were able to remove significant amounts of crude oil from contaminated soil. Rhamnolipid removed up to 80% oil and lecithin about 42%. In comparison with distilled water washing, crude oil removal from soil using aescin (mixture of saponins), lecithin, saponin and tannin was not effective. These investigators propose that the removal was due to mobilization, caused by the reduction of surface and interfacial tensions. On the other hand, Kang et al. (2010) investigated the effectiveness of sophorolipid in washing and biodegradation of hydrocarbons and crude oil in soil on a laboratory scale. The results showed that the addition of this biosurfactant to soil enhanced the washing and the biodegradation of the tested hydrocarbons.

Another important aspect to consider is the sorption of biosurfactants onto soil, a condition that can cause natural surfactant losses, which in turn reduce the performance of the solubilization of hydrophobic contaminants (Chu, 2003; Zhou and Zhu, 2007), decrease the remediation efficiency and result in an increase in remediation time and costs (Yu et al., 2007; Zhou and Zhu, 2008). At biosurfactant concentrations below the CMC, competitive adsorption of an organic compound by soil and by a biosurfactant in solution may occur, which may cause an increase or a decrease in the desorption of the contaminant from soil, depending on the characteristics of the soil and the organic compound, as has been reviewed by Rodríguez-Cruz et al. (2004). Pei et al. (2009) examined the effect of biosurfactant on the sorption of phenanthrene onto the original or H_2O_2-treated black loamy soil and red sandy soil. The result showed that organic matter played an important role in phenanthrene sorption onto the soil evaluated. The changes values of partition coefficient suggested that biosurfactant inhibited phenanthrene sorption onto the black loamy soil, however facilitated phenanthrene, sorption onto the red sandy soil. On the other hand, was observed that biosurfactant could also be sorbed onto soils. The maximal sorption capacity of the red sandy soil was 76.9 μg g^{-1}, which was 1.31 times that of black loamy soil. Moreover, biosurfactant was degraded in the two selected soils, and 92% was mineralized after 7 days of incubation. It implied that biosurfactant should be added frequently in remediation process of PAH-contaminated soils. The research of Van Dyke et al. [1993] showed that the ability of UG2 rhamnolipid, produced by *P. aeruginosa* UG2, to enhance removal of PAHs into the aqueous phase was affected by the soil type, hydrocarbon equilibration time, and biosurfactant adsorption to soil.

2.3.3 Influence of biosurfactants on the desorption and solubilization of aged chemicals in soil

Organic compounds freshly added to soils are bound almost exclusively to the soil particle surfaces (adsorption) and their desorption is almost complete after a short period of time (Hatzinger and Alexander, 1995; Alexander, 2000). However, if the time of contact between a pollutant and soil increases, decrease in chemical and biological availability occurs, called “ageing” or “sequestration” (Alexander, 1995; Hatzinger and Alexander,

1995; Semple et al., 2003). In the process of ageing, the interactions between soil and HOCs are affected by: the soil organic matter, both its amount and its nature; inorganic components with particular consideration to pore size and structure; microbial activity; and pollutant concentration, as it has been reviewed by Semple et al. (2003).

Over the past few years, numerous researchers have studied the biosurfactant-enhanced desorption of organic contaminants adsorbed onto soil. However, few works reported in the literature have addressed the influence of the time of residence in the soil, or ageing time, of organic pollutants on their desorption in soil-water-surfactant systems. Fava et al. (2004) evaluated the effects of soya lecithin (SL) on the desorption of PAHs in an aged-contaminated soil and they obtained a faster and more extensive overall removal of PAHs accompanied by a large soil detoxification under slurry-phase conditions. After 150 days of incubation at room temperature, about 60% of the original PAHs were biodegraded. Berselli et al. (2004) investigated the effects of TX100 and the biogenic agents: cyclodextrins, humic substances, and rhamnolipids, on the washing of a soil historically contaminated with PAHs. The soil was washed in water with 1% of biogenic agents or TX100 and both the biogenic agents and the synthetic surfactant enhanced the capacity of water to elute organic contaminants from the soil. The biogenic agents sustained the biodegradation of contaminants by enhancing the availability to bacteria; in contrast, TX100 affected the bioremediation due to their toxic effects on bacterial biomass.

The results of Leonardi et al. (2007) showed that the addition of several surfactants (soybean oil, Tween 20, TW80 and olive-mill wastewater) to an aged soil with a negligible amount of the non-bioavailable fraction of PAHs had either a limited or even a negative impact on PAH degradation by *Irpex lacteus* and *Pleurotus ostreatus*.

Fava et al. (2003) studied the effects of methyl-β-cyclodextrins on the solubilization of polychlorinated biphenyls (PCBs) of two different real, aged contaminated soils in bench-scale reactors. They found that the addition of 0.5 and 0.1% of methyl-β-cyclodextrins increased the concentration of PCBs in the water phase in slurry-phase reactors.

In general, most pesticides used in agriculture are moderately hydrophobic compounds, with complex molecular structures that differ from hydrocarbons in their lower hydrophobicity and in the presence of a polar functional group. These compounds are also

strongly adsorbed by soil organic matter and desorption is limited (Rodríguez-Cruz et al., 2004). Their desorption rate decreased with an increase in ageing time. Wattanaphon et al. (2008) evaluated the ability of a BS biosurfactant produced by *Burkholderia cenocepacia* BSP3 to enhance pesticide solubilization for further application in environmental remediation. The BS biosurfactant was identified as a glucolipid, having a CMC of 316 mg l^{-1}. Moreover, it lowered the surface tension of deionized water to 25 ± 0.2 mN m^{-1} and exhibited good emulsion stability. The results showed that the application of the BS biosurfactant to facilitate pesticide solubilization demonstrated that this biosurfactant at concentrations below and above its CMC could enhance the apparent water solubility of methyl parathion, ethyl parathion and trifluralin.

2.4 Influence of biosurfactants on the degradation of hydrophobic organic contaminants by microorganisms

2.4.1 Biosurfactant-microorganism interactions

Biosurfactants have the potential to enhance the bioavailability of HOCs in contaminated sites, and therefore enhance the efficiency biodegradation processes. In general, the biosurfactants are considered as low or non-toxic (Desai and Banat, 1997). Soeder et al. (1996) showed that the SL had a lower bacterial toxicity than quillaya saponin. Flasz et al. (1998) determined that the synthetic surfactants presented higher toxicity and mutagenic effect, whereas that the natural surfactants were considered slightly non-toxic and non-mutagenic. Boyle (2006) evaluated the effects of various cyclodextrins (Gamma W8, Beta W7 M1.8 and Alpha W6 M1.8) and pentachlorophenol (PCP) on the radial growth of the white-rot fungus *Trametes hirsute*. The results at pH 4.9 showed that the cyclodextrin Gamma W8 eliminated the inhibitory effects of 10 mg L^{-1} of PCP and partially overcame those at 50 mg L^{-1}. Beta W7 M1.8 also alleviated inhibition, but the effect was less pronounced and Alpha W6 M1.8 had little effect. In control assays, without PCP, cyclodextrins did not affect radial growth of *T. hirsute.* On the other hand, Bustamante et al. (2011) found that the increase in SL concentration from 0 to 10 g L^{-1} caused an increase in mycelia growth of *Anthracophyllum discolor,* a white-rot fungus isolated from a Chilean

forest. However, biosurfactants can be exerting negative effects on the biodegrading microorganisms (Fava and Di Gioia, 2001), bacteria, fungi, algae, and virus, as it has been by Volkering et al. (1995), Muthusamy et al. (2008), and Banat et al. (2010). Some biosurfactants have antibiotic properties, which can inhibit spore germination, hyphal growth of some fungi. Das et al. (2008) showed that a biosurfactant from *Bacilus circulans,* different from surfactin produced from *B. subtilis,* was effective for Gram- negative and Gram-positive pathogenic and semipathogenic microorganisms. In the medical, cosmetics, and pharmaceutical areas, biosurfactant have been used to inhibit bacterial growth, cell lysis, tumor growth, synthesis of cell wall, and to stimulate enzymes, inhibit the adhesion of pathogenic organisms to solid surfaces and the recovery of purified intracellular proteins. More applications are reviewed in the articles published by Muthusamy et al. (2008) and Gharaei-Fathabad (2011). The effect of biosurfactants on the microorganisms will depended of factors, such as: biosurfactant concentration and bioavailability, environmental and cultural conditions, charge type of biosurfactants, and characteristic and properties of microorganisms as cellular ultraestructure (Van Hamme et al., 2006).

2.4.2 Effect of biosurfactants on the degradation of hydrophobic organic pollutants by white-rot fungi

Most of the studies have been directed towards removal of HOCs from soil using biosurfactants and added bacteria or indigenous soil microorganisms; but few studies have addressed the removal of hydrophobic pollutants from soil using biosurfactants and white-rot fungi, which indicates the necessity of evaluating the potential ligninolytic effect of this combination.

White-rot fungi have the ability to degrade a wide range of persistent or toxic environmental contaminants, such as PCBs, PAHs, PCP, pesticides, dioxins, and synthetic dyes, among others, which makes them good candidates for use in processes of bioremediation (Barr and Aust, 1994; Pointing, 2001; Eichlerová et al., 2005; Tortella et al., 2005; Rubilar et al., 2008; Rubilar et al., 2011). The potential of white-rot fungi resides in their enzymatic system, which is nonspecific, and secreted into the extracellular environment (Durán and Esposito, 2000). White-rot fungi secrete one or more of the three

enzymes that are essential for the degradation of lignin: laccase (Lac), lignin peroxidase (LiP), and Mn dependant peroxidase (MnP) (Reddy, 1995; Pointing, 2001; Eichlerová et al., 2005). These enzymes are secreted during secondary metabolism, as a result of nutrient depletion of carbon or sulphur, or especially, manganese and nitrogen (Hamman et al., 1997; Leung and Pointing, 2002). Other factors are also implicated, such as initial pH of the medium and incubation temperature (Bustamante et al., 2011), the level of aeration and agitation during incubation, the availability of mediator compounds and metals (Leung and Pointing, 2002; Mouso et al., 2003; Cordi et al., 2007), or the presence and concentration of surfactants (Rodríguez Couto et al., 2000; Ürek and Pazarlioğlu, 2005; Wang et al., 2008). Recently, Bustamante et al. (2011) determined that *A. discolor* produced more activity of MnP when SL was included in the growth medium. Yamanaka et al. (2008) found that supplementation of a *Trametes villosa* culture medium with a vegetable oil-surfactant emulsion induced MnP activity and higher Lac activity when copper was added. On the other hand, Zhou et al. (2007) showed that white-rot fungi degraded decabromodiphenyl ether (BDE-2009, a widely used flame retardant) and that TW80 and β-cyclodextrin could both increase the biodegradation.

2.5 Remediation applications

The addition of biosurfactants, bioemulsifiers, and/or biosurfactant-producing microorganisms can be used in soil biodegradation techniques, soil washing, and water and waste treatment (*in situ* and *ex situ*) (Urum and Pekdemir, 2004; Zhou and Zhu, 2008). Biosurfactants have also been found to be useful for oil spill remediation and for dispersing oil slicks into fine droplets and converting mousse oil into an oil-in-water emulsion (Toledo et al., 2008).

Barkay et al. (1999) examined the influence of the bioemulsifier alasan on the biodegradation fates of PAHs. The presence of alasan (500 μg mL^{-1}) more than doubled the rate of [^{14}C]fluoranthene mineralization and significantly increased the rate of [^{14}C]phenanthrene mineralization by *Sphingomonas paucimobilis* EPA505. Shin et al. (2006) used a rhamnolipid from *Pseudomonas* to remediate soil contaminated with phenanthrene by the combined solubilization-biodegradation process. They reported a high

percentage of removal in the solubilization step and a significant decrease of phenanthrene in the soil sample during the biodegradation. From their results, they suggest that the degradation of contaminants by specific species might not be affected by the residual biosurfactants following application of the solubilization process that they would not present negative effects to the environment, and that they could be combined with the biodegradation process to improve the removal efficiency.

Soeder et al. (1996) studied the influence of two phytogenic surfactants, quillaya saponin and SL, on the biodegradation of PAHs. They found that high concentrations of phytogenic surfactants efficiently solubilized phenanthrene and fluoranthene. On the other hand, Fava and Di Gioia (2001) evaluated the effects of SL on the bioremediation of PCBs in an artificially contaminated soil and, in this work; the SL enhanced the availability of PCBs, while SL was also found to be an excellent carbon source for the microorganisms. Similar results were obtained by Fava et al. (2004) when studying the influence of SL on the bioremediation of an aged- PAHs contaminated soil. These results suggest the capacity of a pythogenic surfactant, SL, to improve the bioavailability of HOCs in contaminated sites. In addition, they have other properties, such as being nontoxic and biodegradable.

The bioremediation of petroleum is carried out by microorganisms capable of utilizing hydrocarbons as a source of energy and carbon. These microorganisms are ubiquitous in nature and are capable of degrading various types of hydrocarbons, all with low solubility in water. The hydrocarbon-degrading microorganisms generally produce emulsifiers, so biosurfactants help to disperse the oil, increase the surface area for growth, remove the bacteria from the oil droplets after the utilizable hydrocarbon has been depleted (Ron and Rosenberg, 2002), and stimulate the indigenous bacterial population to degrade hydrocarbons at rates higher than those which could be achieved through addition of nutrients alone (Desai and Banat, 1997). Whang et al. (2008) investigated the application of a biosurfactant, rhamnolipid and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. Their results in diesel/water batch experiments showed that with the addition of 40 mg L^{-1} of surfactin, there was significantly enhanced biomass growth as well as increased diesel biodegradation (94%), compared with 40% in batch experiments without surfactin. A concentration of biosurfactant more than 40 mg L^{-1} decreased both biomass growth and diesel biodegradation. Addition of rhamnolipid to the

diesel-water systems from 0 to 80 mg L^{-1} increased biomass growth and diesel biodegradation. The application of surfactin and rhamnolipid stimulated the indigenous microorganisms for enhanced biodegradation of diesel-contaminated soil. On the other hand, *Ralstonia picketti* and *Alcaligenes piechaudii,* which are producers of biosurfactants, degraded crude oil over 80% in 20 days incubation (Plaza et al., 2008).

The EPS AD2 produced by *O. anthropi* strain AD2 was tested in soil microcosms and experimental biopiles. Also, its efficiency in mixtures with activated sludge from an oil refinery was tested. In soil microcosms the EPS emulsifier together with an oleophilic fertilizer (S200 C) increased the indigenous microbial populations as well as hydrocarbon degradation and therefore decreased the amount of hydrocarbon remaining. Similar effects were obtained in biopile assays amended with EPS emulsifier plus activated sludge (Calvo et al., 2008).

In soil washing, the recovery and reuse of biosurfactants would be the preferred option because of the expense involved in production, although, if they are labile, this may not be an option (Christofi and Ivshina, 2002). Rhamnolipids have been proposed as soil washing agents for an enhanced removal of organic pollutants and metals from soil. A potential limitation to the application of biosurfactants is sorption by soil matrix components. *Pseudomonas* spp. produce rhamnolipids, either in the monorhamnolipid form or, more frequently, as a mixture of the mono- and dirhamnolipid forms. Ochoa-Loza et al. (2007) demonstrated that monorhamnolipid sorption on soil matrix components is concentration-dependent, and that the monorhamnolipid form sorbs more strongly alone than when in a mixture of forms. Conte et al. (2005) compared the efficiency of a humic acid with that of common surfactants, SDS and TX100, and water in the washing of polluted soil in the contaminated industrial area of a chemical plant. The results showed that the water was unable to fully remove pollutants from the soil, whereas all the organic surfactants revealed similar efficiencies (up to 90%) in the removal of the pollutants from the soils. Hence, the use of solutions of natural humic acids appears to be a better choice for soil washings of highly contaminated soils due to their additional capacity to promote microbial activity, in contrast to chemical surfactants.

Biosurfactants may have applications in metal treatment (Soeder et al., 1996; Zouboulis et al., 2003; Aşçi et al., 2007). Heavy metals along with other metals and

minerals have been released from their natural chemical compounds through industrial activities and processes into rivers, lakes and other surface waters. Since the sediments that have become contaminated below these surface waters have large quantities of water after dredging, dewatering is necessary before using treatment techniques (Dahrazma and Mulligan, 2007). Rhamnolipids, due to their anionic nature, are able to remove metals from soil and ions such as cadmium, copper, lanthanum, lead and zinc due to their complexation ability (Mulligan, 2005). Dahrazma and Mulligan (2007) demonstrated that rhamnolipids have the capacity to remove heavy metals from sediments; the removal was up to 37% of Cu, 13% of Zn, and 27% of Ni, when the biosurfactant was applied in a continuous flow configuration. Rhamnolipids also presented the capacity for enhanced recovery of Cd(II) from kaolin, a soil component (Aşçi et al., 2007).

2.6 Future prospects

The continuous release of contaminants, organic and inorganic, through either natural or industrial processes, has led to the accumulation and contamination of soils and sediments, surface and groundwater. Several strategies have been studied and developed to decontaminate and restore these sites. However, the efficiency of these processes is limited principally due to low aqueous solubility of contaminants and, therefore, low availability to both physical-chemical processes and microorganism degraders.

The use of biosurfactants is presented as an attractive option because of its versatility, biodegradability, ecological safety and environmental acceptance. However, their high production cost limits their use in bioremediation processes. In this context, it is necessary to evaluate the culturing conditions that optimize their production, assess the economic use of new substrates, such as those arising from industrial waste, and to evaluate techniques of isolation and purification to make production more economically feasible.

The available information is related to studies under laboratory conditions, and little work has been done on a field scale. Therefore, more efforts are required to evaluate biosurfactant production *in situ* and their effect on the indigenous microorganisms, and to evaluate the efficiency and effectiveness of a bioremediation processes *in situ*.

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Chapter **3**

Effect of soya lecithin on the enzymatic system of the white-rot fungi *Anthracophyllum discolor*

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Effect of soya lecithin on the enzymatic system of the white-rot fungi *Anthracophyllum discolor*

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Abstract

The present work optimized the initial pH of the medium and the incubation temperature for ligninolytic enzymes produced by the white-rot fungus *Anthracophyllum discolor*. Additionally, the effect of soya lecithin on mycelial growth and the production of ligninolytic enzymes in static batch cultures were evaluated. The critical micelle concentration of soya lecithin was also studied by conductivity. The effects of the initial pH (3, 4 and 5) and incubation temperature (20, 25 and 30ºC) on different enzymatic activities revealed that the optimum conditions to maximize ligninolytic activity were 26ºC and pH 5.5 for laccase and manganese peroxidase and 30ºC and pH 5.5 for manganese-independent peroxidase. Under these culture conditions, the maximum enzyme production was 10.2, 484.5 and 112.5 U L^{-1} for laccase, manganese peroxidase, and manganese-independent peroxidase, respectively. During the study of the effect of soya lecithin on *A. discolor*, we found that the increase in soya lecithin concentration from 0 to 10 g L^{-1} caused an increase in mycelial growth. On the other hand, in the presence of soya lecithin, *A. discolor* produced mainly manganese peroxidase, which reached a maximum concentration of 30.6 ± 4.6 U L^{-1} after 25 days of incubation with 1 g L^{-1} of the surfactant. The other enzymes were produced but to a lesser extent. The enzymatic activity of *A. discolor* was decreased

when Tween 80 was used as a surfactant. The critical micelle concentration of soya lecithin calculated in our study was 0.61 g L^{-1}.

Keywords: Soya lecithin, white-rot fungi, *Anthracophyllum discolor*, ligninolytic enzymes, phytogenic surfactant

3.1 Introduction

Ligninolytic enzyme production in white-rot fungi is affected by many factors, such as the presence of inductors, temperature, pH, type and concentration of nutrients, and others (Kirk et al., 1978; Leung and Pointing, 2002; Wang et al., 2008). Some studies have demonstrated that the presence of a surfactant may increase extracellular enzyme production in various filamentous fungi, including the white-rot fungi (Grgič and Perdih, 2003; Jäger et al., 1985; Rodríguez Couto et al., 2000). Additionally, its application in bioremediation processes may allow for an increase in mass transfer and the availability of hydrophobic organic contaminants (Gao et al., 2007).

Surfactants are organic molecules with a polar or ionic hydrophilic group and a nonpolar or hydrophobic chain, known as the head and tail groups, respectively (Christofi and Ivshina, 2002; West and Harwell, 1992). At low concentrations in aqueous solutions, the surfactants are present as single molecules (Volkering et al., 1998; West and Harwell, 1992). Above the critical micelle concentration (CMC), surfactants form micelles that consist of monomers organized in a more or less spherical structure. The ability to form micelles in solution confers certain properties on surfactants such as emulsifying, foaming, dispersing, and the ability to act as a detergent, as has been reviewed by Desai and Banat (1997). The CMC depends on surfactant structure, composition, temperature, ionic strength, and the presence and types of organic additives in the solution (Fuguet et al., 2005**)**. At the CMC of surfactant solutions, a drastic change occurs in many physicochemical properties (surface tension, conductivity, or turbidity) (Hanna et al., 2005; Zana, 2005). Surfactants can be biologically produced by a wide variety of microorganisms, such as bacteria, yeast, and fungi. This group of surface-active agents includes phytogenic surfactants such as saponins and lecithins (Soeder et al., 1996).

The response of certain microorganisms to a surfactant will depend on several factors, such as cellular ultrastructure, surfactant concentration and bioavailability, and environmental and culture conditions (Van Hamme et al., 2006**)**. Soeder et al. (1996) showed that soya lecithin (SL) had a lower bacterial toxicity than quillaya saponin.

Several studies have reported that the addition of some surfactants increases extracellular enzyme production in various filamentous fungi (Grgič and Perdih, 2003; Jäger et al., 1985**)**. Nevertheless, the mechanism by which surfactants enhance enzyme production has not been established. Garon et al. (2002) evaluated the effect of surfactants on different fungal strains; their results showed the inhibition of fungal growth at the CMC by an anionic surfactant, while a nonionic surfactant was well tolerated at doses far above the CMC in most tested fungi.

White-rot fungi are widely studied for their ability to degrade a wide range of persistent or toxic environmental contaminants, such as chlorophenols, polycyclic aromatic hydrocarbons (PHAs), pesticides, synthetic dyes, and others (Barr and Aust, 1994; Reddy, 1995; Rubilar et al., 2008; Svobodová et al., 2006; Tortella et al., 2008). The potential of these microorganisms is based on their enzymatic system, which is unique, nonspecific, and secreted into the extracellular environment (Durán and Esposito, 2000). White-rot fungi secrete one or more of three extracellular enzymes that are essential for lignin degradation; those enzymes include lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) (Eichlerová et al., 2005; Reddy, 1995), and are often referred to as lignin-modifying enzymes. Ligninolytic systems have been widely studied in several white-rot fungi, such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pycnoporus cinnabarinus*, *Pleurotus ostreatus*, and *Bjerkandera adusta* (Leung and Pointing, 2002; Rubilar et al., 2007; Ürek and Pazarlioğlu, 2005; Wang et al., 2008). However, increasing attention has recently been paid to the evaluation of the lignin-modifying enzymes of new fungal strains that would not alter ecosystems. In preliminary studies, *Anthracophyllum discolor*, a native Chilean fungus, demonstrated ligninolytic activity with a high level of MnP production and pentachlorophenol degradation potential (Rubilar et al., 2007).

In this study, the initial pH of the medium and the incubation temperature for ligninolytic enzyme production were optimized by response surface methodology (RSM). Also, the effect of the natural surfactant SL on mycelial growth and ligninolytic enzyme

production by the white-rot fungi *A. discolor* was evaluated. The CMC of SL was experimentally determined.

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Microorganism and growth conditions

The white-rot fungi *A. discolor* was obtained from the culture collection of the Environmental Biotechnology Laboratory of the Universidad de La Frontera. The fungus was transferred from slant tubes to glucose malt extract agar plates (agar 15 g L^{-1}, glucose 10 g L^{-1}, malt extract 30 g L^{-1}, pH 5.2), maintained at 4ºC for each plate and then incubated at 30 ± 2ºC for 7 days. *A. discolor* was first grown in 1-L Erlenmeyer flasks containing 100 mL of modified Kirk medium (Tien and Kirk, 1988) and five 6-mm diameter agar plugs of active mycelium. The culture was incubated statically at 30 ± 2ºC for 7 days in darkness. After this, the fungal culture was homogenized in a sterilized blender for 1 min. The modified Kirk medium contained (per liter of distilled water) 10 g of glucose, 0.2 g of $C_4H_{12}N_2O_6$, 2 g of KH_2PO_4, 0.59 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$, 3.3 g of sodium acetate, and 10 mL of mineral salts. The initial pH of the medium was adjusted to 4.5 with either 1 N NaOH or 1 N HCl.

3.2.1.2 Surfactants

Natural soya lecithin (SL) surfactant was purchased from PRINAL®, contain 1.99 Mn mg L^{-1} analyzed by Mn as measured by atomic spectrophotometry in our laboratory. Tween 80 (TW80) is a synthetic surfactant that was purchased from Merck; it has a molecular weight of 1,310 g mol^{-1} and a density of 1.07 mg L^{-1}.

Soya lecithin and TW80 were chosen for this study due to the fact that no studies had evaluated the effect of SL on the enzyme production of white-rot fungi. TW80 demonstrates no toxic effect on either fungal growth or the stimulation of ligninolytic

enzyme production (Ding et al., 2008); both are nonionic surfactants, which means that they are nontoxic (Van Hamme et al., 2006; Volkering et al., 1998).

3.2.2 Methods

3.2.2.1 Optimization of pH and temperature on the production of ligninolytic enzymes of A. discolor by response surface methodology (RSM)

Effects of the initial pH of the medium (4, 5 and 6) and the temperature (20, 25 and 30ºC) on the ligninolytic activity of *A. discolor* were evaluated using 50 mL of modified Kirk medium in 100-mL Erlenmeyer flasks. The initial pH of the medium was adjusted with either 1 N NaOH or 1 N HCl. The culture medium was autoclaved at 121 ± 2ºC for 15 min, and 2 mL of homogenized mycelium was used as the inoculum. Ligninolytic activities of laccase (Lac), lignin peroxidase (LiP), manganese-independent peroxidase (MiP), and manganese peroxidase (MnP) were evaluated periodically for up to 22 days. These assays were carried out in triplicate for each treatment.

3.2.2.2 Effect of SL on the mycelial growth of A. discolor

This test was used to determine the effect of SL (0, 0.3, 0.4, 1, and 10 g L^{-1}) on the mycelial growth of *A. discolor* on potato dextrose agar (PDA). Several SL concentrations were added to the PDA medium, which was autoclaved at 121 ± 2ºC for 15 min and aseptically transferred to Petri dishes. Plates were inoculated at the plate center with one 6-mm agar plug of active *A. discolor* mycelia containing a 7-day-old culture on PDA medium. The plates were incubated in the dark at 26 ± 2ºC. Mycelia growth was measured daily from the edge of the agar disk to the inner edge of the Petri dish. PDA plates without SL and inoculated with *A. discolor* were used as a control. The results are reported as the mean of four replicates for each SL concentration.

3.2.2.3 Experimental determination of the critical micelle concentration of SL by conductivity

The critical micelle concentration (CMC) was determined by the breakpoint of conductivity versus the surfactant concentration curve (0.1–1.5 g L^{-1} SL). The electrical conductivity of each surfactant solution was measured with a HACH sensION5 conductivity meter at 25 ± 2ºC. All solutions were prepared in 50 ml of modified Kirk medium at the optimized pH of 5.5. Deionized water was used to prepare the SL solution and modified Kirk medium in these experiments. The results are reported as the mean of three replicates.

3.2.2.4 Effect of SL and TW 80 on the production of ligninolytic enzymes by A. discolor

The effects of SL and TW80 were evaluated at concentrations of 0–1 g L^{-1} using modified Kirk medium (50 mL) in 100-mL Erlenmeyer flasks. The culture medium was autoclaved at 121 ± 2ºC for 15 min, and 2 mL of homogenized mycelium was used as the inoculum. The cultures were statically incubated at 26 ± 2ºC. The ligninolytic activity of Lac, LiP, MiP, and MnP were evaluated periodically for up to 33 days. The results are reported as the mean of three replicates per treatment, with the standard deviation of the mean represented by error bars.

3.2.3 Analytical determinations

3.2.3.1 Enzyme assays

The enzymatic activities were measured from the supernatant of a previously centrifuged sample (2 min at 3,000 rpm at 4ºC) of the culture medium.

The Lac activity was determined with 2,6-dimethoxyphenol (DMP) as the substrate in sodium malonate (pH 4.5). The enzyme activity unit was defined as an increase in absorbance per minute at 468 nm and 30ºC (deJong et al., 1994). The MnP activity was determined by monitoring the oxidation of 2,6-DMP spectrophotometrically at 30ºC. The reaction mixture (1 mL) contained 200 µL of 250 mM sodium malonate (pH 4.5), 50 µL of

20 mM 2,6-DMP, 50 µL of 20 mM $MnSO_4 \cdot H_2O$, and 600 µL of supernatant. The reaction was initiated by adding 100 µL of 4 mM H_2O_2, monitored at 468 nm and corrected by the Lac activity. The MiP activity was determined in a reaction mixture containing 200 µL of 250 mM sodium malonate (pH 4.5), 50 µL of 20 mM 2,6-DMP, 100 µL of 20 mM EDTA, and 550 µL of supernatant. The reaction was initiated by adding 100 µL of 4 mM H_2O_2, monitored at 468 nm, and corrected by the Lac activity (deJong et al., 1994). The extinction coefficient was 49,600 M^{-1} cm^{-1}. The LiP activity is based on the oxidation of veratryl alcohol. The reaction mixture contained 1,420 µL of disodium tartrate dihydrate (0.1 M, pH 3.0), 400 µL of veratryl alcohol (20 mM), and 100 µL of supernatant. The reaction was initiated by adding 80 µL of H_2O_2 (10 mM) (Castillo et al., 1997) and was monitored at 310 nm for 2 min. The extinction coefficient was 93,000 M^{-1} cm^{-1}.

3.2.3.2 Experimental design and statistical analysis

The independent variables selected in this study were the initial pH of the medium (4, 5, and 6) and the temperature (20, 25, and 30ºC). Central composite methodology was applied according to the Design Expert 6.0.6 statistical software (Stat-Ease, Minneapolis, MN, USA). Selection of the low and high levels of each variable was based on the results of a single-factor method investigation.

The experimental design consisted of 11 trials, including three replications at the central point. All experiments were performed in triplicate, and the average of each enzymatic activity was taken as the dependent variable. Statistical analysis and analyses of variance (ANOVA) were carried out using the Design Expert 6.0.6 statistical software (Stat- Ease, Minneapolis, MN, USA).

Where required, the data were discussed using the ANOVA and the Duncan test was used to compare means. The statistical significance level for all treatments was 5%.

3.3 Results

3.3.1 Optimization of pH and temperature on the production of ligninolytic enzymes of *A. discolor* by RSM

To examine the combined effect of different culture conditions (initial pH of the medium and the incubation temperature) on ligninolytic enzyme production, a central composite design of two variables at three levels of work each plus three center points, which led to a total of 11 trials, was performed.

The everyday values of different enzyme activities (Lac, LiP, MnP, and MiP) were used to determine the accumulated enzyme activity. The values of the variables analyzed and the responses measured are shown in Table 3.1.

The accumulated enzyme activity data were fitted by a polynomial quadratic equation (Table 3.2). The lack-of-fit value indicates variation due to model inadequacy. Under the conditions tested, the lack-of-fit test was not significant for the experimental data for LiP activities, which were, therefore, not adjusted to any type of equation.

Table 3.1 Accumulated ligninolytic activity (U L^{-1} ± SD) of *A. discolor* in liquid medium obtained under different culture conditions after 22 days of incubation

Temperature (ºC)	pH	Lac	LiP	MnP	MiP
20	4	0.4 ± 0.1	23.7 ± 2.2	8.1 ± 2.1	11.1 ± 0.7
	5	3.4 ± 0.1	73.1 ± 8.4	236.2 ± 3.2	57.6 ± 3.6
	6	4.1 ± 0.2	22.6 ± 2.8	328.3 ± 5.6	110.2 ± 2.5
25	4	0.2 ± 0.1	21.5 ± 2.0	2.4 ± 0.2	2.1 ± 1.0
	5	12.2 ± 0.5	24.7 ± 5.4	408.2 ± 4.4	98.8 ± 7.2
	6	2.1 ± 0.1	69.4 ± 6.0	585.0 ± 6.0	98.5 ± 2.7
30	4	1.0 ± 0.3	32.3 ± 2.2	2.3± 0.3	6.4 ± 4.4
	5	11.8 ± 0.7	10.8 ± 2.2	596.4 ± 6.7	170.5 ± 4.9
	6	2.1 ± 0.2	32.2 ± 4.3	186.2 ± 5.4	47.1 ± 4.1

Standard deviation (SD) is the mean of three replicates

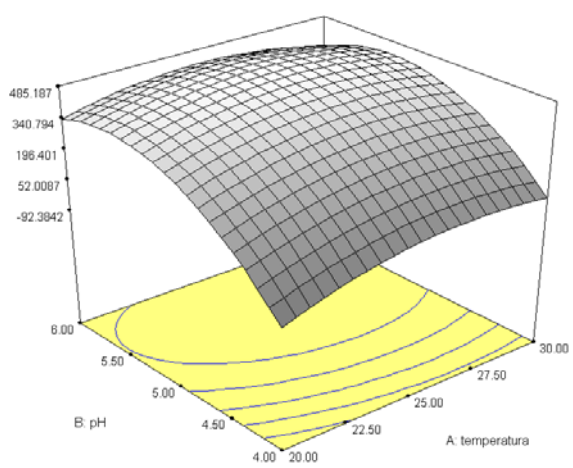
The equations show that there were negative interactions between the independent variables for enzyme production (-0.1, -6.8 and -2.9 for Lac, MnP and MiP, respectively). However, it also showed a positive effect for both variables in the linear term, but with a greater influence of pH. This result indicates that the production of Lac, MnP and MiP increased with increasing values of initial pH of the medium and incubation temperature. On the other hand, the negative quadratic coefficient of pH indicates the existence of a maximum activity of Lac, MnP and MiP as a function of pH. The response surface plot obtained as a function of the initial pH of the medium versus the incubation temperature is shown in Fig. 3.1.

Table 3.2 Experimental equations of Lac, MnP, and MiP produced by *A. discolor* to evaluate the combined effect of initial pH of the medium and incubation temperature

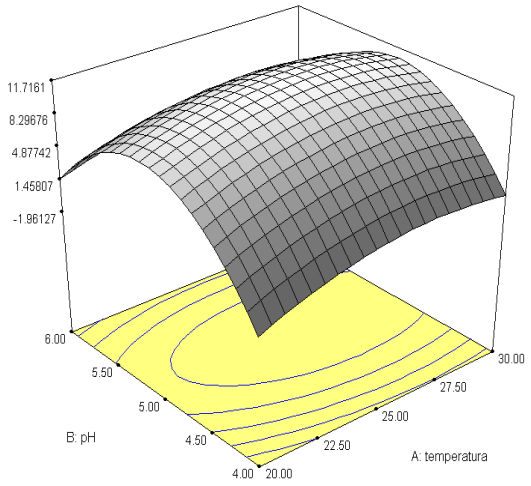
Enzymatic activity	Experimental equations	R^2
Lac	$-280.2 + 5.0\ T + 89.3\ pH - 0.1\ T^2 - 8.5\ pH^2 - 0.1\ T\ pH$	0.8
MnP	$-8637.8 + 204.4\ T + 2394.0\ pH - 3.3\ T^2 - 204.2\ pH^2 - 6.8\ T\ pH$	0.7
MiP	$-1901.8 + 8.6\ T + 712.4\ pH + 0.2\ T^2 - 60.0\ pH^2 - 2.9\ T\ pH$	0.8

Figure 3.1 shows that MnP, Lac and MiP demonstrated no variations when the incubation temperature varied between 20 and 30ºC. However, it is shown that the initial pH of the medium has a greater effect on the ligninolytic activity of *A. discolor*.

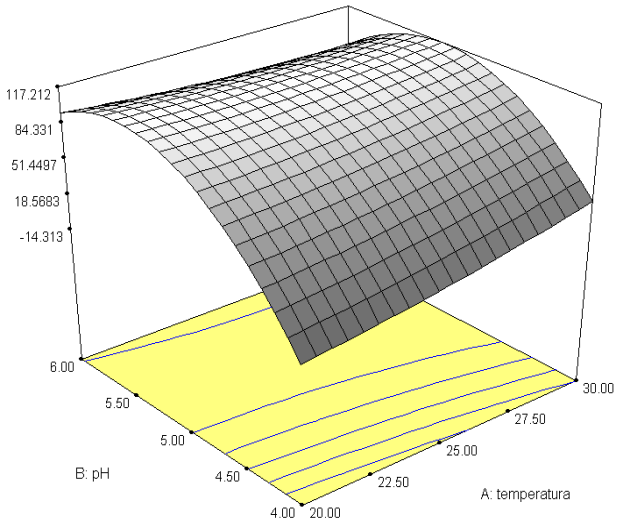
The optimal conditions to obtain maximal ligninolytic enzyme production were an initial pH of the medium of 5.5 and an incubation temperature of 26ºC for MnP and Lac. For MiP, those conditions were pH 5.5 and 30ºC. The modeled values predict that the maximum enzyme productions that can be obtained by using the abovementioned, optimized conditions of the variables are 484.4 U L^{-1} for MnP, 10.2 U L^{-1} for Lac, and 112.5 U L^{-1} for MiP.



a. MnP activity (U L^{-1})



b. Lac activity (U L^{-1})



c. MiP activity (U L^{-1})

Fig. 3.1 Response surface plot showing the effects of the initial pH of the medium and the incubation temperature and their mutual effect on the production of MnP (a), Lac (b) and MiP (c) by *A. discolor*.

3.3.2 Effect of SL on the mycelial growth of *A. discolor*

Figure 3.2 presents the results of fungal growth in the presence of different concentrations of SL (0, 0.3, 0.4, 1, and 10 g L^{-1}). As the SL concentration increased from 0 to 10 g L^{-1}, an increase in mycelial growth was observed. After 4 days of incubation, the mycelium of *A. discolor* grew 3.3, 3.7, 3.4, 3.5, and 3.9 cm when PDA was supplemented with 0, 0.3, 0.4, 1, and 10 g L^{-1} SL, respectively. At all of the tested concentrations of SL, including the control with no natural surfactant, the mycelium of *A. discolor* covered the full Petri dish within 5 days.

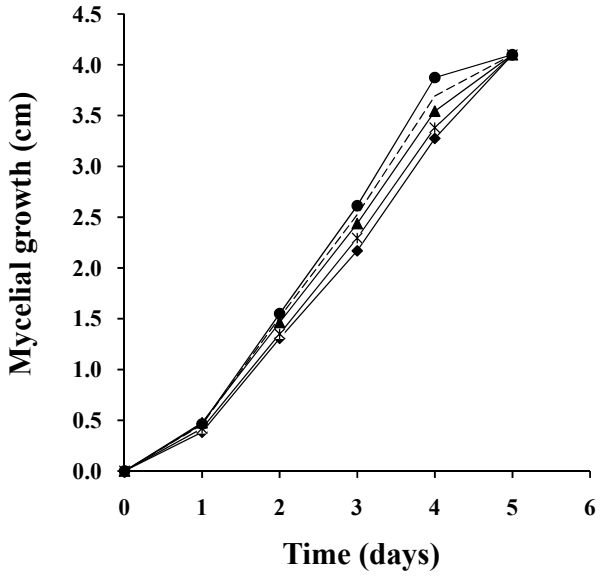


Fig. 3.2 Effects of SL on mycelial growth of *A. discolor* on PDA at 26ºC and pH 5.5. The evaluated SL concentrations were 0 g L^{-1} (*filled diamonds*), 0.3 g L^{-1} (*dashed line*), 0.4 g L^{-1} (*asterisks*), 1 g L^{-1} (*filled triangles*), and 10 g L^{-1} (*filled circles*). The data points represent the average values of four replicates for each SL concentration.

3.3.3 Experimental CMC determination of SL

Figure 3.3 shows the variation in electrical conductivity in response to changing SL concentrations in modified Kirk medium. The specific conductivity (1/K) vs. surfactant concentration (1/SL) plot shows two straight lines with different slopes. The first zone is

characterized by the equation y = -0.0042x + 0.2622, which corresponds to the concentration range above the CMC. At higher surfactant concentrations, micelles start to form and a slope change appears because the conductivity increases in a different manner. The equation y = -0.0002x + 0.2556 represents the concentration range below CMC when only monomers of surfactant exist in the solution. The intersection of these two straight lines is the CMC value of the surfactant (Fuguet et al., 2005; López-Díaz and Velázquez, 2007). The CMC value of SL in modified Kirk medium at 25 ± 2ºC obtained by conductivity measurements in this study was 0.61 g L^{-1}.

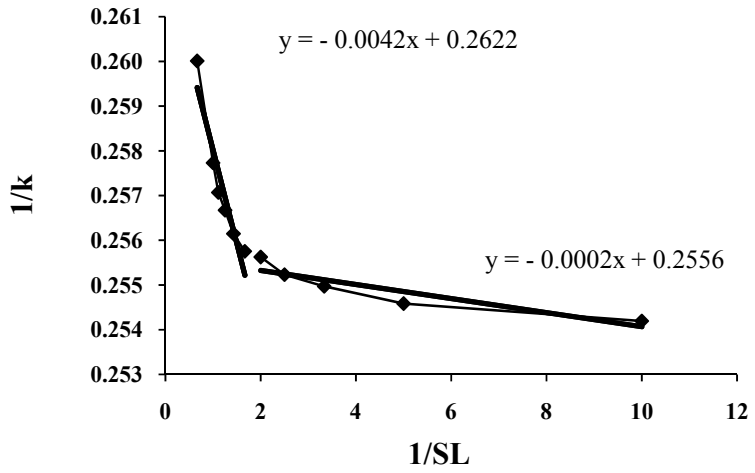


Fig. 3.3 Variation of the electrical conductivity at several concentrations of the nonionic surfactant SL at 25 ± 2ºC and pH 5.5. The points represent the average values of three replicates.

3.3.4. Effect of SL and TW80 on the production of ligninolytic enzymes by *A. discolor*

The SL and TW80 concentrations of 0, 0.15, 0.3, 0.4, 0.5, and 1 g L^{-1} were evaluated. The production profiles of the ligninolytic enzymes in liquid culture of *A. discolor* containing SL and TW80 are depicted in Figs. 4 and 5, respectively. Lac (Fig. 3.4a) showed low activity in all treatments and reached a maximum of 5.3 U L^{-1} after 30 days with 1 g L^{-1} of SL. The LiP (Fig. 3.4b) activity reached a maximum of 30.1 U L^{-1} after 30 days of culture when the medium was supplemented with 0.15 and 0.3 g L^{-1} of SL. MiP (Fig. 3.4c) production increased steadily, although it showed low activity in all treatments, and did not

exceed 5 U L^{-1} with 0.15 g L^{-1} of SL after 17 days of incubation. Finally, MnP (Fig. 3.4d) demonstrated low activity during the first 13 days of incubation, which did not exceed 10 U L^{-1} for all evaluated conditions. The MnP production then increased steadily at different speeds with increasing SL concentration, and reached a maximum of 30.6 ± 4.6 U L^{-1} on day 25 when the medium was supplemented with 1 g L^{-1} of SL. Low Lac production (Fig. 3.5a) under all of the tested concentrations of TW80 was observed; production did not exceed 4 U L^{-1}. The LiP production (Fig. 3.5b) demonstrated a maximum of 30.1 U L^{-1} after 30 days of culture when the medium was supplemented with 0.15 and 0.3 g L^{-1} of SL. The MiP activity (Fig. 3.5c) was low but increased steadily, reaching a maximum of 7.2 U L^{-1} with 0.3 g L^{-1} of TW80 after 33 days of incubation. Finally, the MnP activity was low during the first 13 days of incubation and did not exceed 9.1 U L^{-1} for all evaluated conditions. After this period of time, the MnP production increased slowly in all treatments. The maximum MnP production was 42.1 ± 3.1 U L^{-1} with 0.3 g L^{-1} of TW80 on day 33.

Figures 3.4 and 3.5 demonstrate that the ligninolytic activity of *A. discolor* gradually increased after 4 days of incubation when the culture medium was not supplemented with SL or TW80. Under these conditions, the Lac, LiP, and MnP activities did not exceed 12 U L^{-1}. *A. discolor* produced mainly MnP, which reached a maximum of 55.2 U L^{-1} after 33 days of incubation.

Table 3.3 indicates that MnP activity accumulates after 33 days of incubation. At equal concentrations of SL and TW80, the accumulated MnP activity was significantly different. The maximum accumulated activities of MnP in *A. discolor* were 162.5 ± 2.1 and 133.5 ± 3.0 U L^{-1} when the culture medium was supplemented with 1 g L^{-1} of SL and 0.30 g L^{-1} of TW80, respectively. However, the accumulated activity of MnP in culture medium without surfactant was significantly different with respect to those activities obtained with medium supplemented with SL or TW80.

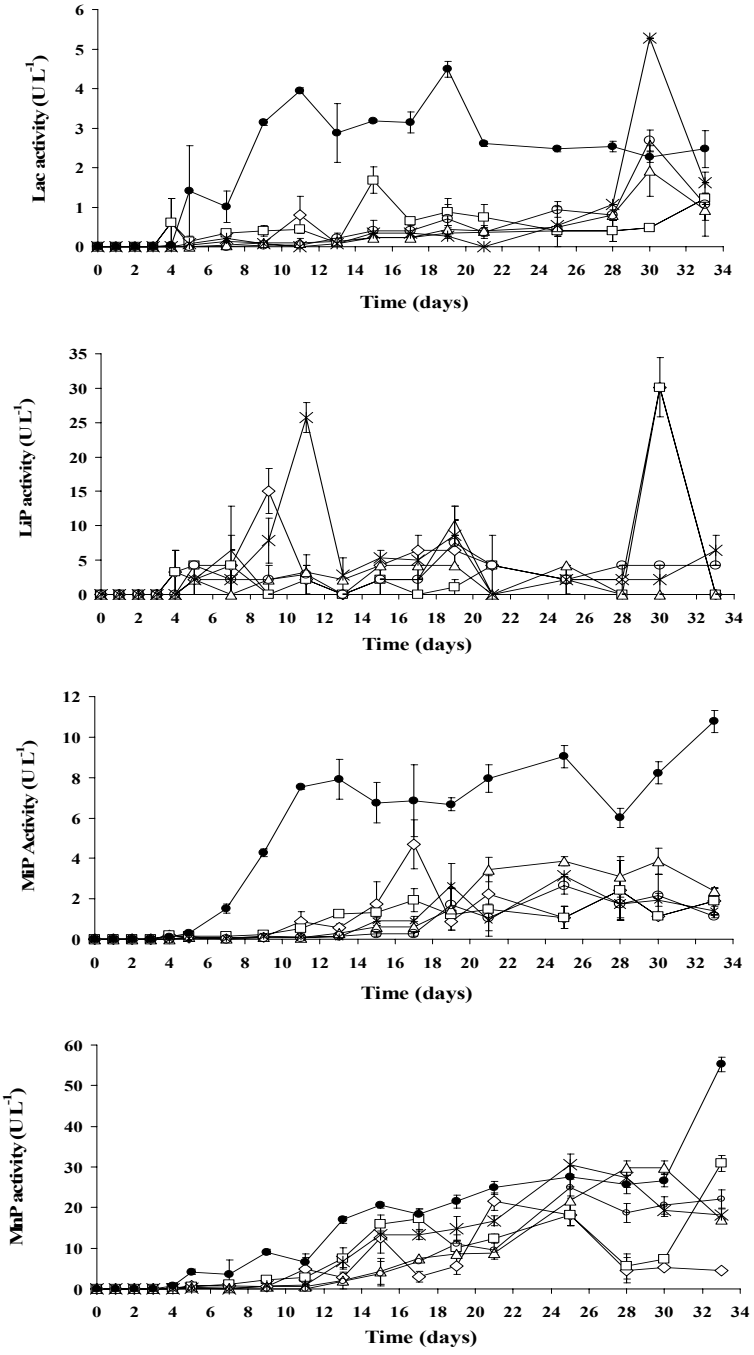


Fig. 3.4 Effect of different SL concentrations on the ligninolytic activity of *A. discolor* in liquid medium at 26ºC and pH 5.5. The points represent the average values of three replicates. The evaluated SL concentrations were 0 g L^{-1} (*filled circles*), 0.15 g L^{-1} (*open diamonds*), 0.3 g L^{-1} (*open squares*), 0.4 g L^{-1} (*open triangles*), 0.5 g L^{-1} (*open circles*), 1 g L^{-1} (*asterisks*).

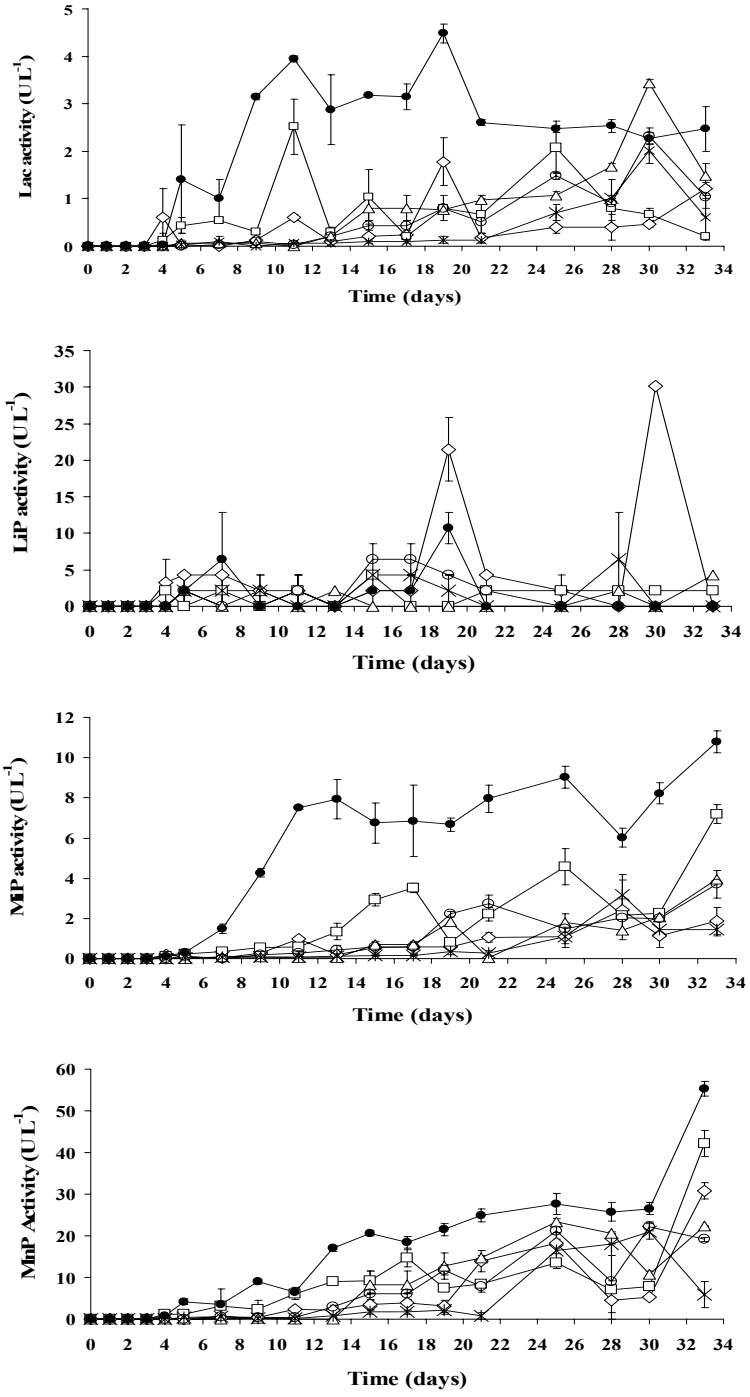


Fig. 3.5 Effect of different TW80 concentrations on the ligninolytic activity of *A. discolor* in liquid medium at 26ºC and pH 5.5. The points represent the average values of three replicates. The evaluated TW80 concentrations were 0 g L^{-1} (*filled circles*), 0.15 g L^{-1} (*open*

diamonds), 0.3 g L^{-1} (*open squares*), 0.4 g L^{-1} (*open triangles*), 0.5 g L^{-1} (*open circles*), 1 g L^{-1} (*asterisks*).

Table 3.3 Accumulated MnP activity (U L^{-1} ± SD) of *A. discolor* in liquid culture with several concentrations of SL or TW80 at pH 5.5 and 26ºC after 33 days of incubation

Surfactant	Surfactant concentration (g L^{-1})					
	0	**0.15**	**0.3**	**0.4**	**0.5**	**1**
SL	216.5 ± 2.1^{a}	111.4 ± 1.2^{g}	128.9 ± 1.1^{d}	127.9 ± 0.3^{d}	116.6 ± 3.8^{f}	162.5 ± 2.1^{b}
TW80		57.6 ± 1.9^{i}	133.5 ± 2.9^{c}	121.1 ± 1.3^{e}	107.7 ± 3.0^{g}	69.8 ± 0.9^{h}

The standard deviation (SD) is the mean of three replicates

Different letters indicate significant differences (Duncan test, $P \leq 0.05$)

3.4 Discussion

The ligninolytic activity of white-rot fungi depends on many factors, and each strain responds in a particular way to each of these factors. The combined study of two or more factors is important not only to evaluate the ligninolytic enzyme but also to explore possible interactions between variables. In order to enhance the production and stability of ligninolytic enzymes of *A. discolor*, several culture conditions were evaluated. First, the initial pH of the medium and the incubation temperature were studied, followed by the effect of SL.

Response surface methodology could be used for the optimization of culture conditions to obtain the maximum production of ligninolytic enzymes by *A. discolor*, which lowers the cost of biotechnological processes. The experimental design employed showed that there was no change in the enzymatic activities of *A. discolor* when the incubation temperature varied between 20 and 30ºC. However, the initial pH of the medium had a greater effect on the ligninolytic activity of these white-rot fungi. When evaluating the combined effect of the initial pH of the medium and the incubation temperature, *A. discolor* was found to mainly produce MnP, with low production of Lac (Table 3.1). In the study by Vukojević et al. (2006), there was no ligninolytic enzyme production by *Ganoderma lucidum* when the pH of the medium varied between 2.0 and 7.0 at room temperature using

a rotary shaker. On the other hand, *Stereum hirsutum* demonstrated high Lac activity at pH 5, while MnP production was high at an initial pH of 6 (Mouso et al., 2003).

Induction of the ligninolytic system is affected by many factors, such as the type of carbon and nitrogen source (Hamman et al., 1997; Leung and Pointing, 2002), the level of aeration and agitation during incubation, and the availability of low molecular weight mediator compounds and metals (Mouso et al., 2003; Soares et al., 2006; Wang et al., 2008). Ligninolytic activity is also affected by some oils and surfactants (Grgič and Perdih, 2003).

Some studies have demonstrated that the use of surfactants can stimulate fungal growth and enhance enzyme production. The results in Fig. 3.1 indicate that SL does not appear to have a negative effect on the fungal growth of *A. discolor*. Nonionic surfactants such as SL are often considered to be nontoxic (Van Hamme, et al., 2006; Volkering et al., 1998), and, therefore, do not affect the fungal growth of *A. discolor*. Several studies of chemical surfactants have shown that charge has an impact on toxicity; cationic surfactants are the most toxic and have been used as antimicrobials. Ding et al. (2008) found no negative effect of TW80 on *P. chrysosporium* growth. Garon et al. (2002) evaluated the toxicity of SDS, TX100, and TW80 on fungal strains. The results showed growth inhibition by SDS (anionic surfactant), whereas TX100 and TW80 (nonionic surfactants) were well tolerated at the doses evaluated in most of the tested fungi.

The CMC value is one of the most important surfactant properties. The CMC value of SL in modified Kirk medium at 25ºC obtained by conductivity was 0.61 g L^{-1}, which is greater than that obtained by Soeder et al. (1996), who reported a value of 0.383 g L^{-1}. Other CMC values of SL have been reported. Wu and Wang (2003) determined that the CMC value was 13.6 mg mL^{-1}, Urum and Pekdemir (2004) obtained a value of 0.4 (mass %), and Wu and Wang (2003) reported CMC values between 1.18 and 4.13 mg mL^{-1}. This difference can be explained because the CMC value depends on many factors, such as the determination method, phospholipid composition, electrolyte addition, buffer pH, solution temperature, ionic strength of the aqueous solutions, and the presence of organic additives, which makes this value different from that determined in pure water (Fuguet et al., 2005). The importance of determining the CMC of SL lies in the fact that surfactant presence, in particular at concentrations above the CMC value, often had a toxic effect on

microorganism degrading, as reported by Laha and Luthy (1992). This negative effect can be explained by disruption of the cell membranes through interactions with structural lipid components (Laha and Luthy, 1992). In this context, the CMC value can help to fix a limit of work when studying the surfactant effect on the enzymatic activity of a microorganism.

In the present work, SL or TW80 was added to the culture medium in order to explore the possibility of enhancing the enzyme production by *A. discolor*. Several investigators (Ding et al., 2008; Jäger et al., 1985; Rodríguez Couto et al., 2000) have shown improvements in enzyme production in the presence of some surfactants in semi-solid, immobilized, submerged, agitated, and shallow stationary liquid cultures of white-rot fungi such as *P. chrysosporium*. We chose SL because it shows lower toxicity in bacteria than quillaya saponin (Garon et al., 2002) and because no studies have evaluated the effect of SL on enzyme production in white-rot fungi. The evaluated concentrations were 0.15, 0.3, 0.4, 0.5, and 1 g L^{-1}. At these concentrations, the detected levels of LiP and MnP were higher than those of Lac and MiP, which might be explained by the SL composition. This phytogenic surfactant contains 1.99 mg L^{-1} of Mn, a known inducer of these enzymes. Studies by Wang et al. (2008) demonstrated that the presence of Mn^{2+} in the culture medium enhanced MnP and LiP production. Moreover, Ürek and Pazarlioğlu (2005) determined that MnP production depended on the Mn^{2+} concentration. The presence of TW 80 stimulated LiP and MnP production with respect to Lac and MiP. Several authors have shown an improvement in enzyme excretion in the presence of certain surfactants such as TW80 in immobilized and submerged cultures of *P. chrysosporium* (Ding et al., 2008; Jäger et al., 1985). The mechanism by which surfactants enhance extracellular enzyme production in filamentous fungi has not been established. Several authors have suggested that surfactants promote both the uptake and exit of compounds from the cell through modification of the plasma membrane permeability (Ding et al., 2008).

The results obtained in our study show that optimal conditions for the production of high levels of Lac and MnP in *A. discolor* are the same, which is advantageous with respect to their possible synergism in bioremediation processes. The SL demonstrated no negative effects on the fungal growth of *A. discolor* and increased the enzyme production of ligninolytic enzymes more than the use of TW80.

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Chapter 4

Effect of soya lecithin on solubilization and biodegradation of pentachlorophenol by *Anthracophyllum discolor*

Paper in preparation

Effect of soya lecithin on biodegradation of pentachlorophenol by *Anthracophyllum discolor* in liquid medium

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Abstract

The effect of soya lecithin (SL) on solubilization and biodegradation of pentachlorophenol (PCP) by *Anthracophyllum discolor* was evaluated. Besides, the PCP effect on critical micelle concentration (CMC) of SL and the effect of PCP and SL on the ligninolytic activity of *A. discolor* were evaluated. The PCP solubilization test was performed for 100, 200 and 400 mg L^{-1} with SL solution varying concentrations of ranging from 0 to 0.9 g L^{-1}. The effect of PCP (5, 10, 15, and 20 mg L^{-1}) on CMC of SL by conductivity was evaluated. The effect of SL (0, 0.3, 0.6, and 0.9 g L^{-1}) on biodegradation of PCP (21.4 mg L^{-1}) in modified Kirk medium was studied. The results showed that SL addition enhanced PCP solubility in water. The solubilities of 100, 200 and 400 mg L^{-1} of PCP was about 4.2, 5.0 and 2.5 times higher than their solubility in pure water when the system was added with 0.9 g L^{-1} of SL. We found that the increase in PCP concentration caused a decrease in the CMC value. In the biodegradation assay, in liquid medium supplemented with 0.9 g L^{-1} of SL, the 94.4% of PCP was removed after 26 days of incubation. In contrast, in the control assay (without SL), the 76.5% of PCP was removed. The PCP degradation was confirmed by the

presence of its metabolites. During biodegradation assay, *A. discolor* mainly produced manganese peroxidase (MnP) reaching a maximum of 96.8 U g^{-1} (dw) when the culture medium was added with 0.6 g L^{-1} of SL. The MnP activity is negatively affected by the presence of PCP and without SL. These results suggest that SL can be applied to increase the bioavailability and biodegradation of PCP.

Key words: Soya lecithin, solubility, biodegradation, white-rot fungi, pentachlorophenol

4.1 Introduction

In the past, pentachlorophenol (PCP) was used extensively for the protection and preservation of wood, as an antifungal agent in the leather industry and as wide-spectrum biocide in industry and agriculture. This fact has led the groundwater, sediments and soil contamination with PCP due to its molecular stability and sorption properties (Männisto et al., 2001). Besides, the exposition to PCP-contaminated soil can result in dermal absorption, which can have adverse health effects (Qiao et al., 1997).

The PCP is a chlorinated organic compound and a weak acid with a pKa of 4.75, with a molecular weight of 266.34 g mol^{-1}, low water solubility (14 mg L^{-1} at 25ºC), and high K_{OW} (1.0 x 10^5) (Shiu et al., 1994). Therefore, PCP adsorbs on the organic fraction of soils (Cea et al., 2005) and can be present as non-aqueous phase liquids due to its low solubility in water (USEPA, 1997). Although PCP persists in many environments, biological methods have been utilized in groundwater and soil bioremediation of PCP (Männisto et al., 2001; Rubilar et al., 2011).

Biodegradation process of PCP by white-rot fungi is well-known. *Bjerkandera adusta, Phanerochaete chrysosporium*, *Trametes versicolor*, and *Trametes hirsute* are some of the white-rot fungi that shown the ability to degrade several contaminants, including PCP (Reddy and Gold, 2000; Sedarati et al., 2003; Boyle, 2006; Rubilar et al., 2007). The potential of these microorganisms resides in its extracellular enzymatic systems, which can includes lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) (Reddy, 1995; Eichlerova et al., 2005). Recent studies have led to find native fungal strains with ligninolytic activity and capacity to degrade several contaminants. Studies realized by

Rubilar et al. (2007, 2011) and Tortella et al. (2008) have demonstrated that *Anthracophyllum discolor*, a native Chilean fungus, presents ligninolytic activity with high level of MnP production and capacity to degrade PCP.

However, biological processes of degradation are negatively affected by the low water solubility of the contaminants, limiting their availability to microorganisms. Several researchers have reported that the addition of surfactant in contaminated sites increases the bioavailability of hydrophobic compounds, allowing an increase of its concentration in the water phase and, therefore, more available for biodegradation (Zhu and Feng, 2003; Zhao et al., 2005; Gao et al., 2007).

Surfactants are organic molecules with a polar or hydrophilic group and a nonpolar or hydrophobic chain (West and Harwell, 1992; Christofi and Ivshina, 2002). An important characteristic of these molecules is the production of micelles, which are capable of dissolving hydrophobic compounds in their hydrophobic core, which results in an apparent increment of its aqueous solubility (Edwards et al., 1991; Prak and Pritchard, 2002). Solubilization depends on the type and dose of the surfactant, and hydrophobic compound properties. Doong and Lei (2003) evaluated the solubilization of four polycyclic aromatic hydrocarbons (PAHs) in a soil system in presence of different surfactants. They found that the solubility of contaminants enhances with the addition of surfactant and, the extent of availability depended of the polyoxyethylene chain present in the surfactant. Besides, Zhu and Feng (2003) demonstrated that the PAHs are solubilized synergistically in mixed anionic-nonionic surfactant solution, especially at low surfactant concentration. Shin et al. (2006) examined the effect of pH on phenanthrene solubilization from soil in presence of rhamnolipid, an anionic biosurfactant produced by *Pseudomonas*. Their results showed that the solubility of the contaminant enhanced in a pH range of 4.5-5.5. Moreover, they concluded that the changes in the apparent solubility of the compound within the pH are attributed to the different pH-depended structures of rhamnolipid. Cort et al. (2002) evaluated the solubility of PCP in minimal medium containing Tergitol NP-10 (nonionic surfactant) adjusted to pH 2. They found that PCP concentration increased linearly as surfactant was added in the range of 50-1256 mg L^{-1}. On the other hand, Hanna et al. (2005) determined that the apparent PCP solubility increases linearly with catioinic surfactant concentration at pH 3.

Soya lecithin (SL) is a slightly studied phytogenic surfactant or biosurfactant on solubilization and biodegradation processes. It has been applied for studied its effects on biodegradation of PAHs and polychlorinated biphenyls. In this context, Soeder et al. (1996) evaluated the effect of SL and quillaya saponin on the aerobic biodegradation of PAHs in shake-batch cultures of bacteria. Their results showed that SL has high PAH-solubilizing activity and a lower bacterial toxicity with respect to quillaya saponin.

Another important surfactants property is their toxicity. In general, biosurfactants are considered as low or non-toxic (Desai and Banat, 1997). However, some biosurfactants can be toxic and exert inhibitory effects on the biodegrading microorganisms (Fava and Di Giogia, 2001; Singh et al., 2007). Flasz et al. (1998) found that the chemical surfactant presented higher toxicity and mutagenic effects, whereas that the natural surfactants were less-toxic and non-mutagenic. Soeder et al. (1996) established that SL enhances growth of bacteria. Besides, Bustamante et al. (2011) demonstrated that when SL is included in the growth medium enhances growth and MnP production of *A. discolor*.

The potential of SL for enhance the solubility of hydrophobic organic compounds and its low toxicity on the degrader microorganisms and the capability of *A. discolor* to produce MnP and to degrade PCP, suggest the opportunity to study the effect of SL on the solubilization and biodegradation of PCP by *A. discolor* in liquid medium. Besides, the effect of several PCP concentrations on critical micelle concentration of SL and the effect of SL on *A. discolor* surface were evaluated.

4.2 Materials and methods

4.2.1 Materials

4.2.1.1 Microorganism and growth conditions

The white-rot fungus *Anthracophyllum discolor* was obtained from the culture collection of the Environmental Biotechnology Laboratory of the Universidad de La Frontera (Chile). The fungus was transferred from slant tubes to glucose malt extract agar plates (per liter of distilled water) 15 g agar, 10 g glucose, 30 g malt extract, pH 5.2, maintained at 4ºC in plate and then incubated at 26 ± 2ºC for 7 days before being used for inoculum preparation.

4.2.1.2 Preparation of the blended mycelium

A. discolor was first grown in 1-L Erlenmeyer flask containing 100 mL of modified Kirk medium (Tien and Kirk, 1988) and five 6-mm diameter agar plugs of active mycelium. The culture was incubated statically at 26 ± 2ºC for 7 days in darkness. After this, the fungal culture was homogenized in a sterilized blender for 1 min. The modified Kirk medium contained (per liter of distilled or deionized water) 10 g glucose, 0.12 g $C_4H_{12}N_2O_6$, 2 g KH_2PO_4, 0.59 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 3.3 g $CH_3COONa \cdot 3\ H_2O$ and 10 mL mineral salts. The initial pH of the medium was adjusted to 5.5 with either 1 N NaOH or 1 N HCl.

4.2.1.3 Chemicals and stock solutions preparation

The 2,4-dicholorophenol (2,4-DCP) was obtained from Sigma-Aldrich and 2,4,6-trichlorophenol (2,4,6-TCP) was purchased from Fluka. Pentachlorophenol (PCP) was obtained from Aldrich Chemical Co., with >98% purity. The other compounds were greater than 97% purity. Soya lecithin (SL) was purchased from PRINAL®.

***For PCP Solubilization test*,** the SL solution was prepared dissolving SL in distilled water to obtain a stock solution of 50 g L^{-1}. Besides, PCP was dissolved in acetone to obtain a stock solution of 5000 mg L^{-1}.

***For the PCP on critical micelle concentration of SL*,** the surfactant stock solution was prepared dissolving SL in modified Kirk medium to obtain a solution of 5 g L^{-1}. The contaminant stock solution was prepared dissolving PCP in deionized water to obtain a stock solution of 1000 mg L^{-1}, adequate quantity of 1 N KOH was added to dissolve PCP.

***For the effect SL on PCP biodegradation by A. discolor in liquid medium*,** the surfactant stock solution was prepared dissolving SL in hexane to obtain a solution of 50 g L^{-1}. The contaminant stock solution was prepared dissolving PCP in acetone to obtain a solution of 5000 mg L^{-1}.

4.2.2 Methods

4.2.2.1 The PCP Solubilization test

The solubility test was performed in a 20-mL glass tube, each tube were added with aliquot of PCP stock solution to reach a final concentration of 100, 200 and 400 mg L^{-1}. After evaporating the acetone, each tube was added with 10 mL SL stock solution varying concentrations of ranging from 0 to 0.9 g L^{-1}. The tubes were homogenized in a mechanical shaker for 24 h at 150 rpm at 25 ± 2ºC. After this time period, the samples were centrifuged for 15 min at 3000 rpm to separate the undisolved portion of PCP. Then supernatants were filtered through 0.2 μm diameter pore. The PCP was quantified by high performance liquid chromatography (HPLC). Triplicates tube were run for each concentration.

4.2.2.2 Effect of PCP on critical micelle concentration of SL

The effect of PCP on critical micelle concentration (CMC) of SL in modified Kirk medium was determined by the breakpoint of conductivity versus surfactant concentration curve. From the SL stock solution was prepared 50 mL of modified Kirk medium, where the SL concentration ranged from 0.2 to 2 g L^{-1}. After this, each solution was added in 100-mL Erlenmeyer flask and added with aliquot of PCP stock solution to obtain a final concentration of 5, 10, 15 and 20 mg L^{-1}. The electrical conductivity of each solution was measured with a HACH Sens ion5 conductivimeter at 25 ± 2 ºC. The results of CMC are reported as the mean of three replicates.

4.2.2.3 Effect of SL on PCP biodegradation by A. discolor in liquid medium

Effect of SL on PCP biodegradation by *A. discolor* was evaluated using 10 mL of modified Kirk medium in 100-mL Erlenmeyer flask. Aliquot of PCP and SL stock solution were aseptically added to a sterile Erlenmeyer flask to give a final concentration of 20 mg L^{-1} of PCP and 0, 0.3, 0.6, or 0.9 g L^{-1} of SL. Then, organic solvents were allowed to evaporate for re-crystalization of SL and PCP under aseptic conditions. After this, each Erlenmeyer flask received an inoculum formed by 10 mL of modified Kirk medium and 0.4 mL of blended mycelium of *A. discolor* (4.2.1.2), grown for 14 days at 26 ± 2ºC. The flask were incubated under static conditions in darkness for 26 days at 26 ± 2ºC. PCP and its metabolites (2,4-DCP and 2,4,6-TCP) were quantified periodically (1, 5, 10, 15, and 26 days) in the liquid medium and in the fungal mycelium by HPLC. Besides, enzymatic activities of Lac, MnP, lignin peroxidase (LiP) and manganese-independent peroxidase (MiP), pH variation and glucose consumption were evaluated

4.2.2.4 Effect of SL on A. discolor surface visualized by electronic microscopy

The scanning electron microscopy (SEM) was used for detecting some SL effect on the surface of *A. discolor*. Aliquot of SL stock solution (50 g L^{-1} in hexane) was added in to a sterile 100-mL Erlenmeyer flask to give a final concentration of 0.6 g L^{-1}. Then, hexane

was allowed to evaporate. After this, each flask was supplemented with 10 mL of modified Kirk medium and 0.4 mL of blended mycelium of *A. discolor*. Biotic control (liquid medium with SL and *A. discolor*) was established in parallel. Each experiment was carried out under destructive sampling mode. All flasks were incubated at 26 ± 2ºC in darkness for 20 days. The fungal surface of *A. discolor* was visualized by SEM after 5 and 20 days of incubation. The analyses were carried out with a Jeol JSM-6360LV microscope.

4.2.2.5 Statistical analysis of data

The Duncan Test was used to compare means. The statistical significance level for all treatments was 5%.

4.2.3 Analytical procedures

4.2.3.1 Determination of enzymatic activities in the liquid medium

The enzymatic activities (Lac, LiP, MiP and MnP) were measured from the supernatant of a previously centrifuged sample (2 min at 3000 rpm) of liquid medium.

Laccase activity was determined with 2,6-dimethoxyphenol (2,6-DMP) as the substrate in sodium malonate (pH 4.5). The enzyme activity unit was defined as an increase in absorbance per minute at 468 nm and 30ºC (deJong et al., 1994). MnP activity was determined by monitoring the oxidation of 2,6-DMP spectrophotometrically at 30ºC. The reaction mixture (1 mL) contained 200 µL of 250 mM sodium malonate (pH 4.5), 50 µL of 20 mM 2,6-DMP, 50 µL of 20 mM $MnSO_4 \cdot H_2O$ and 600 µL of supernatant. The reaction was initiated by adding 100 µL of 4 mM H_2O_2, monitored at 468 nm and corrected by Lac activity. MiP activity was determined in a reaction mixture containing 200 µL of 250 mM sodium malonate (pH 4.5), 50 µL of 20 mM 2,6-DMP, 100 µL of 20 mM EDTA, and 600 µL of supernatant. This reaction was initiated by adding 100 µL of 4 mM H_2O_2, monitored at 468 nm and corrected by the Lac activity (deJong et al., 1994). The molar extinction coefficient was 49600 M^{-1} cm^{-1}. LiP activity is based on the oxidation of veratryl alcohol. The reaction mixture contained 1420 µL di-sodium tartrate dihydrate (0.1 M, pH 3.0), 400

μL veratryl alcohol (20 mM) and 100 μL of supernatant. The reaction was initiated by adding 80 μL of H_2O_2 (10 mM) (Castillo et al., 1997) and was monitored at 310 nm for 2 min. The molar extinction coefficient was 93000 M^{-1} cm^{-1}. The absorbance was read using Spectronic Genesys 2PC model spectrophotometer and expressed in U g^{-1} dry weight (U g^{-1} dw) of mycelium.

4.2.3.2 Extraction and quantification of PCP from the liquid medium and fungal mycelium

The liquid medium and fungal mycelium of *A. discolor* were vacuum-filtered through 0.2 μm pore size of nitrocellulose membrane. The filtered liquid medium was analyzed by HPLC. The biomass retained in the membrane was transferred to 100-mL Erlenmeyer flasks, diluted with 5 mL hexane:acetone mixture (1:1) and adjusted at pH 2 with 0.1 N H_2SO_4. The Erlenmeyer flasks were hermetically sealed and shaken for 2 h at 150 rpm. After shaking, Erlenmeyer flasks were taken to a sonic bath for 30 min. The mixture was vacuum-filtered through 0.22 um pore size of polytetrafluoroethylene membrane. The retained biomass was dried at 105ºC for 3 h to determine dry weight. The extract of PCP adsorbed in fungal mycelium was analyzed by HPLC.

The PCP concentrations were quantified by HPLC with a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with 20 μL loop diode array detector. The column was a reverse phase (Lichrosphere 60RP select B, 5 μm). Detection of PCP and metabolites (2,4-DCP and 2,4,6-TCP) were at 210 nm and the mobile phase consisted of 1% phosphoric acid/acetonitrile (40:60, v/v). Determinations were with a flow rate of 1 mL min^{-1}, at room temperature (22 ± 2ºC). The presence of surfactant, SL, did not interfere with the detection of PCP and their metabolites. The procedure described was checked for PCP recovery (which ranged from 94 to 100%). The retention time were 3.79, 4.49 and 6.11 min for 2,4-DCP, 2,4,6-TCP and PCP, respectively.

4.2.3.3 Preparation and inspection of mycelium of A. discolor for SEM

Mycelium of *A. discolor* for SEM was obtained from cultures with 5 and 20 days of incubation. The mycelium sample was centrifuged at room temperature by 15 min at 4000 rpm. After this, the mycelium was fixed with a 2% v/v glutaraldehyde in 0.1M potassium phosphate buffer (pH 7) for 18 h at 4ºC. Then, mycelium was centrifuged (15 min at 4000 rpm at room temperature) and washed three times with distilled water in 30 min. Afterwards, the mycelium sample was fixed with a 4% v/v osmium tetraoxide in 0.1M potassium phosphate buffer (pH 7) for 2 h at room temperature. Besides this treatment, the samples were dehydrated by immersion in ascending series solution of ethanol (70, 80, 90, and 100%). Apart from this treatment, the mycelium was washed with potassium phosphate buffer (pH 7) for 30 min followed by three changes with distilled water. Finally, the mycelium was re-suspended in distilled water, and a drop of suspension was deposited over coverslips and air-dried overnight at room temperature. The completely dried mycelium was coated with Au/Pd with BAL-TEC to make observations using a scanning electron microscope.

4.2.3.4 Quantification of glucose

The glucose concentration was evaluated from the liquid medium. The liquid medium was filtered through 0.2 μm pore size of nitrocellulose membrane. The reaction mixture (1 mL) contained 250 μL of liquid medium and 750 μL of 3,5-dinitrosalicylic acid (DNSA). The mixture was heated for 5 min in a boiling water batch and then 4 mL of distilled water was added. The samples were measured in a Spectronic Genesys 2PC model spectrophotometer at 540 nm. The calibration curve was prepared with standard D-glucose, where the D-glucose concentration ranged from 0 to 1 g L^{-1}.

4.2.3.5 Quantification of fungal biomass

The fungal biomass was determined by measuring the dry weight of fungal mycelium. The culture medium was vacuum-filtered through a 0.22 μm pore size of nitrocellulose

membrane. The retained biomass was dried at 105ºC until reaching a constant weight. The yield was expressed as g dry weight of mycelium L^{-1} of liquid medium.

4.2.3.6 The pH measurement

The pH measurement was determined in liquid medium after vacuum-filtered with Orion 420-A, pH-meter.

4.3 Results and discussion

4.3.1 The PCP solubilization test

The effect of SL on the aqueous solubility of PCP was evaluated by test tube solubilization assays in the presence of increasing concentrations of SL (0.3 to 0.9 g L^{-1}). In general, the concentration of solubilized PCP in the SL solutions increased with the addition of SL (Fig. 4.1). The PCP solubility in pure water, without SL, reached values of 16.2, 15.0 and 24.0 mg L^{-1} when PCP concentrations were 100, 200 and 400 mg L^{-1}, respectively. The contaminant solubility enhanced slowly when SL increased at 0.3 g L^{-1}, reaching values between 20.8 and 24.8 mg L^{-1}. An increase of solubility was observed with 0.6 g L^{-1} of SL, reaching values of 48.8, 33.4 and 35.8 mg L^{-1} in presence of 100, 200 and 400 mg L^{-1} of PCP, respectively. Finally, PCP solubility with 0.9 g L^{-1} of SL were about 4.2, 5.0 and 2.5 folds higher than the pure water solubilities for 100, 200 and 400 mg L^{-1} of initial concentration of PCP, respectively.

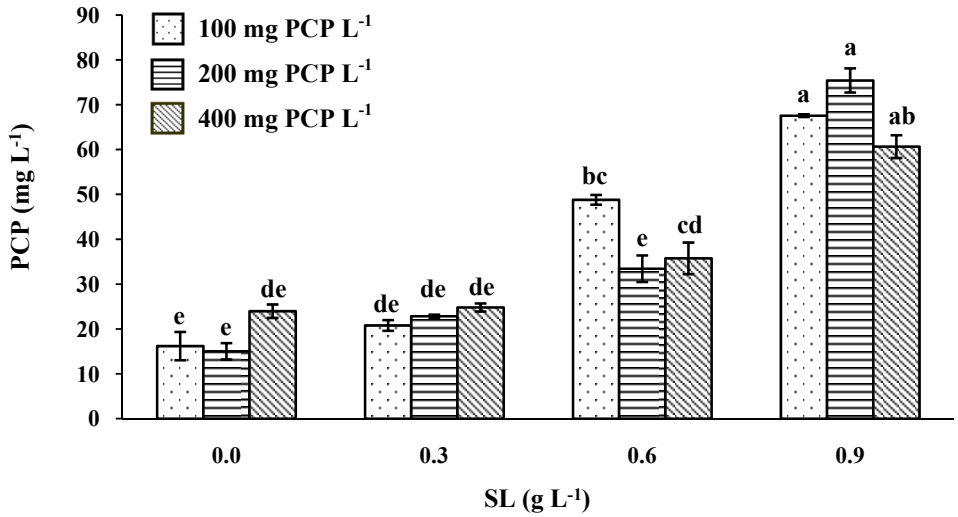

Fig. 4.1 Solubilization of pentachlorophenol (PCP) by soya lecithin (SL) solutions prepared with distilled water, at 25 ± 2ºC and pH 5.5. Different letters refer to significant differences mean values (n = 3) with Duncan Test ($p < 0.05$).

Besides, when PCP initial concentration was of 100 mg L^{-1}, the concentration of PCP solubilized increased linearly with SL addition and the relationship was given by the equation y = 60.7x + 11.0 (R^2 = 0.94), where *x* is the SL concentration and *y* is solubilized PCP (Table 4.1). When the PCP initial concentration was of 200 and 400 mg L^{-1}, values of R^2 were 0.85 and 0.83, respectively. The effectiveness of a surfactant in solubilizing a given solute is known as the molar solubilization ratio (Edwards et al., 1991). The molar solubilization ratio is defined as the number of moles of compound solubilized per number of moles of surfactant added to the solution, and can be experimentally determined as of slop of the curve solute concentration *vs*. surfactant solution. In this context, the effectiveness of SL for 100 mg L^{-1} of PCP was 60.7 mg g^{-1}. However, the effectiveness of SL decreases to 63.94 and 40.35 when the PCP concentration increases to 200 and 400 mg L^{-1}, respectively. Edwards et al. (1991) evaluated the solubility of PAH in nonionic surfactant solution. Their result demonstrated that the solubility of PAH increases linearly with surfactant concentration above the CMC, similar to the results above presented.

Table 4.1 The effect of SL on aqueous solubility of three initial PCP concentrations in water-SL system

Initial PCP concentration (mg L^{-1})	**Experimental equation[(a)]**	**R^2**
100	y = 60.72 x + 11.01	0.94
200	y = 63.94 x + 7.91	0.85
400	y = 40.35 x + 18.14	0.83

(a) Where: *x* is the SL concentration and *y* is solubilized PCP

The according to the result presented in Fig. 4.1 solubility of PCP was not affected significantly ($p < 0.05$) in pure water, without SL, and when SL concentration was 0.3g L^{-1}. However, significant differences ($p < 0.05$) were detected when SL concentration was 0.6 and 0.9 g L^{-1} respect to when SL concentration was 0 and 0.3 g L^{-1}. On the other hand, solubility of PCP was not affected significantly ($p < 0.05$) in pure water, without SL, and when SL concentration was 0.3 and 0.9 g L^{-1} for 100, 200 and 400 mg L^{-1} of PCP.

However, significant differences ($p < 0.05$) were detected when SL concentration was 0.6 g L^{-1} for the three PCP concentrations. The results shows that SL had certain effect on the solubilization of PCP either below or above its CMC, 0.61 g L^{-1} (Bustamante et al., 2011); the solubilization was higher than when the SL concentration was above its CMC. Similar result was reported by Eddouaouda et al. (2011). They found that the solubility of phenanthrene in water was higher when the biosurfactant concentration was above its CMC. This situation can be explained due to that surfactant molecules exist as monomers below its CMC and, as was described by Eddouaouda et al. (2011) and Li and Chen (2009) monomeric molecules have minimal partition effect on solute. In contrast, contaminant solubility is increased when surfactant concentration is above its CMC. On the other hand, Cort et al. (2002) evaluated the aqueous solubility of PCP in the presence of surfactant nonionic, Tergitol NP-10 found that PCP concentration increased linearly as surfactant was added. Garon et al. (2002) quantified the extent of fluorene solubilization in presence of three surfactants. Their results showed that fluorine dissolution was increased in the presence of surfactant, and the increasing solubility was dependent on the type of surfactant. They obtained higher solubilization with Tween 80 with respect to SDS. The effect of biosurfactants (produced by *Pseudomona aeruginosa* strains) on the solubility of polycyclic aromatic hydrocarbons was evaluated by Bordoli and Konwar (2009). They reported that solubility of contaminants was enhanced. Besides, it depended on contaminant and *P. aeruginosa* strains.

4.3.2 Effect of PCP on CMC of SL

Table 4.2 presents the effect of several PCP concentrations on CMC of SL. This Table shows that CMC value decreases as the concentration of PCP increases. In a previous study, we determined that CMC of SL in modified Kirk medium without PCP was 0.61 g L^{-1} (Bustamante et al. 2011). However, the CMC of surfactant depends on many factors, such as determination method (Nesměrák and Němcová, 2006; Fuget et al., 2005), phospholipid composition, electrolyte addition, solution temperature, presence of organic additives (Hassan and Yakhmi, 2000) or conditions of salinity (Choi et al., 2000), among other; making this value different from the one determined in pure water (Fuguet et al., 2005).

As reported in literature CMC value decreases as the concentration of solute increases (Abu-Hamdiyyah, 1986). This result can be due to a neutralization of the surface charge of the surfactant by the contaminant, affecting the micelle formation. A reduction of the thickness of the ionic atmosphere around the surfactant ionic heads, produce electrostatic repulsions between them, helping in this way the micellization process. This

Table 4.2 The CMC of SL determined through electrical conductivity measurements in modified Kirk medium at pH 5.5 and 25 ± 2ºC

PCP ($mg\ L^{-1}$)	CMC ($g\ L^{-1}$)
0	0.61 †
5	1.27
10	1.00
15	0.88
20	0.39

†Source: Bustamante et al. (2011).

behavior is followed by all kinds of surfactants, independent from their chemical nature (Fuguet et al., 2005).

4.3.3 Effect of SL on biodegradation of PCP by *A. discolor* in liquid medium

A variety of surfactants have been applied for the biodegradation of compound with low solubility in liquid medium, groundwater aquifers, and contaminated soils, among others. Nevertheless, few studies have addressed the removal of these compounds using phytogenic surfactant, as SL, in combination with white-rot fungi. In this context, the SL effect on the PCP degradation by *A. discolor* in liquid medium was evaluated. Considering the lack of information about the SL effects on PCP biodegradation, SL was tested at

concentrations of 0, 0.3, 0.6, and 0.9 g L^{-1}, which were below and above the previously determined CMC (0.6 g L^{-1}).

The medium was contaminated with 21.4 mg L^{-1} of PCP. The degradation was established by both the disappearance of PCP (Table 4.3) and the production of metabolites, as 2,4-dicholorophenol (2,4-DCP) and 2,4,6-trichlorophenol (2,4,6-TCP) (Table 4.4). After 26 days of incubation, the 94.4% of PCP was removed, when the culture medium was supplemented with 0.9 g L^{-1} of SL. In contrast, in the control assay (without SL) the 76.5% of PCP was removed. These results show that the presence of SL improved PCP degradation by *A. discolor*. The PCP degradation with 0.9 g L^{-1} of SL was 18% higher than control test. Similar results were obtained by Cort et al. (2002), they evaluated the effect of nonionic surfactant on PCP biodegradation. Their result showed a faster PCP degradation rates at higher concentrations of nonionic surfactant, Tergitol NP-10. These results can be explained by surfactant reducing the substrate inhibition. On the other hand, some studies have shown that the toxicity of PCP in white-rot fungi is not related to the concentration of contaminant in liquid medium, it is given by the dose, expressed as the ratio of the mass of chemical to the mass of the mycelium (Mileski et al., 1988; Alleman et

Table 4.3 Residual concentration of PCP (mg L^{-1}) and PCP removed (%) by *A. discolor*, in modified Kirk medium with several concentrations of SL, at pH 5.5 and 25 ± 2ºC, after 26 day of incubations†.

SL (g L^{-1})	Residual PCP (mg L^{-1})		PCP (%)		
	Liquid medium	Mycelium	Liquid medium	Mycelium	Removed
0	3.5	1.6	16.1	7.4	76.5
0.3	1.4	0.4	6.5	1.7	91.8
0.6	1.2	0.5	5.5	2.3	92.2
0.9	0.5	0.6	2.6	2.9	94.4

† Initial concentration of PCP = 21.4 mg L^{-1}

al., 1992). Nonionic surfactants, such as Tween 20, Tergitol NP-10, among others, are good candidates to enhance in situ remediation, based on their low biotoxicity and protective effect (Park and Bielefeldt, 2003).

In relation to the biomass sorption of PCP (Table 4.3), the result shows that the increase in SL concentration caused an increase in the biomass sorption of contaminant, 1.7, 2.3 and 2.9 % for 0.3, 0.6 and 0.9 g L^{-1} of SL, respectively. This fact can be explained by that the surfactants may have an effect on the permeability of membrane for contaminants with low solubility in water, due to biological membranes present phospholipid equal that the surfactants (Van der Werf et al., 1995). However, in the control assay, without SL, the biomass sorption of PCP was greater than in presence of SL, and can explain the low percent of PCP removal (76.5%). Besides, Table 4.3 shows that the percent of PCP removed increase with increase in SL concentration. In this context, the SL presence can eliminate the inhibitory effect of PCP.

Table 4.4 Degradation products from PCP degradation by *A. discolor* after 26 days of incubation. The metabolites are expressed as mean of three replicates ± SD.

SL (g L^{-1})	**2,4-DCP** (mg L^{-1})	**2,4,6-TCP** (mg L^{-1})
0	1.0 ± 0.1	-
0.3	1.4 ± 0.2	-
0.6	2.0 ± 0.3	-
0.9	3.7 ± 0.1	0.2 ± 0.0

On the other hand, the concentrations of metabolites in the culture medium were quantified after 26 days of incubations (Table 4.4). The highest concentration of metabolites of PCP was detected when the culture medium was supplemented with 0.9 g L^{-1} of SL, with 3.7 of 2,4-DCP and 0.2 mg L^{-1} of 2,4,6-TCP. Besides, 2,4-DCP concentration increased when the SL concentration increased in liquid medium from 0 at 0.9 g L^{-1} SL. However, the percent

of metabolites produced was low respect to the PCP initial concentration and did not exceed the 10% for 2,4-DCP and 2,4,6-TCP when the SL concentration was from 0 to 0.6 g L^{-1} and 0.9 g L^{-1}, respectively.

4.3.4 Enzyme production in the presence of SL and PCP

The enzymatic activity of *A. discolor* was evaluated during the PCP biodegradation assay. After 26 days, the amount of Lac produced did not exceed 4 U g^{-1} (Fig. 4.2a). Besides, the highest MnP activities were detected at 26 days (Fig. 4.2b). The MnP activity presented a maximum of 96.8 U g^{-1} when 0.6 g L^{-1} of SL was incorporated into the culture medium (day 1). After this stage, MnP activity went down until 21.7 U g^{-1} (day 5). The MnP activity of *A. discolor* after of 26 days of incubation was 29.5, 37.1 and 42.2 U g^{-1} when liquid medium was added with 0.3, 0.6 and 0.9 g SL L^{-1}, respectively. *A discolor* only produced high levels of MiP when 0.6 g L^{-1} of SL were added to liquid medium, reaching a maximum of 9.7 U g^{-1} at 26 days (Fig. 4.2c). In other conditions, MiP activity did not exceed 4 U g^{-1}. Finally, LiP activity did not exceed 5.7 U g^{-1}, date no shown.

4.3.5 Variation of pH, biomass, and consumption of glucose in the presence of SL and PCP

The pH variation, biomass production, and glucose consumption are presented in Fig. 4.3. After 26 days of incubation, the pH of liquid medium was between 5.2 and 5.7 units for all the conditions, with and without SL (Fig. 4.3a). In this assays the initial concentration of biomass for the biodegradation test was 1.4 g L^{-1} (Fig. 4.3b). The PCP addition affected negatively the biomass production of *A. discolor*, diminished until 0.5 g L^{-1}, approximately. After 5 days, the fungal biomass increased slowly when the liquid medium was added with SL. Besides, an increase on biomass production was observed with the increase in SL concentration, however was not higher than 0.7 g L^{-1} in presence of 0.9 g L^{-1} of SL, after 26 days of incubation. In contrast, the fungal biomass production was constant in the control assay, without contaminant and without SL, reached a value of 1.9 g L^{-1} after 26 days of culture. Boyle (2006) evaluated the effects of three cyclodextrins and PCP on radial growth

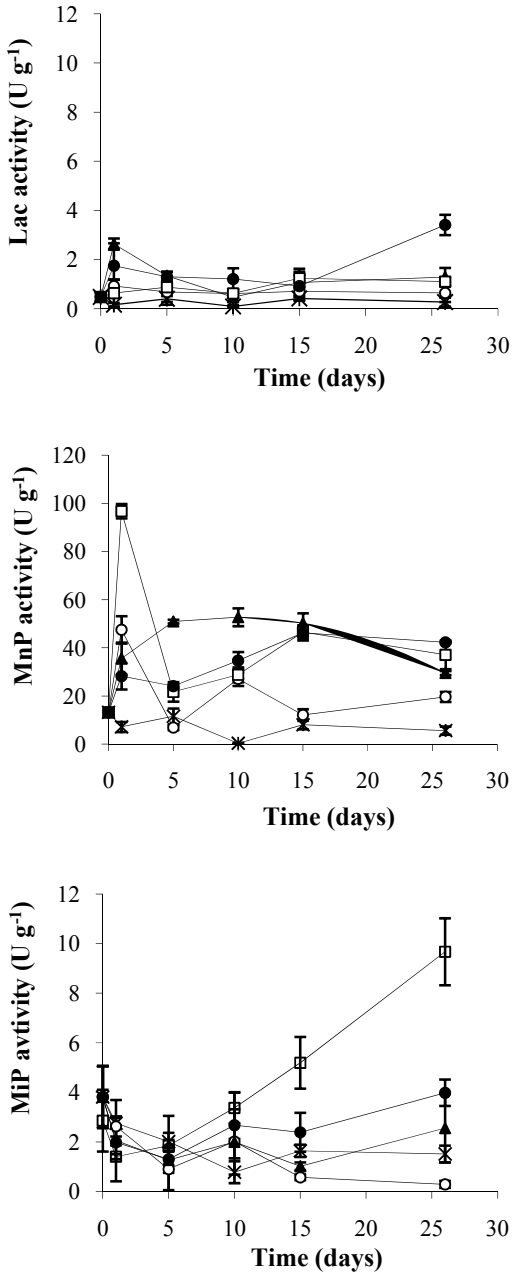

Fig. 4.2 Effect of PCP and SL on the ligninolytic activity of *A. discolor* in liquid medium. Lac activity (a), MnP activity (b) and MiP activity (c). Values are expressed as mean ± SD of three replicates. Where: (∗) *A. discolor*, (○) *A. discolor* + PCP + 0 g SL L^{-1}, (▲) *A. discolo*r + PCP + 0.3 g SL L^{-1}, (□) *A. discolor* + PCP + 0.6 g SL L^{-1}, (•) *A. discolor* + PCP + 0.9 g SL L^{-1}.

of the *T. hirsute*. He reported that at pH 4.9 Gamma 8 eliminated the inhibitory effects of 10 mg L^{-1} of contaminant and partially overcome those at 50 mg L^{-1} Beta W7 M1.8 also alleviated inhibition, but less pronounced. Alpha W6 M1.8 had little effect and cyclodextrins did not affect radial growth of *T. hirsute*. The PCP effects on growth of several white-rot fungi have been well studied. In this context, Tortella et al. (2008) studied the tolerance of fungi to mycelia growth in the presence of PCP. They found inhibition mycelia growth when the fungi were exposed at 25 mg L^{-1} of PCP. Besides, Walter et al. (2003) informed that the most strains of white-rot fungi studied tolerant 20 mg L^{-1} of PCP. However, Alleman et al. (1993) detected inhibition of growth with 5-10 mg L^{-1} of PCP for *P. chrysosporium*, *T. versicolor*, *Inonotus* sp, *Ganoderma* sp. The several responses found could be due to composition of liquid medium and growth conditions (Mendoza-Cantú et al., 2002), PCP doses (Alleman et al., 1992), or fungal strains adapted to PCP (Tortella et al., 2008).

The consumption of glucose decreased slowly when liquid medium was added with SL. However, an increase in the glucose consumption was observed with the increase in the SL concentration, situation related with a high biomass production when the SL concentration increases. After 26 days of incubation, the final glucose concentration for 0.9, 0.6, 0.3 and 0 g $L^{-1}$of SL was of 3.9, 4.1, 4.4 and 4.5 g L^{-1}, respectively. In contrast, glucose consumption in control culture, without SL and without PCP, was higher than in all other conditions. After 26 days of incubation, glucose concentration in the control assay reached 3 g L^{-1}, which represent a 39% of glucose consumption (Fig. 4.3c). The higher PCP biodegradation by *A. discolor* was observed with the higher SL concentration, 0.9 g L^{-1}. In this condition of culture, was observed higher biomass production, higher glucose consumption and a higher MnP production of *A. discolor*.

4.3.6 Effect of SL on fungal surface of *A. discolor* visualized by SEM

Fungal hyphae of *A. discolor* were observed by SEM to evaluate SL effect on their surface. Figure 4.4 shows SEM images of fungal hyphae with 5 and 20 days of incubation in modified Kirk medium at pH 5.5 and 25 ± 2ºC. These images show that *A. discolor*

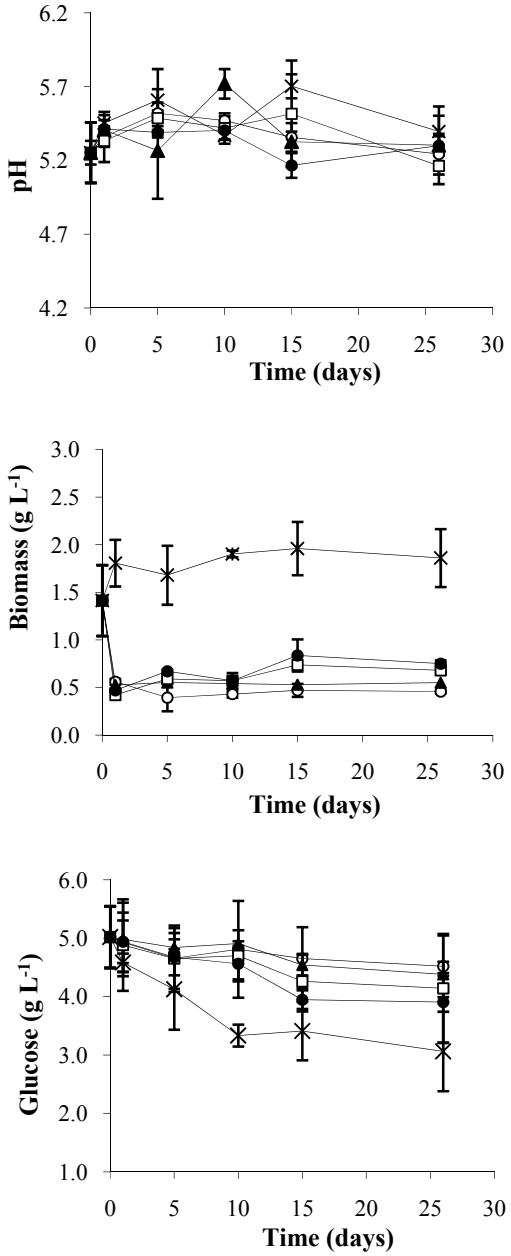

Fig. 4.3 Profiles of pH (a), biomass of *A. discolor* (b) and glucose consumption (c) in liquid medium after of the contamination with PCP and addition of SL. Values are expressed as mean of three replicates ± SD. Where: (∗) *A. discolor*, (○) *A. discolor* + PCP + 0 g SL L^{-1}, (▲) *A. discolo*r + PCP + 0.3 g SL L^{-1}, (□) *A. discolor* + PCP + 0.6 g SL L^{-1}, (•) *A. discolor* + PCP + 0.9 g SL L^{-1}.

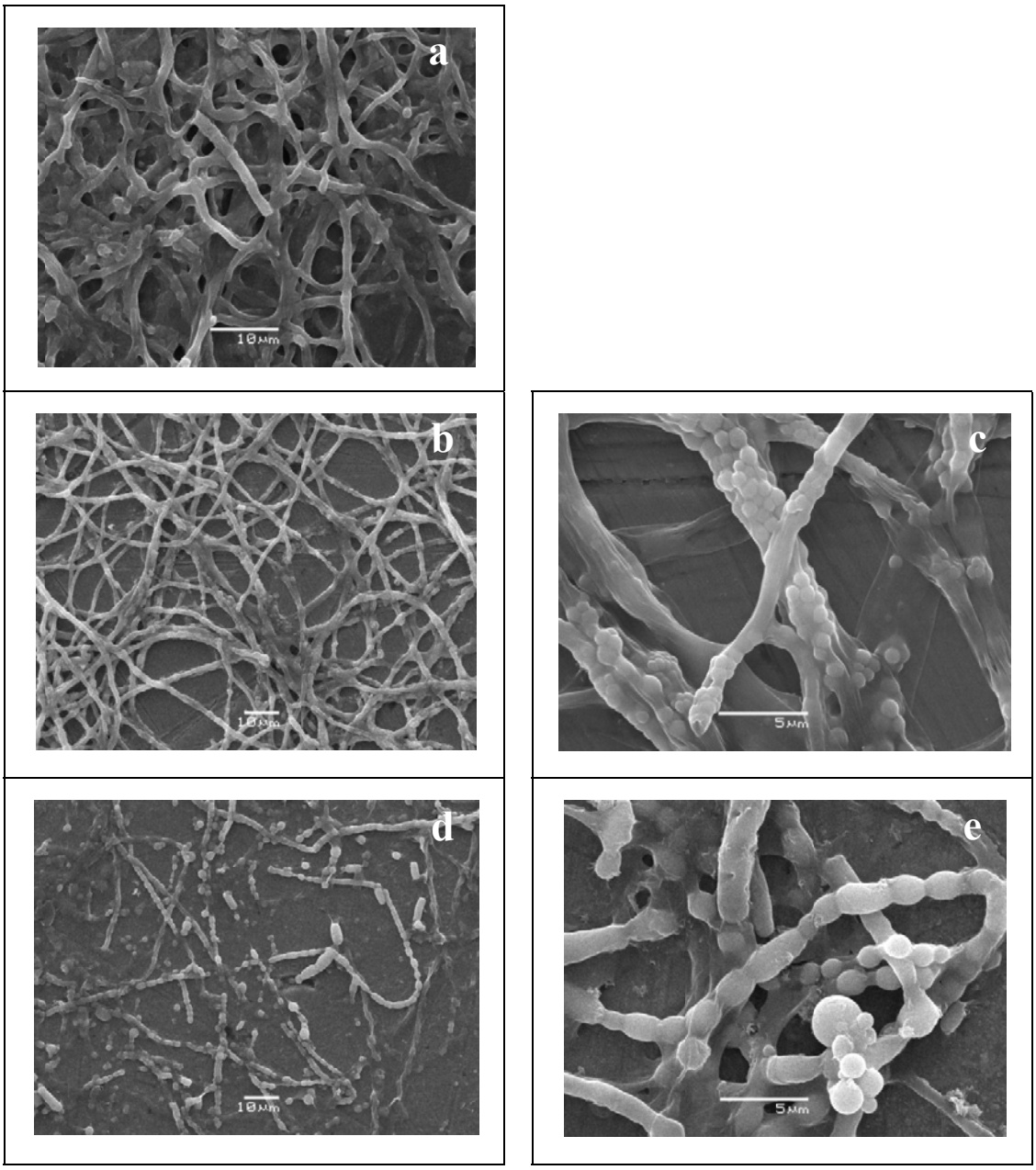

Fig. 4.4 The SEM micrograph of *A. discolor*. (a) *A. discolor* after 20 days of incubation in culture medium without SL, (b) and (c) *A. discolor* after 5 days of incubation, (d) and (e) *A. discolor* with 20 days of incubation. Liquid medium added with 0.6 g SL L^{-1} for (b), (c), (d) and (e).

presents septate hyphae (Fig. 4.4a) and their conidia are spherical and oval. Spherical conidia have 2.4 ± 0.8 µm diameters (n=5) and oval conidia have 3.7 ± 1.2 µm long (n=5). The SEM micrograph shows that SL presents no negative effect on fungal surface of *A. discolor* at the evaluated concentration after 5 and 20-day culture (Fig. 4c and 4e). Information about the effect of SL on degrading microorganisms is limited. However, Bustamante et al. (2011) found that the increase in SL concentration caused an increase in mycelia growth of *A. discolor*. Besides, Soeder et al. (1996) found that SL enhanced growth of the bacteria under all conditions evaluated of surfactant concentrations. Nevertheless, it is known that surfactants can have toxic effect on microorganisms. This negative effect can be related to damage on the cellular membranes or reaction of surfactant with proteins essential to cell functioning, it as was reviewed by Volkering et al. (1998). Garon et al. (2002) evaluated the toxicity of three surfactants on several fungal strains. They found that Triton X-100 and Tween 80 were well tolerated by fungi. However, growth inhibition was observed in presence of SDS (sodium dodecyl sulfate). They explained that the low SDS tolerance is possibly due to physic-chemical interactions between surfactants and fungal structures such as membranes and walls. Besides, some studies have shown that charge has a toxic effect; cationic surfactants are more toxic and are applied as antimicrobials, as it has been reviewed by Van Hamme et al. (2006).

4.4. Conclusions

In conclusion, it could be said that PCP solubility enhances with increasing SL concentration. Besides, the CMC values decreased when PCP concentration was increased. The biodegradation assay showed a positive SL effect, with high degradation obtained during fungal culture. The SL addition in liquid medium enhanced PCP biodegradation in 18% with respect to culture control. *A. discolor* does not diminish its ligninolytic activity in presence of PCP and SL, mainly producing MnP. The PCP removal (%) obtained and the ligninolytic activity, represented by high MnP activity, suggest that SL can be applied for increasing bioavailability and biodegradation of contaminants with low solubility in water. Finally, the SEM micrograph shows that the fungal surface of *A. discolor* was not affected by the SL concentration added to culture medium

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Chapter **5**

Effect of soya lecithin on the biodegradation of chlorpyrifos and pentachlorophenol in a contaminated soil by *Anthracophyllum discolor*

Paper in preparation

Effect of soya lecithin on the biodegradation of chlorpyrifos and pentachlorophenol by *Anthracophyllum discolor* in a contaminated soil

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Abstract

The effect of soya lecithin (SL) on the biodegradation of chlorpyrifos (CLP) and pentachlorophenol (PCP) by *Anthracophyllum discolor* in a contaminated soil was evaluated. The assays were performed using an Andisol collected from Temuco Series. Contaminants were added to the soil at final concentration of 50 mg kg^{-1} of CLP or 250 mg kg^{-1} of PCP. The SL was added to the soil at final concentration of 1.5 and 3.0 g kg^{-1}. The results showed that the 77% of CLP in natural soil inoculated with *A. discolor* and added with 3.0 g kg^{-1} of SL was degraded after 40 days of incubation. On the other hand, the 96.4% of PCP in natural soil inoculated with *A. discolor* and added with 3.0 g kg soil of SL was degradation after 90 days of incubation. Organic matter concentration of soil could increase the adsorption of CLP and PCP, which allowed an additional removal. *A. discolor* and autochthonous microflora are capable of degrading the CLP and PCP metabolites. The CLP and PCP degradation increased with *A. discolor* and with 3.0 g kg^{-1} of SL. The results indicate the potential SL use to facilitate the CLP and PCP degradation.

Keywords: Biodegradation, chlorpyrifos, pentachlorophenol, soya lecithin, white-rot fungi, *Anthracophyllum discolor*, contaminated soil

5.1 Introduction

Soil contamination with hydrophobic compounds may be due to several factors such as natural processes (Gribble, 1994), anthropogenic activities (Venkataraman et al., 2002), application of pesticides (Gilani et al., 2010), and industrial accidents (Kiem et al., 2003). Various technologies have been applied to remediate hydrophobic compounds in contaminated soil. Biodegradation method is often ineffective due to the low solubility of these compounds in water and their slow desorption from soils, which make the pollutants unavailable to the microorganism degraders.

The addition of surfactant has been suggested as a promising technology for remediation of contaminated soils as those surfactants can increase the solubilities of hydrophobic compounds (Edwards et al., 1991), enhance the desorption of low solubility compounds from solid into aqueous phases (Fava et al., 1998; Cort et al., 2002; Zhou and Zhu, 2007), and improve the biodegradation of these pollutants in soils (Soeder et al., 1996).

Surfactants are organic molecules with a polar or ionic hydrophilic group and a nonpolar or hydrophobic chain, known as head and tail groups, respectively (West and Harwell, 1992). An important characteristic is the production of small aggregates called micelles. Micelles are capable of dissolving hydrophobic compounds in their hydrophobic core, which results in an increased apparent aqueous solubility of the compounds (Edwards et al., 1991; Prak and Pritchard, 2002). Tween 80, Triton X-100, Tergitol NP-10 and Tween 20 are the most studied synthetic surfactant in biodegradation processes (Fava and Di Giogia, 1998; Cort et al., 2002; Park and Bielefeldt, 2003). However, increasing attention has been paid to the evaluation of natural surfactants, as humic substances, cyclodextrins and phytogenic surfactants (Fava and Di Gioia, 2001; Conte et al., 2005; Fava et al., 2004; Boyle, 2006). Soya lecithin (SL) is a natural surfactant widely used in the food, cosmetic, and pharmaceutical industries as an emulsifier, lubricant, and release agent (Wu and Wang, 2003). The SL is a mixture of phospholipids, which has been little studied in the biodegradation processes; Soeder et al. (1996) evaluated the effect of SL and quillaya saponin on the aerobic biodegradation of polycyclic aromatic hydrocarbons (PAHs) in

shake-batch cultures of bacteria. The result showed that SL has high PAH-solubilizing activity and a lower bacterial toxicity with respect to quillaya saponin.

The use of surfactants result attractive for soil remediation when present low toxicity and can be degrades by soil microorganisms. However, soil characteristics and desorbed organic compound, type and concentration of surfactant, interaction between surfactant and soil, are some of the factors that can affect the efficiency of surfactant-enhanced biodegradation. Therefore, each surfactant-soil-contaminant system must be particularly analyzed.

Chlorophenols were defined as dangerous pollutants because of their toxicity and persistence in the environment therefore they are priority pollutants in the U. S. EPA list. Pentachlorophenol (PCP) is an organochlorine compound with low water solubility (18 mg L^{-1} at 25ºC) and high K_{OW} (102330) (log K_{ow} 5.01) (Shiu et al., 1994), therefore, PCP adsorbs on the organic fraction of soils (Cea et al., 2005) or into light non-aqueous phase liquids (USEPA, 1997). The PCP was the main component of wood preservative and a microbial breakdown product of pesticides therefore widely used. As at result of this, PCP is present in more than 700 preserving wood sites in the U.S. (USEPA, 1997). In Chile, importation, manufacture, sale, distribution, and use of PCP are prohibited since 1988. Widespread contamination of the environment with PCP has motivated research on microorganisms able to degrade PCP under a variety of conditions (McAllister et al., 1996). However, the degradation of PCP in soil is affected mainly by their low solubility in water; hence tending to bind with organic matter, limiting their availability to microorganisms, which are primarily active in the water phase of the soil (Cea et al., 2005).

On the other hand, chlorpyrifos (O, O-diethyl-o-(3,5,6-thrichloro-2-pyridyl)) (CLP) is a broad-spectrum organophosphate insecticide and acaricide, which has been widely used for agricultural and household pest control since 1965 (Singh et al., 2004). In Chile, CLP was the second best-selling active ingredient 2006 with 1.046.550 L (9.86%) (SAG, 2006). The solubility of CLP is 1.4 mg L^{-1} at 25ºC and K_{OW} 2042 to 186209 (log Kow 3.31 to 5.27), therefore it is strongly adsorb on the organic fraction of soils and affinity for lipids and thus a potential of bioaccumulation in aquatic organisms. Its high soil adsorption coefficient or affinity for soil organic carbon K_{OC} 41 to 52481 (log K_{OC} 40 to 52481) and low solubility indicates a low partitioning by desorption from soil matter to soil water

(Racke et al., 1996). The half-life of CLP in soil varies from less than 1 day to more than 100 days depending on the soil type, soil microorganisms, climatic condition, and clay and organic content (Singh et al., 2002). A reduction of soil microorganisms had been observed when soil or biobed is contaminated with CLP (Shan et al., 2006; Vischetti et al., 2007). Its primary degradation product is 3,5,6-trichloro-2-pyridinol (3,5,6-TCP), which is classified as persistent and mobile due to its solubility in water (49,1 g L^{-1} at 25ºC and pH 7), leading to widespread contamination of soils, sediments and water (Shemer et al., 2005). The accumulation of 3,5,6-TCP, which has anti-microbial properties, prevents the proliferation of chlorpyrifos-degrading microbes (Singh et al., 2004).

Biodegradation with microorganisms has been applied for reducing the level of pesticides in the environment. However, a few chlorpyrifos-degrading microorganisms have been reported. A bacterial strain, *Serratia* sp., could transform CLP to 3,5,6-TCP and *Trichosporo*n sp. could mineralize 3,5,6-TCP (Xu et al., 2007). On the other hand, biodegradation processes are negatively affected due to low solubility in water and strong adsorption in organic matter of CLP soil. Several studies have been demonstrated that the addition of surfactant in contaminated sites enhances solubility, desorption and biodegradation of these compounds.

White-rot fungi are known by capacity of degrading a wide range of contaminants such as PCP (Rubilar et al., 2007; Tortella et al., 2008), pesticides, synthetic dyes (Eichlerová et al., 2005), polycyclic aromatic hydrocarbons (Nikiforova et al., 2009). Their capacity for degrading toxic compounds has been associated with the extracellular ligninolytic system; this system includes lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) (Eichlerová et al., 2005; Barr and Aust, 1994). In preliminary studies, *Anthracophyllum discolor* presented ligninolytic activity with high level of MnP production and PCP degradation potential (Rubilar et al., 2007; Tortella et al. 2008) and SL tolerant in solid medium (Bustamante et al., 2011).

In this work, the effect of SL on the biodegradation of CLP and PCP by *A. discolor* in an artificially contaminated soil was evaluated.

5.2 Material and methods

5.2.1 Materials

5.2.1.1 Microorganism and growth conditions

White-rot fungus *Anthracophyllum discolor* was obtained from the culture collection of the Environmental Biotechnology Laboratory of Universidad de La Frontera (Chile). The fungus was transferred from slant culture tubes (maintained at 4ºC and transferred every 6 months) to glucose malt extract agar plates (per liter of distilled water) 10 g glucose, 30 g malt extract, 15 g agar, pH 5.2 and incubated at 26 ± 2ºC for 7 days before being used for inoculum preparation.

5.2.1.2 Preparation of the blended mycelium

White-rot fungus was first grown in 1-L Erlenmeyer flask containing 100 mL of modified Kirk medium (Tien and Kirk, 1988). The liquid culture medium was inoculated with five glucose malt extract agar plugs (6 mm diameter). The culture was incubated statically at 26 ± 2ºC for 7 days in darkness. After this, the fungal culture was homogenized in a sterilized blender for 1 min. The blended mycelium was used as inoculum. The modified Kirk medium contained (per liter of distilled water) 10 g glucose, 0.12 g $C_4H_{12}N_2O_6$, 2 g KH_2PO_4, 0.59 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 3.3 g $CH3COONa \cdot 3H_2O$ and 10 mL mineral salts. The initial pH of the medium was adjusted to 5.5 with either 1 N NaOH or 1 N HCl.

5.2.1.3 Preparation of the A. discolor inoculum immobilized in lignocellulosic material

A. discolor was grown on wheat grains, which were used as lignocellulosic material, as described by Rubilar et al. (2011). Two grams of this material was transferred to glass tubes and 2 mL of distilled water were added. The tubes were autoclaved at 121 ± 2ºC for 15 min. Then, the tubes with 4 mL of blended mycelium of *A. discolor* were inoculated and

incubated at 26 ± 2ºC for 7 days in darkness. This material was used as inoculum for the bioremediation of soil contaminated with CLP or PCP.

5.2.1.4 Preparation of soil

An Andisol collected from Temuco Series, located in southern Chile, was used for the experiments. The soil was collected at 0-20 cm depth, air-dried and sieved to select a particle size lower than 2 mm. Afterwards, 25 g soil in a 125-mL glass Erlenmeyer flask were fumigated with 2 mL ethanol-free chloroform. After this, the soil was maintained at 30 ± 2ºC for 7 days and placed in vacuum desiccators to remove all traces of chloroform. The fumigated soil was used as abiotic control. The major physical-chemical properties of the soil are summarized in Table 5.1.

Table 5.1 Physical-chemical properties of soil

Parameter	Value
pH (in water)	6.0 ± 0.1
N (mg kg^{-1})†	29.3 ± 1.5
P (mg kg^{-1})†	23.7 ± 1.2
Organic matter (%)	13.7 ± 1.5

† Available

5.2.2 Methods

5.2.2.1. Effect of SL on CLP and PCP biodegradation by A. discolor in a contaminated soil

The assay of CLP biodegradation was performed in 100-mL Erlenmeyer flask containing 10 g soil (dw). The soil was contaminated with CLP (Sigma-Aldrich, >98% of purity) diluted in acetone to reach a final concentration of 50 mg kg^{-1} of soil. The solvent was allowed to evaporate before SL addition. Then, the soil was atomized with an SL stock solution (PRINAL®) diluted in hexane to reach concentration of 1.5 and 3.0 g kg^{-1} of soil. Once the solvent evaporated, the soil was inoculated with *A. discolor* immobilized in wheat grains, as described in *5.2.1.3*. Similarly, it was performed for the assay of PCP biodegradation, however in this case the soil was contaminated with a PCP stock solution (Aldrich Chemical Co., >98% purity) diluted in acetone to reach a final concentration of 250 mg kg^{-1} of soil.

The assays were performed under static conditions, in darkness, maintaining soil moisture to 40% with sterile distilled water, and incubated at 25 ± 2ºC. Besides, each assay was carried out in triplicate under destructive sampling mode. The residual contaminants concentration, ligninolytic enzymatic activity and fluorescein diacetate (FDA) hydrolysis were analyzed by 40 and 90 days of incubation for CLP and PCP, respectively. The results are reported as the mean of three replicates per treatment, with a standard deviation of the mean represented by error bars. Moreover, biotic (natural soil without fungus) and abiotic control (fumigated soil without fungus) were incubated with sterile distilled water to maintain soil moisture at 40%.

First-order dissipation kinetics ($C = C_o\, e^{-kt}$, where C = concentration after time t, C_o = apparent initial concentration, and k = removal rate constant) were used for interpreting the CLP and PCP extractable residues data. From the data obtained, the $t_{1/2}$ (half-dissipation time or the time required to reach a contaminant removal of 50%) was calculated for each treatment.

5.2.2.2. Statistical analysis of data

Duncan Test was used to compare means. Statistical significance level for all treatments was 5%.

5.2.3 Analytical procedures

5.2.3.1 Extraction and quantification of CLP, PCP and their metabolites

The residual concentration of CLP and 3,5,6-TCP from soil was determined after extraction with 6 mL of acidified acetone (acetone:water:concentrated phosphoric acid, 98:1:1, v/v/v) per gram of substrate (Racke et al., 1996). Each soil sample was homogenized using an orbital shaker for 2 h at 350 rpm. Later, samples were sonicated for 30 min and centrifuged for 7 min at 10000 rpm. A second extraction under the same conditions as described previously was made to soil. The collected supernatants were filtered using a membrane of 0.2 μm pore size and stored in amber glass flasks at -18 ± 2°C.

The PCP extraction from soil was performed from flasks after enzymatic activity assays. Each flask containing 10 g of soil were adjusted to pH 2 with 0.1 N H_2SO_4 and homogenized with 20 mL hexane:acetone mixture (1:1, v/v) using an orbital shaker for 2 h at 200 rpm in order to attain transport of PCP and its metabolites from water and soil to the organic phase. After this, the soil homogenate was sonicated for 30 min and centrifuged for 10 min at 2500 rpm for separation of the organic and aqueous phases. An aliquot of the organic phase was filtered for 0.2 μm diameter pore and used for determining the residual PCP and its metabolites.

The contaminants concentration were quantified by HPLC with a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with 20 μL loop diode array detector. The column was a reverse phase (Lichrosphere 60RP select B, 5 μm). Detection of PCP and metabolites were at 210 nm and the mobile phase consisted of 1% phosphoric acid:acetonitrile (40:60, v/v). The CLP and metabolites detection were 290 nm and the mobile phase consisted of 1% phosphoric acid:acetonitrile (45:55, v/v between 0-10 min; and 60:40, v/v between 11 – 20 min). Both determinations were with a flow rate of 1 mL min^{-1}, at room temperature (22

± 2ºC). The SL presence did not interfere with the detection of CLP, PCP and metabolites. The described procedures were checked for CLP and PCP recovery, which ranged 93 to 100% and 86 to 100%, respectively. The retention times were 3.98 and 13.46 min for 3,5,6-TCP and CLP, respectively. Besides, the retention times for 2,4-DCP, 2,4,6-TCP and PCP were 3.79, 4.49 and 6.11 min, respectively.

5.2.3.2 Preparation of enzymatic extract from soil sample

The enzymatic extract from contaminated soil with CLP was determined after extraction with 12.5 mL of buffer succinate–lactate 0.1 M (pH 4.5) per 5 g of soil. Each sample was homogenized using an orbital shaker for 1 h at 150 rpm. After this, the samples were centrifuged for 10 min at 5000 rpm. Enzymatic extract from contaminated soil with PCP was determined in 10 g of soil contained in 100 mL Erlenmeyer flask was homogenized in 10 mL of 0.1 M sodium tartrate buffer (pH 4.5). The flasks were shaken at 200 rpm for 30 min in an orbital shaker. Then, an aliquot of 3.0 mL was centrifuged at 10000 rpm for 7 min at 4ºC.

Enzymatic activities were measured from the supernatant and the mixture of reactions was performed at 30ºC. The absorbance was read using Spectronic Genesys 2PC model spectrophotometer and was expressed in U kg $soil^{-1}$.

Laccase activity was determined with 2,6-dimethoxyphenol (DMP) as the substrate in sodium malonate (pH 4.5). The enzyme activity unit was defined as an increase in absorbance per minute at 468 nm and 30ºC (deJong et al., 1994). The MnP activity was determined by monitoring the oxidation of 2,6-DMP spectrophotometrically at 30ºC. The reaction mixture (1 mL) contained 200 µL of 250 mM sodium malonate (pH 4.5), 50 µL of 20 mM 2,6-DMP, 50 µL of 20 mM $MnSO4{\cdot}H_2O$ and 600 µL of supernatant. The reaction was initiated by adding 100 µL of 4 mM H_2O_2, monitored at 468 nm and corrected by the Lac activity. The MiP activity was determined in a reaction mixture containing 200 µL 250 mM sodium malonate (pH 4.5), 50 µL 20 mM 2,6-DMP, 100 µL of 20 mM EDTA, and 600 µL of supernatant. The reaction was initiated by adding 100 µL of 4 mM H_2O_2, monitored at 468 nm and corrected by the Lac activity (deJong et al., 1994). The molar extinction coefficient was 49600 M^{-1} cm^{-1}.

5.2.3.3. Fluorescein diacetace hydrolysis in the soil sample

Hydrolysis of fluorescein diacetate (FDA) was determined according to method proposed by Schnürer and Rosswall (1982). Briefly, 1.0 g of dry soil in a 25 mL flask with 9.9 mL of sodium phosphate buffer was prepared. The reaction was started by adding 0.1 mL of FDA solution (1 mg mL^{-1}). The reaction mixture was incubated at 25 ± 2ºC for 1 h in a thermoregulated bath. After this, 10 mL of acetone were added to stop the reaction. A sample was filtered (Whatman Nº 40) and the absorbance in the supernatant measured at 490 nm. The concentration of the released fluorescein by a calibration curve with standard quantities of fluorescein was calculated and the results expressed as μg FDA g^{-1}.

5.3 Results

5.3.1 Effect of SL on biodegradation of CLP by *A. discolor* in a contaminated soil

5.3.1.1. Degradation of CLP by A. discolor in soil

The effect of SL on CLP biodegradation in a artificially contaminated soil by *A. discolor* was evaluated. The effect of the autochthonous soil microflora (soil non-inoculated with *A. discolor*) on CLP degradation and CLP adsorption in the soil (non-inoculated sterile soil) was also evaluated. Fig. 5.1 shows that the residual CLP concentration presents two phases, except Fig. 5.1d. The first rapid degradation (until 14 days of incubation) was followed by a slower stage (Figs. 5.1a, b and c). In general, CLP concentration decreased in all treatments. The residual CLP concentration in soil inoculated with *A. discolor* and added with 0, 1.5 and 3.0 g kg^{-1} of SL was 15.8, 15.0 and 11.5 mg kg^{-1}, respectively (Fig. 5.1a). In the biotic control added with 0, 1.5 and 3.0 g kg^{-1} of SL, residual CLP concentration was 27.1, 24.6 and 20.2 mg kg^{-1}, respectively (Fig. 5.1b). Moreover, in sterile soil inoculated with *A. discolor* and supplemented with 0, 3.0 and 1.5 g kg^{-1} of SL , the residual CLP concentration was 23.1, 17.7 and 16.5 mg kg^{-1}, respectively (Fig. 5.1c). Finally, the effect of SL on CLP removal in the abiotic control is exposed in Fig. 5.1d. This Fig. shows that in soil supplemented with 0, 1.5 and 3.0 g kg^{-1} of SL, the residual CLP concentration was 37.7, 40.7 and 36.8 mg kg^{-1} of soil, respectively. After 40 days of culture, the 77% of CLP in natural soil inoculated with *A. discolor* and added with 3.0 g kg^{-1} of SL was degraded, while the 26.4% of CLP in the abiotic control and soil supplemented with 3.0 g kg^{-1} of SL was removal.

Fig. 5.1 shows that the CLP concentration decrease over time in the different treatments and could be well described using first-order kinetics. In the abiotic control with 3.0 g kg^{-1} of SL was obtained the low correlation coefficient (0.72). In contrast, a high correlation coefficient (0.94) was obtained in sterile soil inoculated with *A. discolor* and added with 1.5 g kg^{-1} of SL (Table 5.2). Table 5.2 shows that $t_{1/2}$ of CLP removal varies greatly among the soil treatments from 17.9 to 127.5 days, when the soil was inoculated with *A. discolor* and supplemented with 3.0 g kg^{-1} of SL and in the abiotic control added

with 1.5 g kg^{-1} of SL, respectively. The CLP degradation was faster in natural soil inoculated with *A. discolor* and added with 3.0 g kg^{-1} of SL with 17.9 days. Besides, the degradation time of CLP decreases proportionally when the SL concentration increase. This condition is favored when the soil was inoculated with *A. discolor*. In general, the removal rate (*k*) increases to the higher the SL concentration.

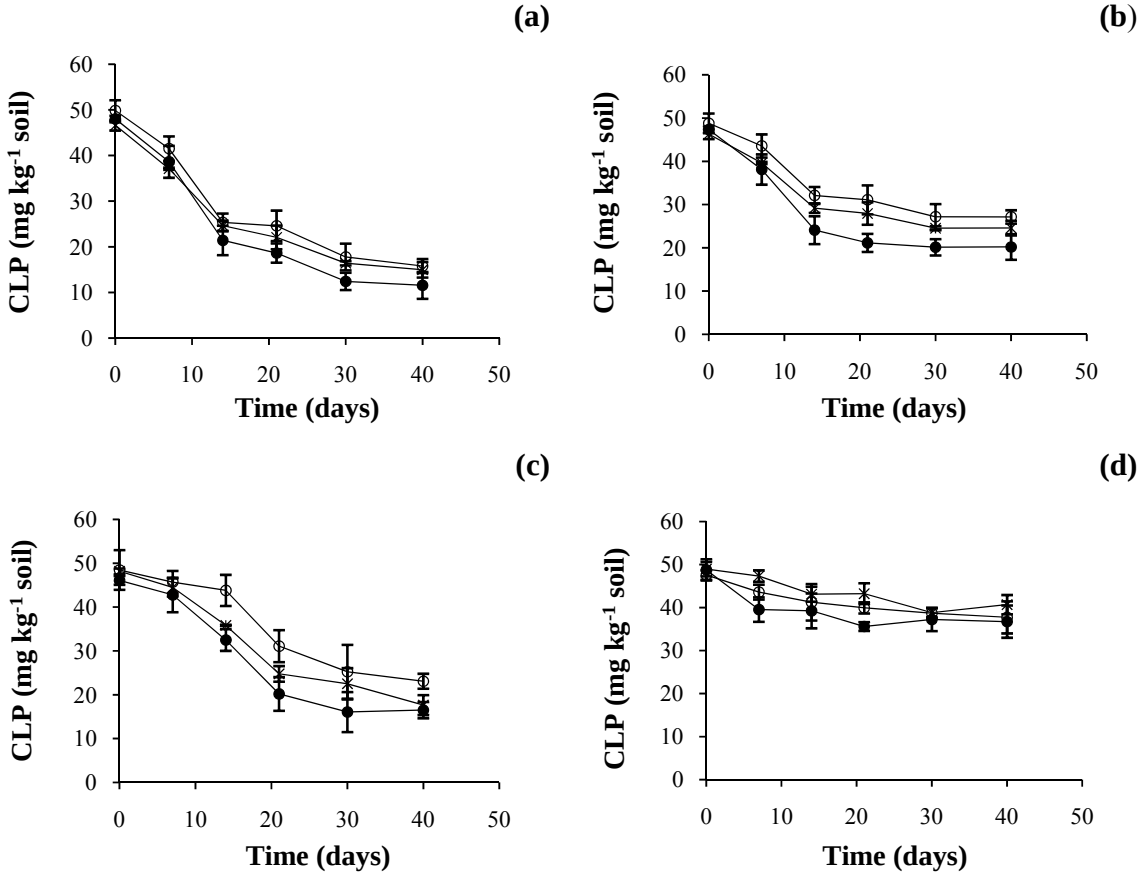

Fig. 5.1 Residual CLP concentrations in soil. (a) Natural soil inoculated with *A. discolor*, (b) natural soil without *A. discolor* (biotic control), (c) sterile soil with *A. discolor* and (d) abiotic control. The symbols represent: (○) without SL, (∗) 1.5 g SL kg^{-1} of soil and (●) 3.0 g SL kg^{-1} of soil. Values are mean of three replicates ± SD.

According to the results obtained CLP removal was significantly higher ($p < 0.05$) in soil supplemented with 3.0 g kg^{-1} of SL and inoculated with *A. discolor*, when is compared with abiotic control. In natural soil inoculated with *A. discolor* were detected significant differences ($p < 0.05$) where soil was supplemented with 3.0 g kg^{-1} of SL respect to soil

Table 5.2 Residual CLP concentrations (mg kg^{-1} of soil), removal rate constant (k in $days^{-1}$) and half life values ($t_{1/2}$ in days) in soil by *A. discolor* as mycelium immobilized in wheat grains

Treatments	Residual CLP (mg kg^{-1} soil)	k (d^{-1})	R^2	$t_{1/2}$
Natural soil with *A. discolor*				
Without SL	15.8 ± 0.2 ^{f}	0.0302	0.93	22.9
1.5 g SL kg^{-1} soil	15.0 ± 1.5 ^{f}	0.0298	0.94	23.2
3.0 g SL kg^{-1} of soil	11.5 ± 0.4 ^{g}	0.0386	0.93	17.9
Natural soil without *A. discolor*				
Without SL	27.1 ± 1.5 ^{b}	0.0156	0.85	44.4
1.5 g SL kg^{-1} soil	24.6 ± 1.7 $^{b, c}$	0.0165	0.84	41.9
3.0 g SL kg^{-1} soil	20.2 ± 3.0 $^{d, e}$	0.0222	0.77	31.2
Sterile soil with *A. discolor*				
Without SL	23.1 ± 1.7 $^{c, d}$	0.0215	0.94	32.2
1.5 g SL kg^{-1} soil	17.7 ± 2.3 $^{e, f}$	0.0272	0.97	25.5
3.0 g SL kg^{-1} soil	16.5 ± 1.9 ^{f}	0.0309	0.89	22.4
Sterile soil without *A. discolor*				
Without SL	37.7 ± 3.7 ^{a}	0.0055	0.89	125.0
1.5 g SL kg^{-1} soil	40.7 ± 2.2 ^{a}	0.0054	0.81	127.5
3.0 g SL kg^{-1} soil	36.8 ± 3.8 ^{a}	0.0093	0.72	74.8

Values of mean (n=3) ± SD

Different letters refer to significance differences between mean values (n=3) with Duncan Test ($p < 0.05$).

added with 0 and 1.5 g kg^{-1} of SL. Also, no significant differences ($p < 0.05$) were found in sterile soil without *A. discolor* and with or without SL (Table 5.2).

The CLP degradation by *A. discolor* was confirmed by the generation of its metabolite 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) (Fig. 5.2). In general, this Figure shows that 3,5,6-TCP concentration increases in all treatments except in the abiotic control (Fig. 5.2d). Depending of treatment, the 3,5,6-TCP concentration begins to decrease after 14 or

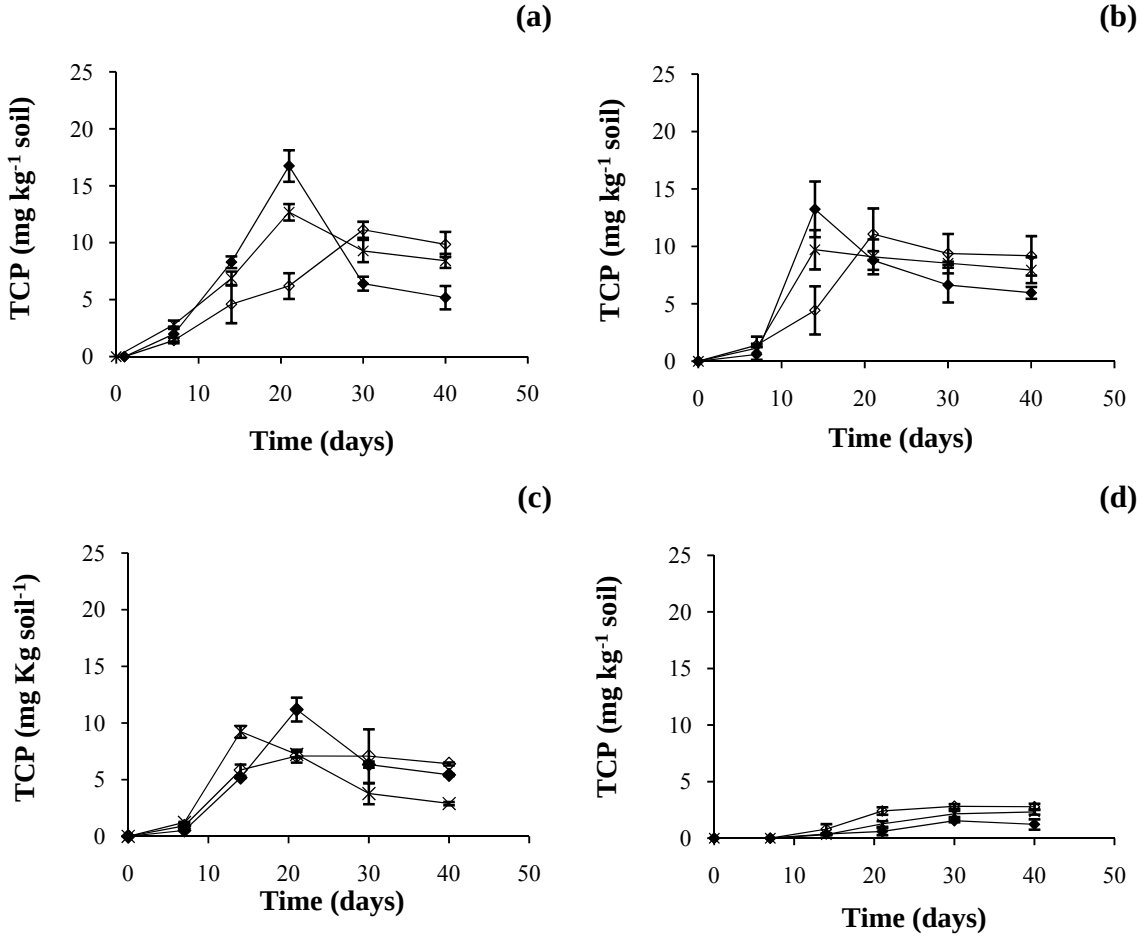

Fig. 5.2 The 3,5,6-TCP concentrations in soil (a) Natural soil inoculated with *A. discolor*, (b) natural soil without *A. discolor* (biotic control), (c) sterile soil with *A. discolor* and (d) abiotic control. The symbols represent: (◊) without SL, (×) 1.5 g SL kg^{-1} of soil and (◆) 3.0 g SL kg^{-1} of soil. Values are mean of three replicates ± SD.

20 days of incubation, this behavior is favored in presence of SL. In addition, the higher 3,5,6-TCP concentration was obtained with an addition of 3.0 g kg^{-1} of SL in the soil. The maximum concentration of metabolite was 16.7 mg kg^{-1} of soil after 21 days of incubation in natural soil inoculated with *A. discolor* and added with 3.0 g kg^{-1} of SL. On the other hand, the metabolite concentration did not exceed 2.8 mg kg^{-1} in the abiotic control (Fig. 5.2d).

5.3.1.2. Effect of SL and CLP on enzyme production in sample soil

Ligninolytic activity of *A. discolor* in presence of SL and CLP was studied (Fig 5.3). *A. discolor* showed low MnP activity during the first 7 days of incubation in both, natural soil and sterile soil, after this MnP activity increased. In natural soil, *A.discolor* reached a maximum of 48.9 U kg^{-1} of soil when 1.5 g kg^{-1} of SL was added, after 14 day culture (Fig.

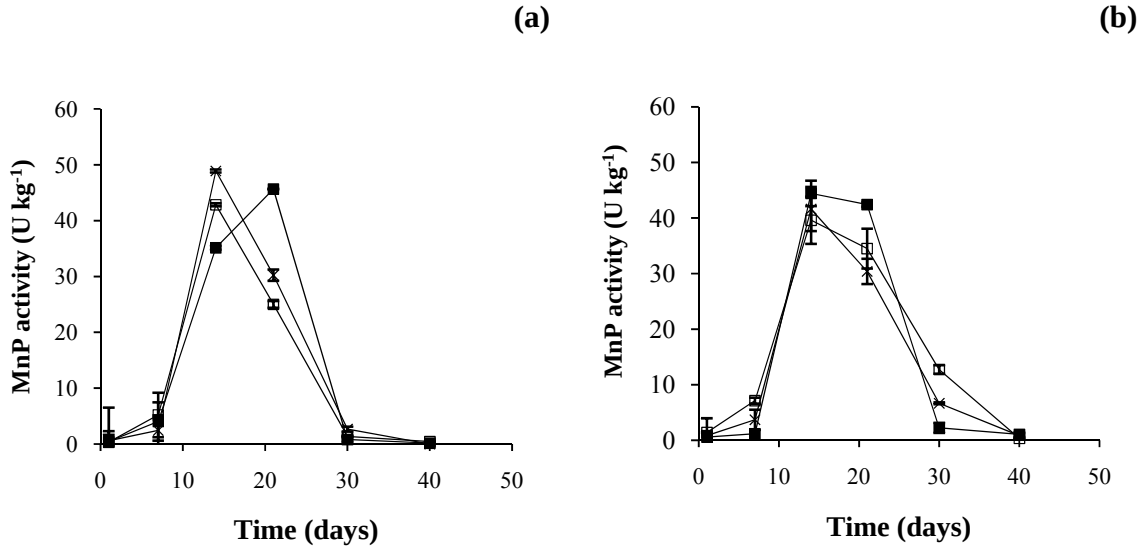

Fig. 5.3 Effect of SL and CLP on MnP activity. (a) Natural soil inoculated with *A. discolor* and (b) sterile soil with *A. discolor*. The symbols represent: (□) without SL, (×) 1.5 g SL kg^{-1} of soil and (■) 3.0 g SL kg^{-1} of soil. Values are mean of three replicates ± SD.

5.3a). On the other hand, in sterile soil, *A. discolor* reached a maximum of 44.4 U kg^{-1} of soil when the assay was supplemented with 3.0 g kg^{-1} of SL, after 14 days of incubation (Fig. 5.3b). Finally, Lac and MiP showed low activity in all treatments and did not exceed 5 U kg^{-1} of soil, data not shown.

5.3.1.3. Effect of SL on total microbial activity in soil added with CLP

The effect of SL and CLP on total microbial activity was evaluated. In general, Fig. 5.4 shows that microbial activity was not affected in any of the treatments.

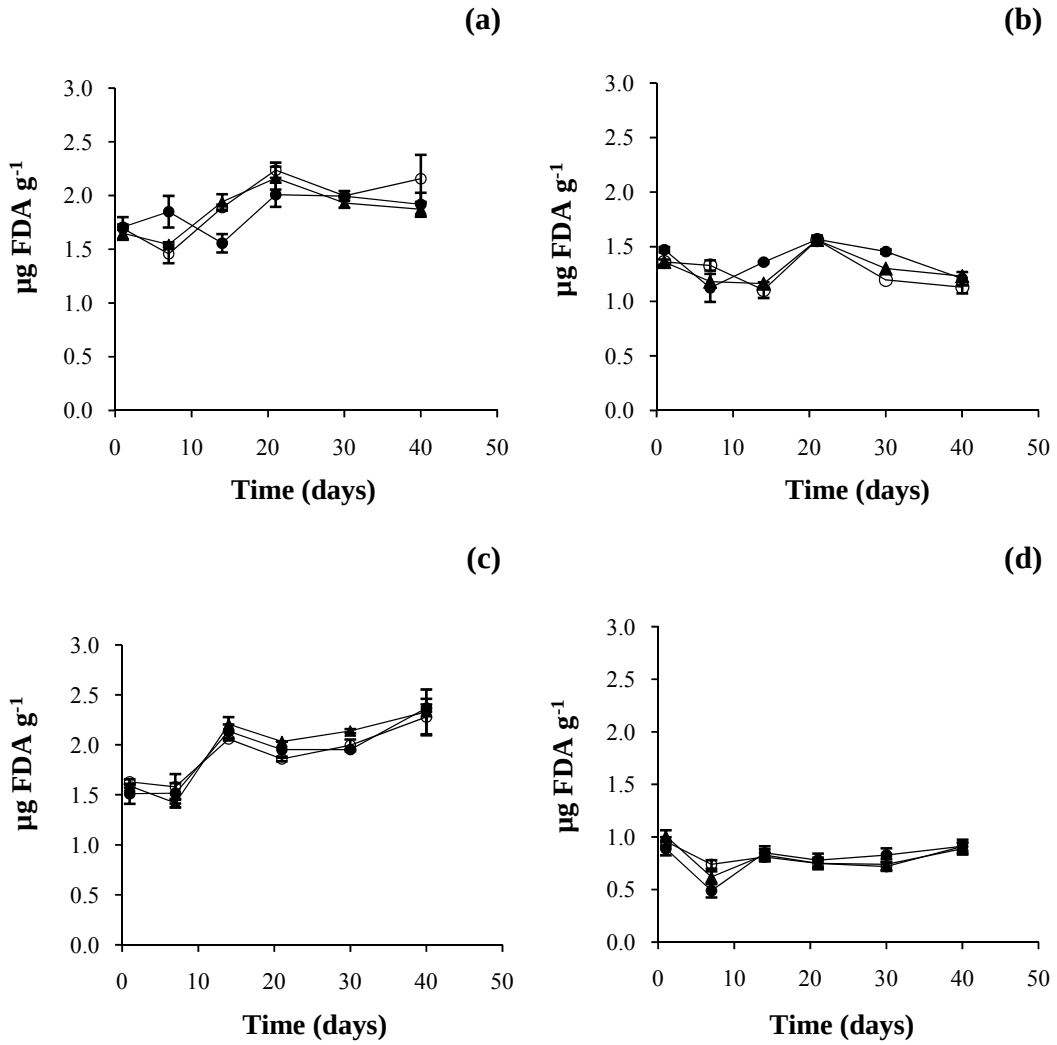

Fig. 5.4 Effect of SL and CLP on the fluorescein diacetate (FDA) hydrolysis. (a) Natural soil inoculated with *A. discolor*, (b) natural soil without *A. discolor* (biotic control), (c) steril soil inoculated with *A. discolor* and (d) Abiotic control. Symbols represent (○) without SL and (▲) 1.5 g SL kg^{-1} of soil and (●) 3.0 g SL kg^{-1} of soil. Values are mean of three replicates ± SD.

5.3.2 Effect of SL on biodegradation of PCP by *A. discolor* in a contaminated soil

5.3.2.1. Degradation of PCP by A. discolor in soil

The SL effect on biodegradation of PCP in an artificially contaminated soil by *A. discolor* was evaluated. The effect of the autochthonous soil microflora (soil non-inoculated with *A. discolor*) on PCP degradation and PCP adsorption in the soil (non-inoculated sterile soil) were also investigated. The Figure 5.5 shows that the residual PCP concentrations in soil present two phases. The first period was rapid degradation and was followed by a slower stage from 30 to 90 days of culture. In soil inoculated with *A. discolor* and added with 0, 1.5 and 3.0 g kg^{-1} of SL the residual PCP was 17.3, 11.5 and 9.0 mg kg^{-1} soil after 90 days of incubation, respectively (Fig. 5.5a). In the same time of incubation, in the biotic control added with 0, 1.5 and 3.0 g kg^{-1} of SL, the residual PCP concentration was 37.2; 45.2 and 51.1 mg kg^{-1} soil, respectively (Fig. 5.5b). Besides, in sterile soil inoculated with *A. discolor*, the concentration of PCP in soil added with added 0, 1.5 and 3.0 g kg^{-1} of SL was 63.3, 52.4 and 30.7mg kg^{-1} after 90 days of incubation, respectively (Fig. 5.5c). Finally, the SL effect on PCP removal in the abiotic control is shown in Fig. 5.5d. This Figure shows that residual PCP concentration in soil added with 0, 1.5 and 3.0 g kg^{-1} of SL was 82.2, 74.6 and 97.9 mg kg^{-1} of soil after 90 days of incubation, respectively. Finished the period of incubation, in natural soil inoculated with *A. discolor* and added with 3.0 g kg^{-1} of SL, the 96.4% of PCP was degraded. In contrast, the PCP removal in the abiotic control reached a value of 60.8% when the soil was supplemented with same SL concentration.

The Fig. 5.5 shows that the PCP concentration decrease over time in the different treatments and could be well described using first-order kinetics. In the abiotic control with 3.0 g kg^{-1} of SL was obtained the low correlation coefficient (0.70). However, a high correlation coefficient (0.94) was obtained in biotic control with 1.5 g kg^{-1} of SL (Table 5.3). Table 5.3 shows that $t_{1/2}$ of PCP removal varies greatly among the soil treatments from 23.8 to 236.9 days, when the natural soil was inoculated with *A. discolor* and added with 3.0 g kg^{-1} of SL and in the abiotic control added with 3.0 g kg^{-1} of SL, respectively. On the other hand, the removal rate evaluated in the different treatments was highest in natural soil

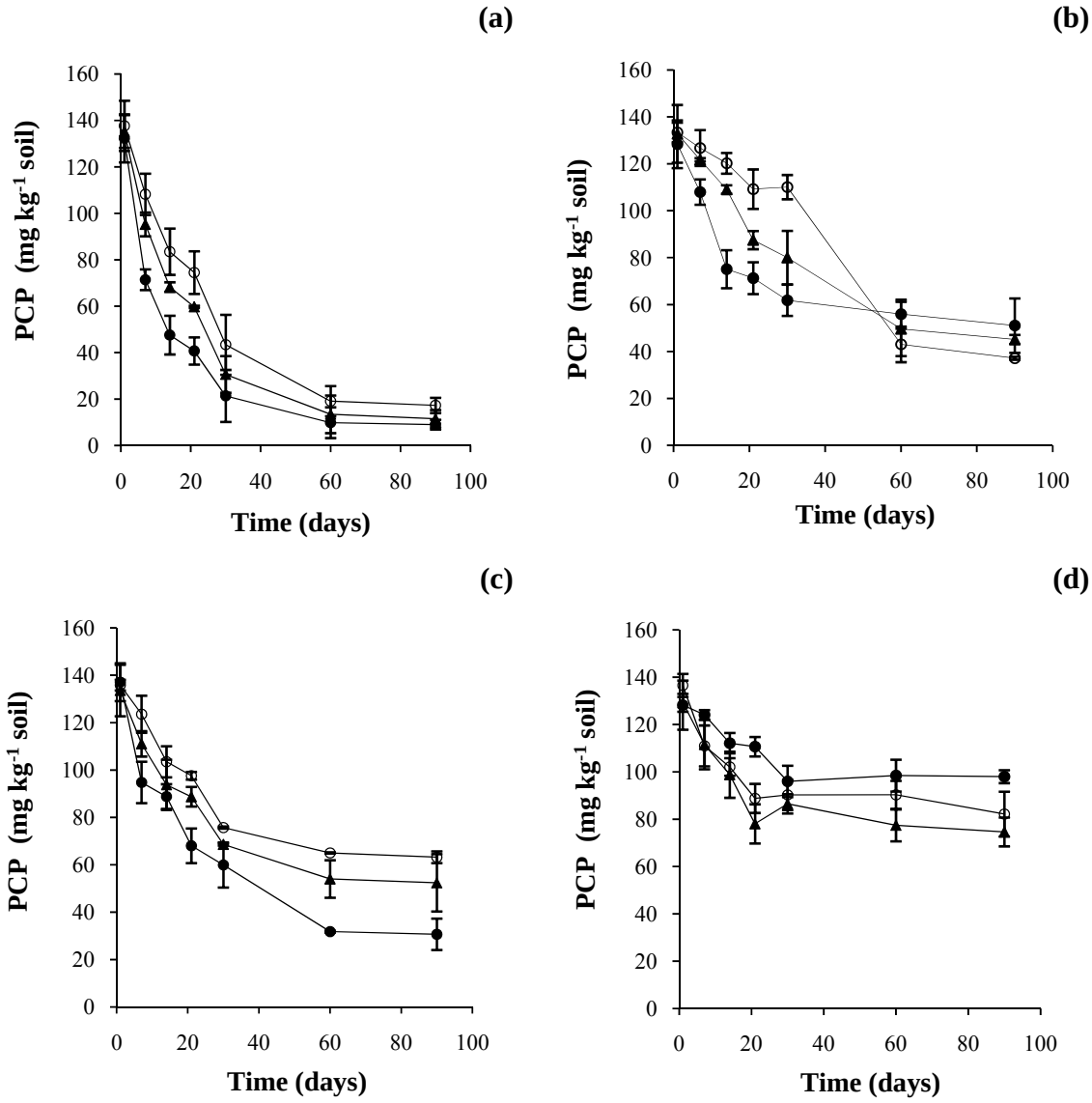

Fig. 5.5 Residual PCP concentrations in soil. (a) Natural soil inoculated with *A. discolor*. (b) Natural soil without *A. discolor* (biotic control). (c) Sterile soil with *A. discolor*. (d) Abiotic control. The symbols represent (○) without SL, (▲) 1.5 g SL kg^{-1} of soil and (●) 3.0 g SL kg^{-1} of soil. Values are mean of three replicates ± SD.

Table 5.3 Residual PCP concentrations (mg kg^{-1} of soil), removal rate constant (*k* in $days^{-1}$) and half life values ($t_{1/2}$ in days) in soil by *A. discolor* as mycelium immobilized in wheat grains

Treatments	Residual PCP (mg kg^{-1} soil)	k (d^{-1})	R^2	$t_{1/2}$
Natural soil with *A. discolor*				
Without SL	17.3 ± 3.3 ^{g}	0.0247	0.92	28.1
1.5 g SL kg^{-1} soil	11.5 ± 3.8 ^{g}	0.0284	0.91	24.4
3.0 g SL kg^{-1} of soil	9.0 ± 2.1 ^{g}	0.0291	0.86	23.8
Natural soil without *A. discolor*				
Without SL	37.2 ± 0.6 $^{e, f}$	0.016	0.93	42.7
1.5 g SL kg^{-1} soil	45.2 ± 1.9 $^{d, e}$	0.013	0.94	54.1
3.0 g SL kg^{-1} soil	51.1 ± 11.6 ^{d}	0.009	0.71	77.4
Sterile soil with *A. discolor*				
Without SL	63.2 ± 2.5 ^{c}	0.0086	0.83	80.3
1.5 g SL kg^{-1} soil	52.4 ± 9.1 ^{d}	0.0102	0.85	67.7
3.0 g SL kg^{-1} soil	30.7 ± 6.6 ^{f}	0.0165	0.90	41.9
Sterile soil without *A. discolor*				
Without SL	82.2 ± 9.5 ^{b}	0.0044	0.71	156.7
1.5 g SL kg^{-1} soil	74.6 ± 6.1 ^{b}	0.0056	0.82	124.2
3.0 g SL kg^{-1} soil	98.0 ± 2.7 ^{a}	0.0029	0.70	236.9

Values of mean (n=3) ± SD

Different letters refer to significant differences between mean values (n=3) with Duncan Test ($p < 0.05$).

inoculated with *A. discolor* and supplemented with 3.0 g kg^{-1} of soil. In contrast, Table 5.3 show significant differences ($p < 0.05$) for all treatments evaluated, however was not detect significant differences ($p < 0.05$) in natural soil inoculated with *A. discolor* for all SL concentrations evaluated.

In general, the results show that the soil inoculated with *A. discolor* and added with 3.0 g kg^{-1} of SL presents a high PCP removal, being 11-fold more effective than in sterile soil without inoculums and added with 3.0 g kg^{-1} of SL. In addition,

The PCP degradation by *A. discolor* was confirmed by the generation of the metabolites such as 2,4-dicholorophenol (2,4-DCP) and 2,4,6-trichlorophenol (2,4,6-TCP) (Fig. 5.6). The highest metabolites concentrations of degradation obtained in the different treatments evaluated were 6.9 mg kg^{-1} of 2,4- DCP in sterile soil inoculated with *A. discolor* and added with 3.0 g kg^{-1} of SL (Fig. 5.6b), and 6.1 mg kg^{-1} of 2,4,6-TCP in natural soil inoculated with *A. discolor* and 1.5 g kg^{-1} of SL (Fig. 5.6c), after 21 and 14 days of incubation, respectively.

5.3.2.2. Effect of SL and PCP on enzyme production in sample soil

The production profiles of the ligninolytic enzymes in soil containing SL and PCP are depicted in Fig. 5.7. The MnP activity was low during the first 14 days of incubation. After this period, MnP production increased reaching a maximum of 114.3 U kg^{-1} of soil on day 21 of inoculating the fungus in soil with 1.5 g of SL kg^{-1} of soil (Fig. 5.7a). On the other hand, in soil without SL, MiP activity reached a maximum of 46.4 U kg^{-1} of soil after 21-day culture (Fig. 5.7b). On the other hand, Lac showed low activity in all treatments and did not exceed 4 U kg^{-1} of soil, data not shown.

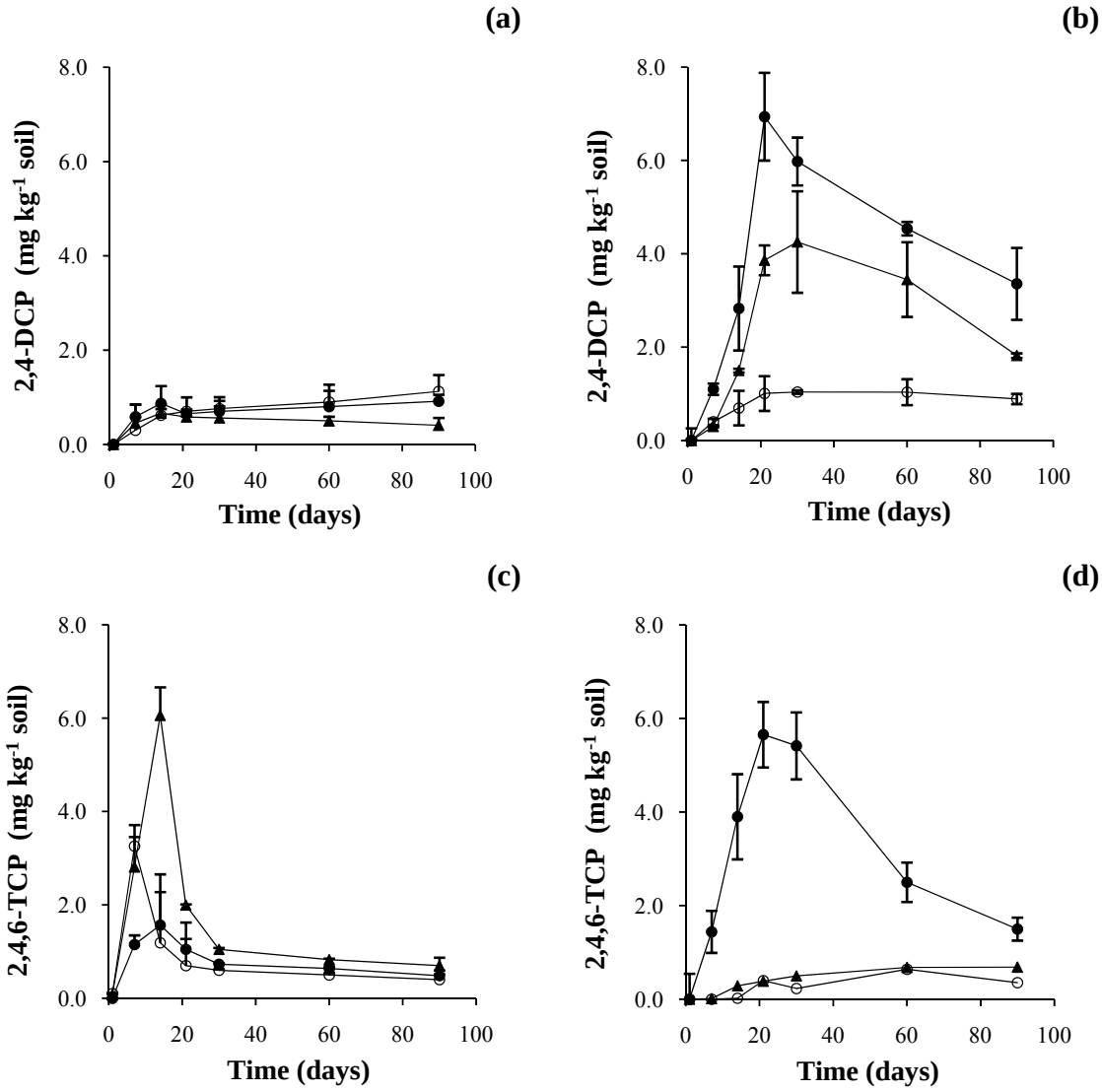

Fig. 5.6 Metabolites of PCP concentrations in soil. (a) and (c) Natural soil inoculated with *A. discolor,* (b) and (d) sterile soil with *A. discolor*. The symbols represent (○) without SL, (▲) 1.5 g SL kg^{-1} of soil and (●) 3.0 g SL kg^{-1} of soil. Values are mean of three replicates ± SD.

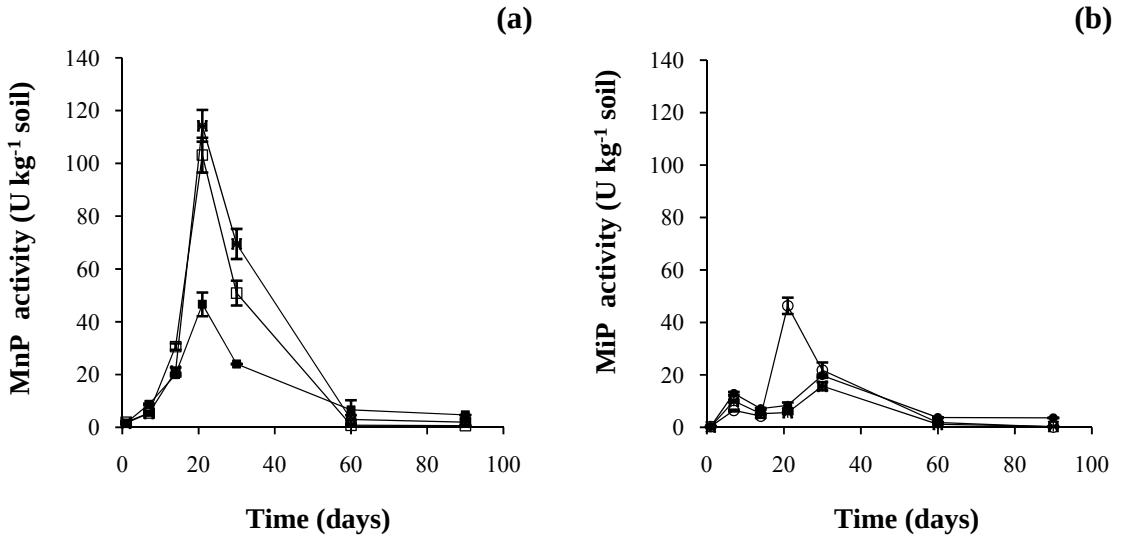

Fig. 5.7 Effect of SL and PCP (250 mg kg^{-1} of soil) on enzyme production in sample soil. (a) MnP activity (b) MiP activity, where: (○ or □) without SL, (∗ or ×) 1.5 g of SL kg $soil^{-1}$ and (● or ■) 3.0 g of SL kg^{-1} of soil. The points represent the means of three replicates ± SD.

5.3.2.3. Effect of SL on total microbial activity in soil added with PCP

The SL effect on total microbial activity was evaluated (Fig. 5.8). In general, this Figure shows that the microbial activity decreased in all treatments after of the PCP contamination. After this, the hydrolytic activity increase reaching a maximum of 4.9 µg FDA g^{-1} when the natural soil was inoculated with *A. discolor* and without SL after 21 days of incubation with. Interestingly, the FDA in all the treatments declined until the 90 days of incubation.

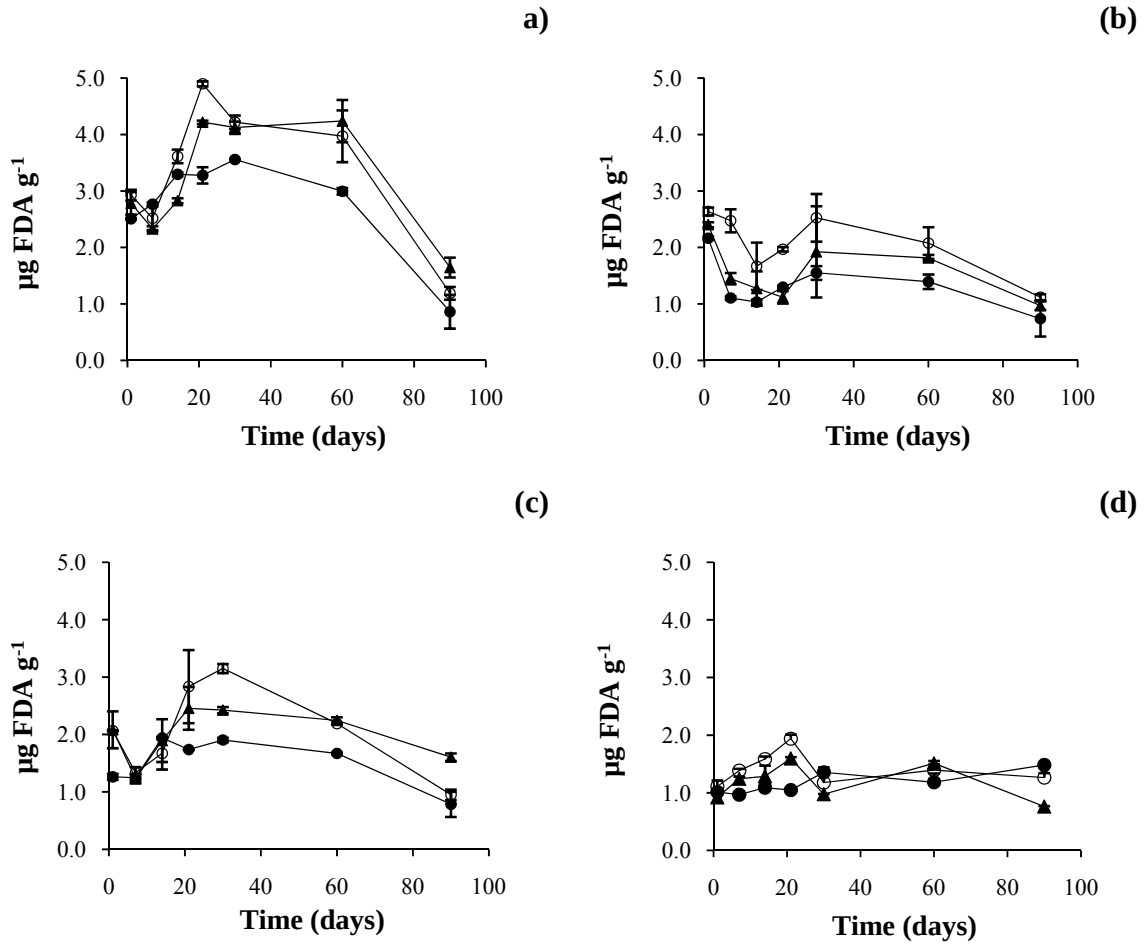

Fig. 5.8 Effect of SL and PCP on the fluorescein diacetate (FDA) hydrolysis. (a) Natural soil inoculated with *A. discolor*, (b) natural soil without *A. discolor* (biotic control), (c) steril soil inoculated with *A. discolor* and (d) abiotic control. Symbols represent (○) without SL, (▲) 1.5 g SL kg^{-1} of $soil^{-1}$ and (●) 3.0 g SL kg^{-1} of soil. Values are mean of three replicates ± SD.

5.4 Discussion

Several researches have demonstrated that surfactants have the potential to increase the desorption of pollutants from soil due to enhanced solubility (Fava et al., 1998; Cort et al., 2002; Zhou and Zhu, 2007). In this context, the SL effect on biodegradation of CLP and PCP by *A. discolor* in an artificially contaminated soil was evaluated.

The SL effect on the CLP biodegradation in a contaminated soil is presented in Fig. 5.1. This Figure shows that SL presence has a positive effect on CLP biodegradation and 3,5,6-TCP in respect to the assays without SL. Moreover, biodegradation increased with the rise of SL concentration. Fava et al. (2004) studied the effects of SL and humic sustances on aerobic bioremediation of an aged-contaminated soil. Their result showed that both biogenic materials are capable to enhance both bioavailability and biodegradation of polycyclic aromatic hydrocarbons in soil. Moreover, their result demonstrated that these surfactants could be metabolized by soil microorganisms and enhanced the persistence of degrading bacteria. Similar, results were obtained by Fava and Di Gioia (2001), they observed that in SL presence of the availability and biodegradation of polychlorinated biphenyls were enhanced, the effects increased with the increase in SL concentration applied.

The results presented here show that *A. discolor* has the capability of degrading both CLP and its primary metabolite 3,5,6-TCP (Fig. 5.2) under the conditions of the experiments described above. Bacteria and fungi, such as *Alcaligenes faecalis DSP3*, *Enterobacter B-14*, *Fusarium WZ-I* and *Acremonium* sp. strain (GFRC-1), are capable of degrading CLP (Kulshrestha and Kumari, 2011; Xie et al., 2005; Yang et al. 2005; Singh et al., 2004). Feng et al. (1997) showed that 3,5,6-TCP could be degraded by a *Pseudomonas* species. Moreover, Xu et al. (2007) showed that *Serratia* sp. could transform CLP into 3,5,6-TCP and that *Trichosporon* sp. could mineralize 3,5,6-TCP. A decrease of degradation was observed after 14 days of incubation, this fact can be explained by the accumulation of 3,5,6-TCP in the soil, which can cause an inhibition of CLP degrading microorganism due to antimicrobial properties of 3,5,6-TCP, as it was described by Sing et al. (2004). *A. discolor* and soil microorganisms have the ability of degrading 3,5,6-TCP

(Fig. 5.2b and 5.2c). Tortella et al. (2012) reported the degradation of 3,5,6-TCP by microbial biomass in the biomixture of a biobed.

The CLP degradation and 3,5,6-TCP formation observed in the abiotic control (Fig. 5.1d) suggests a nonmicrobial process and can be explained due to hydrolysis resulting from moisture of soil under influence of temperature and pH, as it was reviewed by Murray et al. (2001).

The soil used in this study contains 15% of organic matter, which could increase the CLP adsorption and allow a further degradation. Besides, the soil with high organic matter content could promote the development of microbial communities and facilitate the CLP degradation.

In relation to the PCP biodegradation assay, the Fig. 5.5 shows that PCP removal occurred in two stages: a rapid removal (until 30 days of incubation) followed by a slower stage (from 30 to 90 days of culture). This result is similar to the obtained by Rubilar et al. (2011) for *A. discolor* and Rubilar et al. (2007) for *Bjerkandera adusta*. The fact that PCP degradation in soil by white-rot fungi presents two phases has been related to PCP adsorption or polymerization into soil organic matter (McGrath and Singleton, 2000), possibly associated with the bond type (strong or weak) between PCP and organic matter (DiVicenzo and Sparks, 2001). Similar situation occurred in natural soil without *A. discolor* (Fig. 5.5b), due to PCP degradation by autochthonous microorganisms of the soil in two phases, the former phase is fast and the latter phase is slow. Fig. 5.5d shows the extractable PCP residues in the abiotic control, the dissipation curves show that PCP concentration decreased when increasing incubation time. The PCP concentration was moderately constant, i.e. the desorption resistance was constant after 30 days of incubation in soil with and without SL. The desorption resistance increased with the contact time between organic contaminant and soil, this fact is known as "aging" (Hatzinger and Alexander, 1995). However, the extractable PCP residues in soil added with 3.0 g kg^{-1} of SL were higher than in soil without SL, which demonstrated that the addition of SL increased the PCP removal from contaminated soil. Similar results were obtained by Conte et al. (2005) when evaluating the efficiencies of humic acid, as natural surfactant, in removal of the pollutant from contaminated soil.

The extent to which a surfactant influences hydrophobic contaminant distribution in environmental depends of hydrophobic contaminant sorption to solid phases (Chiou et al. 1998), hydrophobic contaminant solubilization by micellar or monomeric surfactant, and surfactant interaction with sediment or soil (Liu et al., 1992). In this context, the sorption of surfactant onto soil may result in surfactant loss and reduced performance for the solubilization of contaminant in water (Liu et al., 1992). Mata-Sandoval et al. (2002) determined that when surfactant dosages were high enough to reach soil saturation and maintain an aqueous micellar phase, pesticide desorption was enhanced.

The presence of *A. discolor* promotes the PCP degradation in respect to biotic control, as was demonstrated by Tortella et al. (2008), Rubilar et al. (2007). This ability of degradation has been attributed to the ligninolytic activity (Eichlerová et al., 2005; Barr and Aust, 1994). In this study, ligninolytic activity is represented by MiP and MnP, both enzymes reaching a maximum after 21 days of culture in soil without and with SL, respectively. The PCP degradation in natural soil is related to both, *A. discolor* activity and autochthonous microorganisms of soil (Cea et al., 2010). Other studies have found positive and negative interactions between white-rot fungi and soil microorganisms with other pollutants. A positive interaction between *Bjerkandera adusta* and autochthonous microorganisms of soil was found in benzo(a)pyrene degradation (Kotterman et al., 1998). However, soil microorganisms can affect the colonization of the soil by white-rot fungi preventing their growth (Radtke et al., 1994).

The PCP removal in biotic control (Fig. 5.5b) may be attributed to the action of other extracellular enzymes secreted by autochthonous microorganisms of soil with ability to degrade PCP, as it was demonstrated by McGrath and Singleton (2000) and Rubilar et al. (2007). Study of Cea et al. (2010) revealed the presence of *Burkholderia* spp in an Andisol from Temuco series, which has been demonstrated that some species are able to produce PCP hydroxylases, enzyme with capacity to degrade chlorinated compounds (Manickam et al., 2007). Cea et al. (2010) also demonstrated the presence of *Rhizopus oryzae*, fungus that has been reported with the capable of tolerating and removing PCP from contaminated soil (León-Santiesban et al., 2008) .On the other hand, PCP removal in soil inoculated with *A. discolor* and added with SL was 96.3% after 90 days. This higher PCP removal in presence

of *A. discolor* and autochthonous microorganisms of soil shows the positive effect of bioaugmentation.

The quantification of microbial activity of soil is an important factor for evaluating variations in management and soil alteration by contaminants (Perucci et al., 2000). The hydrolysis of the FDA is related to several soil hydrolases, such as: esterases, proteases, and lipases, all these ezymes are involved in the microbial decomposition of organic matter in soil (Schnürer and Rosswall, 1982). The FDA hydrolysis has been correlated with microbial biomass in pure and mixed microbial cultures, in soil used for cultivating or pasture (Vekemans et al., 1989). In this context, the SL effect on microbial activity in soil added with PCP was studied. The addition of PCP caused diminishes of the microbial activity; therefore, a decrease of FDA hydrolysis occurred, which is not influenced by SL presence. Perucci et al. (2000) found an impairment of the microbial activity due to the presence of xenobiotics, due to that addition of contaminants causes a soil disturbance or stress at the soil microorganisms.

5.5 Conclusions

The obtained results suggest that CLP and PCP biodegradation increased proportionally with the concentration of the applied SL. Besides, CLP and PCP biodegradation in biotic control may be attributed to the action of autochthonous microorganisms of soil with ability to degrade these contaminants. On the other hand, CLP degradation in abiotic control may be related to the hydrolysis due to the presence of humidity in soil. The obtained data also show that CLP and PCP desorption decreases if the contact time between both, contaminant and soil, increases. However, the extractable CLP and PCP residues in soil with SL were higher than in soil without SL. Moreover, CLP and PCP degradation, both by *A. discolor* and autochthonous soil microorganisms, was confirmed by the formation of metabolites. Nevertheless, the highest metabolites concentration was obtained when the soil was supplemented with SL. Therefore, although our result demonstrated that CLP and PCP can be degraded by *A. discolor* in presence of 3.0 g kg^{-1} of SL. However, are necessary further studies such as determining the extent of SL sorption in a Andisol from Temuco series, to

evaluate the effect of SL on the autochthonous microorganisms of soil, to evaluate as the different soil constituents contribute to SL sorption, among others.

Acknowledgements

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Chapter 6

General discussion

General discussion

In the present study we aimed to evaluate the effect of SL on the removal and biodegradation of CLP and PCP by *A. discolor* in liquid medium and soil. In order to achieve the above we divided the subject on three main areas.

In Chapter 3, the effect of SL addition on production of ligninolytic enzymes by *A. discolor* in liquid culture was studied. In this context, response surface methodology for optimizing the initial pH of the medium and the incubation temperature for ligninolytic enzymes produced by *A. discolor* was applied. Besides, the SL effect on mycelial growth and the production of ligninolytic enzymes in static batch cultures were studied. Effects of the initial pH of the medium and incubation temperature on different enzymatic activities revealed that the optimum conditions to maximize ligninolytic activity were pH 5.5 and 26ºC for Lac and MnP and pH 5.5 and 30ºC for MiP. On the other hand, we found that the increase in SL concentration caused an increase in mycelial growth. Moreover, when the culture medium was added with SL, *A. discolor* produced mainly MnP. The results presented above show that the production of high levels of Lac and MnP in *A. discolor* are the same conditions, which is advantageous with respect to their possible synergism in bioremediation processes. The SL demonstrated no negative effects on the fungal growth of *A. discolor* and increased the MnP production of *A. discolor*, which might be due to that SL present Mn, a known inducer of these enzyme.

The effect of SL on biodegradation of PCP by *A. discolor* in liquid medium was evaluated and results are presented in Chapter 4. Moreover, the effect of SL on solubilization of PCP was studied. In relate to last activity, we determined that the concentration of solubilized PCP in water-SL system increased with the SL addition and that solubilization was higher when the SL concentration was above its CMC. This fact can be due to monomeric molecules of surfactants, below its CMC (0.6 g L^{-1}), which have minimal partition effect on solute. However, when surfactant concentration is above its CMC the solubility of hydrophobic compound increased. Finally, PCP degradation was established by both disappearance of contaminant and production of metabolites as 2,4-DCP and 2,4,6-TCP. The increase in SL concentration caused an increase in PCP

degradation by *A. discolor* and a higher production of 2,4-DCP in liquid medium. Taking into account the lack of information about the SL on the PCP biodegradation, it is possible to hypothesize that this biosurfactant can reduce the contaminant inhibition, i.e. SL could present a protective effect on *A. discolor* such as nonionic surfactants.

In this research, the SL effect on the biodegradation of CLP and PCP by *A. discolor* in a contaminated soil was evaluated in Chapter 5. Their result shows that the microbial activity of both, *A. discolor* and autochthonous microorganisms of soil, were sensitive at the contamination. The half live value of CLP and PCP removal decreased when the soil was inoculated with *A. discolor* and the SL concentration was of 3.0 g kg^{-1}. Additionally, the assays demonstrated that SL presence promoted the CLP and PCP biodegradation and their metabolites biodegradation. The biodegradation processes increased in *A. discolor* presence. However, the presence of high organic matter concentration of soil contributes to increase the adsorption of CLP and PCP, which allowed an additional degradation.

Chapter 7

General conclusions and outlook

General conclusions

Taking into account the main results, it can be concluded that:

- The experimental design, response surface methodology, applied on the initial pH of the culture medium and the incubation temperature, showed that the optimum conditions to maximize ligninolytic activity of *A. discolor* were pH 5.5 and 26ºC for Lac and MnP and pH 5.5 and 30ºC for MiP.

- The addition of soya lecithin in solid cultures presented no inhibitory effect on mycelial growth of *A. discolor*. In addition, when the liquid culture medium was supplemented with soya lecithin, *A. discolor* mainly produced MnP, which might be explained by the composition of soya lecithin. This phytogenic surfactant contains 2 mg L^{-1} of Mn, a well known inducer of this enzyme.

- Soya lecithin was found to increase the PCP solubility. Moreover, the increase in soya lecithin concentration caused an increase in the concentration of PCP solubilized in the water-soya lecithin system. The solubilization was higher when soya lecithin concentration was above its CMC (0.6 g L^{-1}).

- Soya lecithin enhanced the PCP biodegradation by *A. discolor* in liquid medium. Besides, the increase in soya lecithin concentration caused an increase in the PCP biodegradation. The PCP biodegradation is related to the presence of MnP, which presented a high activity in the assay.

- The presence of soya lecithin enhances the CLP and PCP biodegradation by *A. discolor* in contaminated soil. Moreover, the residual concentration of contaminants decreased when the soya lecithin concentration was increased.

- The desorption-resistance of CLP and PCP increases with the contact time between contaminants and soil. However, the extractable residues of CLP and PCP in soil added with soya lecithin were higher than in soil without surfactant.

- The CLP and PCP removal in contaminated soil can be related to adsorption processes due to the high organic matter concentration of soil used in this study. In the case of CLP, it can also be related to hydrolytic degradation due to moisture of the soil.

Outlook

The results obtained in this doctoral thesis suggest that soya lecithin can be applied for increasing the bioavailability and biodegradation of PCP in both, liquid medium and contaminated soil. Besides, soya lecithin can be applied for enhancing the CLP biodegradation. Soya lecithin is non-toxic for white-rot fungus *A. discolor* and did not produce inhibition in the production of MnP, in the evaluated concentrations of soya lecithin. However, more studies are necessary such as determining the extent to which different soil constituents contribute to soya lecithin sorption. Such information will have practical application for studying on the basis of soil properties, the suitability and amounts of soya lecithin required for treatment. Besides, it is necessary to evaluate if soya lecithin sorption is a reversible process. It is important to evaluate the effect of soya lecithin on the autochthonous microflora of soil and determine mechanisms of interactions among contaminants, soya lecithin and degrader microorganisms. Several researches have demonstrated that the mixed surfactants enhance solubilization and may improve the performance of surfactant-enhanced remediation of contaminated sites by decreasing the applied surfactant level. In this context, it is interesting to evaluate the capability of soya lecithin with another surfactant for enhancing water solubility of contaminant. Besides, it is interesting to evaluate the effects of typical inorganic ions, coexisting with contaminants in soil, on water solubility of the contaminant in the presence of soya lecithin alone or mixed with another surfactant. These proposals could be an interesting subject of study in the near future.