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PELLETS FORMULATION FOR WHITE-ROT FUNGI IMMOBILIZATION FOR POLLUTANTS DEGRADATION

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SEBASTIAN ELGUETA PALMA

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PELLETS FORMULATION FOR WHITE-ROT FUNGI IMMOBILIZATION FOR POLLUTANTS DEGRADATION

Dissertation by

Sebastian Elgueta Palma

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Esta tesis fue realizada bajo la supervisión del Director de Tesis, Dra. MARÍA CRISTINA DIEZ JEREZ, del Departamento de Ingeniería Química y ha sido aprobada por los miembros de la comisión examinadora.

SEBASTIAN ELGUETA PALMA

DIRECTOR PROGRAMA DE POSTGRADO EN CIENCIAS DE RECURSOS NATURALES

Dra. MARÍA CRISTINA DIEZ

Dra. CAROLYN PALMA

Dra. GRACIELA PALMA

DIRECCIÓN DE POSTGRADO

UNIVERSIDAD DE LA FRONTERA

Dra. JUANITA FREER

Dr. CESAR ARRIAGADA

“The one thing that matters is the effort”

Antoine de Saint-Exupery

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Abstract

The use of natural support for fungi immobilization performing a satisfactory bioremediation becomes in a critical point to obtain success during the process. Diverse factors such as inoculum quality, production, stability, viability and storage can determinate the success of immobilized fungi to degrade pollutants. White-rot fungi belong to the wood-destroying basidiomycetes responsible for mineralization of all wood polymers due their ligninolytic enzymatic system. However, the use of immobilized white-rot fungi in different support for pollutants degradation requires especial conditions for enhancing their fungal growth and ligninolytic enzyme activity. Therefore, the objective of this thesis was to formulate supports for white-rot fungi immobilization for pollutants degradation.

Six strains of white-rot fungi isolated from southern Chile *Trametes versicolor* (sp and M-107), *Stereum hirsutum*, *Anthracophyllum discolor*, *Inonotus* sp2 and *Galerina patagonica* sp3 were evaluated to select an efficient strain with high ligninolytic activity and high ergosterol/biomass correlation to formulate pellets for further dye decolorization. The strains were evaluated using the standard decolorization method with Poly R-478, which are associated with the ability to produce peroxidases and ABTS oxidation which are associated in turn with the ability to produce laccase. The fungus *A. discolor* was selected for: high ligninolytic activity and high correlation between ergosterol and biomass to formulate: complex pellets (fungal mycelium, sawdust, and activated carbon), coated pellets (complex pellet + alginate) and simple pellets (fungal mycelium) for reactive orange 165 (RO165) decolorization. The activity of ligninolytic enzymes (laccase, manganese peroxidase, manganese-independent peroxidase, and lignin peroxidase) and dye decolorization were evaluated.

Complex pellets of *A. discolor* showed a higher enzymatic production mainly manganese peroxidase (MnP) (38 U L^{-1} at day 15) compared to coated and simple pellets. Decolorization of RO165 was demonstrated with all the pellets formulated. However, the highest and fastest decolorization was obtained with complex pellets (100% at day 8).

Three formulation supports were evaluated to immobilize *A. discolor*. F1: sawdust 74%, starch 6%, corn meal 2% and flaxseed 15%; F2: sawdust 74%, starch 8%, corn meal 5% and flaxseed 10% and F3:sawdust 74%, starch 10%, corn meal and 8% flaxseed 5%. Supports were formulated with a carrier, nutrient source, binder and lubricant, and then pelletized. One set of pelletized supports was coated with alginate (CPS) and another was uncoated (UPS). Fungal growth and ligninolytic enzyme activities in both CPS and UPS for the three formulations were evaluated at 25 ºC during incubation. Results showed that the fungi grew in all formulations (>75%) however, *A. discolor* growth faster in UPS than in CPS supports during the overgrowth period and no differences were observed at 4 and 25 ºC of storage. On the other hand, the highest enzymatic activity was MnP for all formulations (coated and uncoated) and lowest lacasse activity (Lac).

According to the previous results, uncoated pelletized support with the formulation F1 (UPS-F1) was selected to inoculate a biomixture of a biopurification system to evaluate atrazine degradation, phenoloxidase activity and PCR-DGGE analyses. The atrazine degradation was 95% in the biomixture inoculated with *A. discolor* immobilized in UPS-F1 after 30 days of incubation while, 75% of atrazine was degraded in the biomixture non-inoculated at the same time of incubation.

Phenoloxidase activity was higher in the biomixture inoculated with UPS-F1 than in non inoculated biomixture and the DGGE analysis showed there was no negative effect on native microbial population after 30 days of incubation.

Other white-rot fungi (*S. hirsutum* Ru-104, *Inonotus* sp2 and *T. versicolor*) were immobilized in UPS-F1 and inoculated in a biomixture contaminated with atrazine (80 mg kg^{-1}). The effect of inoculation was evaluated through atrazine degradation and biological activities such as: phenoloxidase activity, fluorescein diacetate activity (FDA) and respiratory activity. In general, the biomixture inoculated with fungi increased the degradation of atrazine and biological activities compared with the biomixture non-inoculated. The highest degradation of atrazine was obtained when biomixture was inoculated by *S. hirsutum* Ru-104 (93%) and the lowest one (78%) was in non-inoculated biomixture, after 60 days of incubation. On the other hand, *T. versicolor* showed highest phenoloxidase activity (0.8 U kg^{-1}) at day 30 and the highest FDA activity (53 μg FDA g^{-1} h^{-1}) at day 45, and *S. hirsutum* Ru-104 showed the highest cumulative respiration activity (155 mg CO_2 g^{-1}). In general, the white-rot fungi immobilized in UPS support can be used to improve the atrazine degradation in biomixture of a biopurification system.

Finally, the results obtained in this dissertation demonstrated that the immobilization of white-rot fungi can increase the capacity to growth and produce ligninolytic enzyme in the degradation of different pollutants.

Resumen

El uso de soportes naturales para la inmovilización fúngica ha permitido una satisfactoria bioremediación convirtiéndose en un punto crítico para el éxito del proceso. Diversos factores tales como la calidad del inoculo, la producción, la estabilidad, viabilidad y el almacenamiento pueden determinar el éxito de hongos inmovilizados para degradar contaminantes. Los hongos de pudrición pertenecen al grupo de basidiomicetes degradadores de la madera los cuales son responsables de la mineralización de los polímeros presentes en ésta debido a su sistema de enzimas ligninolíticas. Sin embargo, el uso de hongos inmovilizados en diferentes soportes para la degradación de contaminantes requiere condiciones especiales para el incremento de la biomasa fúngica y la actividad de enzimas ligninolíticas. Por lo tanto, el objetivo de esta tesis fue formular soportes para la inmovilización de hongos de pudrición blanca para la degradación de contaminantes.

Seis cepas de hongos de pudrición blanca aisladas en el sur de Chile *Trametes versicolor* (sp and M-107), *Stereum hirsutum*, *Anthracophyllum discolor*, *Inonotus* sp2 and *Galerina patagónica* sp3 fueron evaluadas para seleccionar una cepa eficiente con alta actividad ligninolítica y una alta correlación de ergosterol/biomasa para una posterior decoloración de un tinte. Las cepas fueron evaluadas usando el método de decoloración estándar con Poly R-478, el cual se asoció con la habilidad de producir peroxidasas y la oxidación de ABTS la cual fue asociada a la producción de lacasa. El hongo *A. discolor* fue seleccionado por: alta actividad ligninolítica y alta relación entre ergosterol y biomasa para formular: pellet complejos (micelio fúngico, aserrín y carbón activado), pellets revestidos (pellet complejos + alginato), pellets simples (micelio fúngico) para la decoloración de Reactive Orange 165 (RO165).

La actividad de las enzimas ligninolíticas (lacasa, manganeso peroxidasa, peroxidasa independiente de manganeso y lignina peroxidasa) y la decoloración de RO165 fueron evaluadas. Pellet complejos de *A. discolor* mostraron la mayor producción enzimática, principalmente manganeso peroxidasa (MnP) (38 U L^{-1} el día 15) comparados con los revestidos y los simples. La decoloración de RO165 fue demostrada en todos los pellet. Sin embargo, la mayor y más rápida decoloración fue obtenida con pellets complejos (100% el día 8).

Tres formulaciones de soportes fueron evaluadas para inmovilizar *A. discolor*. F1: aserrín 74%, almidón 6%, harina de maíz 2% y harina de linaza 15%, F2: aserrín 74%, almidón 8%, harina de maíz 5% y harina de linaza 10%, F3: aserrín 74%, almidón 10%, harina de maíz 8% y harina de linaza 5%. Los soportes fueron formulados con un transportador, una fuente de nutrientes, un aglomerante y un lubricante para ser luego peletizadas. Un set de soportes peletizados fueron revestidos con alginato (CPS) y otros no revestidos (UPS). El crecimiento fúngico, las condiciones de almacenamiento y la actividad de las enzimas ligninolíticas en soportes revestidos (CPS) y no revestidos (UPS) para las 3 formulaciones, fueron evaluadas a 25 ºC durante la incubación. Los resultados mostraron que el hongo creció en todas las formulaciones (≥75%), sin embargo *A. discolor* creció más rápido en la peletización UPS que la CPS durante el periodo de crecimiento, no se encontraron diferencias en el almacenamiento a 4 ºC y 25 ºC. Por otro lado, la mayor actividad enzimática fue manganeso peroxidasa para todas las formulaciones (UPS and CPS) y menor cantidad lacasa. De acuerdo a los resultados previos, la peletización UPS con la formulación F1 (UPS-F1) fue seleccionada para inocular una biomezcla de un sistema de biopurificación para la degradación de atrazina, actividad fenoloxidasa y un análisis PCR-DGGE.

La degradación de atrazina fue 95% en la biomezcla inoculada con *A. discolor* inmovilizado en UPS-F1 luego de 30 días de incubación, mientras un 75% de atrazina fue degradada en la biomezcla

no inoculada al mismo tiempo de incubación. La actividad fenoloxidasa fue mayor en la biomezcla inoculada con UPS-F1 que la biomezcla no inoculada y el análisis DGGE mostró que no hubo efectos negativos sobre las comunidades de microorganismos en la biomezcla cuando se inoculó con UPS-F1 especialmente luego de 30 días de degradación de atrazina.

Otros hongos de pudrición blanca (*S. hirsutum* Ru-104, *Inonotus* sp2 y *T. versicolor*) fueron inmovilizados en UPS-F1 e inoculados en una biomezcla contaminada con atrazina (80 mg kg^{-1}). El efecto de la inoculación fue evaluado a través de la degradación de atrazina y actividades biológicas tales como: fenoloxidasa, fluoresceína diacetato (FDA) y actividad respiratoria. En general, la biomezcla inoculada con hongos de pudrición blanca incrementó la degradación de atrazina y las actividades biológicas comparadas con la biomezcla no inoculada. La mayor degradación de atrazina fue obtenida cuando la biomezcla fue inoculada con *S. hirsutum* Ru-104 (93%) y la menor (78%) fue en la biomezcla no inoculada luego de 60 días de incubación. Por otro lado, *T. versicolor* mostró la mayor actividad fenoloxidasa (0.8 U kg^{-1}) el día 30, la mayor actividad FDA (53 µg FDA $g^{-1}h^{-1}$) el día 45 y *S. hirsutum* Ru-104 mostró la mayor actividad respiratoria acumulada (155 mg CO_2 g^{-1}).

En general, los hongos de pudrición inmovilizados en el soporte UPS pueden ser usados para mejorar la degradación de atrazina en la biomezcla de un sistema de purificación. Finalmente, los resultados obtenidos en esta tesis demostraron que la inmovilización de hongos de pudrición blanca puede incrementar el crecimiento fúngico y la actividad ligninolítica en la degradación de contaminantes.

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CHAPTER 1.

1. INTRODUCTION AND OBJETIVES

1. INTRODUCTION AND OBJETIVES

1.1 Introduction

The use of white-rot fungi has increased because these fungi can produce many compounds of commercial interest, including extracellular enzymes. There are many reports in the literature about pollutants degradation by white-rot fungi. The ability to degrade different molecules is attributed to their lignin degrading enzymatic system that can degrade a diverse range of pollutants (Gianfreda and Rao, 2004). As a consequence, considerable experimental know-how is available which should really contribute to innovate industrial applications based on these fungi. However, in spite of the many potential advantages of immobilized fungal systems, the industrial world still shows certain reluctance toward their utilization. White-rot fungi can be immobilized using different techniques: fungal inocula, immobilized in lignocellulosic supports coated with mycelium, made from inexpensive agricultural and wood industry by-products and grown in submerged forma producing pellets. For morphological applications the selection of medium composition, pH, culture temperature and agitation can produce changes in the fungal morphology of pellets, which significantly improves the culture producing better biomass, oxygen transfer and low energy consumption to be used in biodegradation process including wastewater treatment of synthetic dyes (Liao et al., 2007).

For the development of fungal inoculums with especial features as uniform quality and resistance to competition by indigenous microbes is essential the selection of suitable carrier to improve the growth capacity and biological potential for applications in the environment, as biological control and bioremediation soils (Lestan et al., 1996). In literature, different types of support for fungal immobilization have been used in bioremediation, such as sawdust, straw, wood chips, bark, flax, and peat. Immobilization on different supports sustains the viability and provides sources of nutrition to improve pollutants degradation, a large biomass produced in fungal inocula is frequently faster in

the establishment in soil (Steffen et al., 2007, Baldrian, 2008). Fungal inoculums could be used in soil (Lestan and Lamar, 1996) and in different biological systems to improve the biodegradation of pollutants, for this reason different technologies have been described such engineered soil cells (Walter et al., 2005) or biobed (Torstensson and Castillo, 1997). Biobed has been described as a cheap construction intended to collect and degrade spills of pesticide on farms, include a biomixture of straw, peat and soil that promote microbial activity, especially white-rot fungi, which produce ligninolytic enzyme.

The main objective of this thesis was to study immobilization of white-rot fungi for pollutants degradation and the bioaugmentation of a biomixture for atrazine degradation. The main activities were to select a white-rot fungus to be used to formulate complex and coated fungal pellets to improve decolorization and degradation of Reactive Orange 165 (azo dye). To formulate a suitable support for immobilization of *Anthracophyllum discolor* and evaluate their effect on biological activities during atrazine degradation in a biomixture of a biopurification system. Finally was necessary was to evaluate the use of immobilized white-rot fungi in a biomixture contaminated with atrazine.

The results obtained in this study increase the scientific and technological knowledge about immobilized white-rot fungi to be used in scale studies. The technology will provide a low cost alternative to reduce the pollutant contamination.

1.2. Hypothesis

Considering:

- ✓ The native white-rot fungi from southern Chile have a potential in bioremediation processes.
- ✓ The immobilization of white-rot fungi increases the growth and ligninolytic enzyme activities.
- ✓ The atrazine is degraded biomixture of biobed.

Therefore, considering the previously mentioned facts the working hypothesis is established as:

1. The formulation of a support for the development of fungal inocula will increase growth, ligninolytic enzyme activities, the degradation of reactive orange 165 and atrazine in a biopurification systems inoculated with immobilized white-rot fungi.

1.3. General objective

To formulate pellets for white-rot fungi immobilization for pollutants degradation

1.4. Specific objectives

- ✓ To select white-rot fungi to formulate complex and coated pellets for reactive orange 165 decolorization.
- ✓ To formulate a support for *A. discolor* immobilization to improve their ligninolytic activity and growth for atrazine degradation in a biomixture of a biopurification system.
- ✓ To evaluate atrazine degradation in a biomixture inoculated with immobilized white-rot fungi.

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CHAPTER 2.

2. USE OF IMMOBILIZED WHITE-ROT FUNGI ON LIGNOCELLULOSIC SUPPORTS FOR POLLUTANT BIODEGRADATION

USE OF IMMOBILIZED WHITE-ROT FUNGI ON LIGNOCELLULOSIC SUPPORTS FOR POLLUTANT BIODEGRADATION

2.1 ABSTRACT

The immobilization of white rot fungi is a promise technology that can be used to improve the biological and physiological functions of these microorganisms. The purpose of immobilization is related to increase the viability and the stability of fungi through the time. The selection of suitable supports allows the immobilization giving a natural environment increasing the fungal biomass and the incorporation of elemental nutrients for white-rot fungi. Different lignocellulosic supports such as sawdust, wheat straw, orange peels, and rice hulls have been tested for different biotechnological applications including wastewater treatments of effluent that contain synthetic dyes, pesticide in water sources and soil contaminated by chlorophenols and polycyclic aromatic hydrocarbon (PAH). In literature many studies have been demonstrated the capacity of white-rot fungi for biodegradation of pollutants due their ligninolytic enzymes systems that are involve in degradation of these compounds. This review is focused in different lignocellulosic supports used for different biodegradation process describing the current investigations and the novel information respect of pollutant degradation.

2.2 INTRODUCTION

White-rot fungi are the only microorganisms that can oxide recalcitrant compounds in the nature due their ligninolytic enzymes system that are involved in lignin degradation. The potential of these fungi has been studied because ligninolytic enzymes are extracellular and non-specific and can degrade a wide range of different pollutants (Gianfreda and Rao, 2004). As consequence of different studies high quality information is available this can contribute to improve industrial applications. The immobilization has many advantages over free white-rot fungi and the presence of high amount of lignocellulosic wastes offer suitable conditions for fungal growth. The immobilization on different supports sustains the viability and provides nutrients that are necessary to stimulate the metabolisms necessary for bioremediation process.

The development of immobilized white-rot fungi with high quality and resistance for the competition by native populations in soil o water contaminated is essential for the selection of suitable supports to improve fungal growth and biological potential to increase the oxidation of many pollutants in soil or water (Lestan and Lamar 1996). However, a large biomass amount after the immobilization is faster for the establishment of white-rot fungi in soil or water (Baldrian, 2008). Different source of contamination such as: the release of synthetic dyes in aquatic environments, the spill of pesticides on farms or the release of many polycyclic aromatic hydrocarbon in soil by industrial activities have been demonstrated the necessity to investigate biological treatments to reduce the environmental contamination. Therefore, the next information will provide the status of this technology used for biodegradation of different pollutants.

2.3. White-rot fungi features

These fungi are basidiomycetes with the capacity to degrade the lignin present in many different lignocellulosic compounds including wood and herbaceous plants (Pointing, 2001). The reason to degrade lignin can be attributed to the oxidization of carbon sources present in lignocellulosic supports and the process occurs when the secondary metabolism of fungi is activated (Reddy, 1995). The potential of white-rot fungi has been described since 1985 using species such as *Phanaerochaete chrysosporium* and *Trametes versicolor* investigated due their ability related to the mechanisms of wood degradation. In literature the main characteristic of these fungi are related to the ligninolytic potential used for biodegradation of pollutants. The main enzymes studied are lignin peroxidase (LiP), manganese peroxidase (MnP) and Laccase (Lac). Not all white-rot fungi produce the same levels of ligninolytic enzymes some fungi can produce only manganese peroxidase that is induced by hydrogen peroxides, lignin and manganese to produce the oxidation of Mn^{+2} to Mn^{+3} (Selvam et al. 2003). In literature the white-rot fungus *Anthracophyllum discolor* has been studied under different conditions to degrade different pollutants and all cases the main enzyme produced is manganese peroxidase (Rubilar et al., 2011, Bustamante et al., 2012, Acevedo et al., 2012). Laccase is a copper-protein able to catalyze the oxidation of different compounds requiring hydroxyl peroxide and other mediators to complete their reaction which can oxidize lignin and a wide range of organic pollutants (Gianfreda et al., 1999).

The secondary metabolism of white-rot fungi is produced and affected by different nutrients especially when nitrogen levels decreasing (Rodriguez-Couto and San Roman, 2005a). However, not all white-rot fungi have the same behavior when are growing in different supports, mediators are necessary for the production of ligninolytic enzymes such as hydrogen peroxide, manganese, copper and organic acid used for the chelation and stability in the reaction (Rodriguez-Couto and Ratto,

1998). To quantify ligninolytic enzymes is necessary the standardization of different methodologies and is necessary different compounds to obtain similar results. Normally the substrates used to determinate lignin peroxidase is veratryl alcohol (Tien and Kirk, 1988) for manganese peroxidase is 2.6 dimethoxyphenol and for laccase is used 2.2 azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ABTS (de Jong et al., 1994).

2.4 Immobilization of white-rot fungi in lignocellulosic supports

The generation of lignocellulosic waste and the increasing of agro-industrial activities have produced a large quantity of valuable supports that can be used to obtain added-value products (Serramiá et al., 2010). These lignocellulosic supports are composed by lignin, cellulose and hemicelluloses that provide carbon sources and stimulate the ligninolytic enzymes to oxidize different compounds.

The immobilization of white-rot fungi have been evaluated as a novel technique that improve the fungal growth from different lignocellulosic supports protecting the microorganism and proving a similar natural environment (Rodriguez-Couto, 2009). The benefits of immobilization when white-rot fungi are introduced in different environment are determinant for the resistance in the time. The main factors are related with the stability in support to increase biomass and the viability to produce necessary oxidative enzymes to obtain elemental nutrients for their metabolism (Rodriguez–Couto and Toca-Herrera, 2007). Normally the entrapment and attachment (Figure 2.1) are used to inoculate different lignocellulosic supports producing the immobilization. The entrapment of white-rot fungi can be occurring inside the porous materials providing a suitable environment to grow. On the other hand, the attachment is produced in the surface of the support by chemical reaction or simple-adhesion by fungal mycelium.

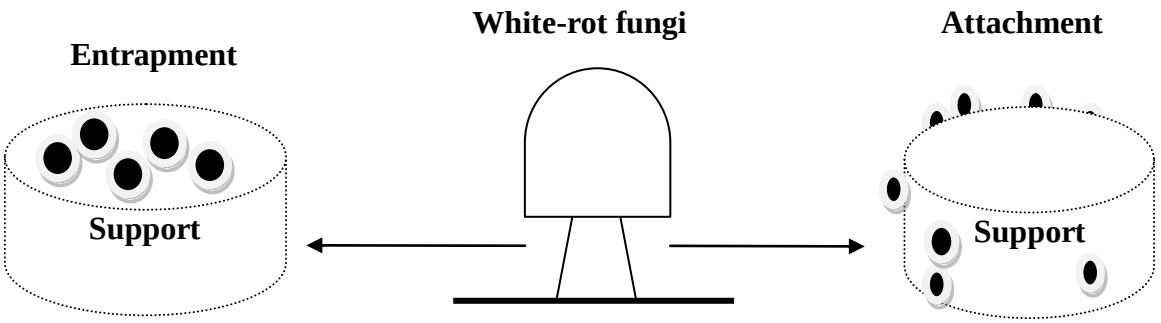

Figure 2.1 Immobilization used for white-rot fungi used in biotechnological applications.

The natural capacity of these fungi has been described as an important factor to consider in biotechnological applications especially for biodegradation of pollutants in soil or water (Gao et al., 2008). The stability of fungi during the immobilization improves the mechanical strength due the natural adhesion of fungal hyphas that can penetrate the interstices of supports, improving the rigidity and the interaction fungus/support. One of the main factors when the immobilization is used is related with the persistence of fungi because the support give to fungus nutrients for growth and stimulation of ligninolytic enzymes, especially when are introduced in soil for bioremediation with purpose to reduce the competition whit native population being critical for the success of process. Interestingly different authors have been described the benefits of immobilization to microorganisms, Federici (1993) described different aspect related with advantages and disadvantages of the technique in a review that are described in the Table 2.1.

Table 2.1 Advantages and disadvantages of immobilization described in a review by Federici (1993).

Advantages	Disadvantages
✓ The biological functions are maintained growing in different supports due supply of nutrients in suitable supports	✓ The growth can be lower due the restriction of nutrients if are not suitable decreasing the viability
✓ The stability is improved by physical-chemical interactions	✓ Secondary reactions can be produced due the interaction of cells and supports.
✓ Re-use of biomass and high density	✓ Sometimes the interaction fungus/support can produce contamination decreasing the viability

In literature different types of lignocellulosic supports have been used for immobilization of white-rot fungi to improve the ligninolytic enzymes activities. Taniguchi et al. (2005) evaluated the pretreatment of rice straw using the native white rot fungi, *Phanaerochaete chrysosporium*, *Trametes versicolor, Ceriporiopsis subvermispora* and *Pleorotus ostreatus* by enzymatic hydrolysis, achieving 41% of lignin degradation during 60 days. Lechner and Papinutti (2006) studied the production of ligninolytic enzymes by the white-rot fungus *Lentinus tigrinus* on wheat straw during 100 days. They

obtained the pick of manganese peroxidase at day 20 and 90 around 750 mU g^{-1}. Aloui et al. (2007) described the capability to grow of four white-rot fungi in olive residues "alperujo" and sugarcane bagasse for decolorization in solid state fermentation. They found in the extracellular fluid lignin peroxidase (LiP) with high capacity to decolorize alperujo. Rubilar et al. (2007) investigated the application of *Anthracophyllum discolor* for the immobilization on wheat grains for pentachlorophenol degradation in soil. Their studies were evaluated at different concentrations 100, 250, and 350 mg of PCP kg^{-1} of soil, achieving 95% of degradation after 28 days. Levin et al. (2008) studied the white-rot fungus *Trametes trogii* for the production of ligninolytic enzymes using wood-base mediums. They found highest laccase and manganese peroxidase 901 and 20 U g^{-1} respectively obtaining crude extracellular extracts. Xu et al. (2009) evaluated the lignocellulose degradation and enzyme production by *Irpex lacteus* in corn stover during 120 days, achieving 63% of hemicellulose degradation after 15 days and 80% of lignin degradation after 60 days. Dias et al. (2010) evaluated the enzymatic saccharification of wheat straw by the strains Euc-1 and *Irpex lacteus* founding low cellulolytic and xylanolytic activity altough high manganese peroxidase, indicating a considerable increase of cellulose accessibility (3-4) times. Isikhuemhen et al. (2012) described the white-rot fungus *Lentinus squarrosulus* Mont for the capacity to grow in cornstalks and produce ligninolytic enzymes achieving after 6 days 154 U L^{-1} of laccase and 27 U L^{-1} of peroxidases. Koyani et al. (2013) investigated the capacity of decolorization of *Phanerochaete chrysosporium* growing in wheat straw mediums reaching 607 IU mL^{-1} of manganese peroxidase and 539 IU mL^{-1} of laccase in 11 days of incubation.

2.5. Applications of immobilized white-rot fungi in lignocellulosic supports for biodegradation of pollutants

2.5.1 Dye decolorization

There are different dyes used in many industries such as textiles, printing, food and paper industries, normally these chemical compounds are used due their easy application over different materials transferring de typical coloration to clothes and textiles. During waste-water treatment different dyes are not degraded due their complex chemical structure and are released to the environment into rivers and lakes. Some dyes when are in contact in aquatic environment can be converted into potentially carcinogenic amines producing serious and negatives impacts in the photosynthesis, amount of oxygen and water quality (Soares et al., 2002).

Currently, different physical-chemical treatments are used to decrease the toxicity of dyes. However, not all the treatments are efficient and environmental friendly, for this reason different low-cost and alternative methods have been investigated for decolorization. The biological treatments have been described as a novel alternative using microorganisms to reduce the toxicity of different dyes present in the effluents of industries. White-rot fungi have been studied for dye decolorization due their capacity to produce ligninolytic enzymes that are non-specific and can degrade a wide range of synthetic dyes (Baldrian, 2004, Eichlerova et al., 2005).

Different lignocellulosic supports have been studied for dye decolorization to improve the ligninolytic enzyme and the ability to grow. Rodriguez-Couto et al. (2001) investigated the ability of *Phanerochaete chrysosporium* using grape seeds, wheat straw and wood shaving for decolorization of Poly R-478 (polyvinylamine sulfonate anthrapyridone) the highest laccase production in grape seed was 1620 U L^{-1} and 74% of decolorization. Lorenzo et al. (2002) evaluated the capacity to grow

of *Trametes versicolor* in different lignocellulosic supports improving the laccase activity in barley bran reaching 639 U L^{-1} and 60% decolorization of Phenol Red in 72 hours. Kasinath et al. (2003) studied the fungus *Irpex Lacteus* immobilized in pine wood for decolorization of 150 µg mL^{-1} of Remazol Brilliant Blue (RBBR), in cultures agitated achieving 100% of decolorization in 6 days by the production of lignin peroxidase and manganese peroxidase. Boer et al. (2004) investigated the application of corn cobs for the immobilization of *Lentinula edodes* for decolorization of synthetic dyes after 18 days, finding the highest level of manganese peroxidase 2600 U L^{-1} and for azo degradation was more than 80 %. Rodriguez-Couto and Sanroman (2005b) studied the potential of coconut flesh as a suitable support for the immobilization of *Trametes hirsuta* reaching the high laccase activity 920 nkat L^{-1} when 2 mM of copper sulphate was added in the culture and the decolorization of Lissamine Green B in vitro and in vivo was 96%. Rodriguez-Couto et al. (2006) used grape seed for the immobilization of *Trametes hirsuta* in scale-bioreactors to improve the laccase activity and dye decolorization, finding the pick of laccase was at day 4 reaching 3.3 X 10^4 nkat L^{-1} and more than 80% of dye decolorization. Osma et al. (2007) investigated the immobilization of *Trametes pubescens* in banana skin for decolorization of Remazol Brilliant Blue (RBBR) and Methyl Green (MG), both decolorizations were more than 40% and pick of laccase activity was 1570 U L^{-1}. Robinson and Nigam (2008) studied the immobilization of *Bjerkandera adusta* in barley husk for waste water decolorization in solid state fermentation, finding more than 80% and a pick of lignin peroxidase of 17000 U Kg dry weight and 510 U Kg dry weight for manganese peroxidase. Sarnthima et al. (2009) used rice straw, sugarcane bagasse and banana peels under solid-state fermentation for growth of *Lentinus polychrous* Lev and Indigo Carmine decolorization, finding after 21 days the pick of laccase 1449 U L^{-1} for rice straw and best conditions for decolorization were at 10 mg L^{-1} and at pH 9. Papinutti and Forchiassin (2010) studied the adsorption and decolorization in waste from *Pleurotus ostreatus*, finding the optimum temperature

between 26-36 °C at pH 3, reaching the highest manganese peroxidase and laccase at 1.39-2.13 fold respectively when Malachite green was decolorized (75%). Rodriguez-Couto (2011) used immobilized *Trametes pubescens* on sunflower-seed shells for Remazol Brilliant Blue (RBRR) decolorization in bioreactors, finding that five successive batches can be used for decolorization of 133 mg L^{-1} of RBRR and the pick of laccase activity was 4000-6000 U L^{-1}. Jonstrup et al. (2012) investigated the capacity of *Phanaerochaete chrysosporium*, *Trametes versicolor* and *Bjerkandera* sp BOL13 to grow on straw, spruce and willow supports for Remazol Red decolorization in bioreactors, after 3 days of continues decolorization (65-90%) the bioreactor was maintained during 12 days inoculated with *Bjerkandera* sp BOL13 and the main enzyme detected was manganese peroxidase. Ryu et al. (2013) evaluated the Remazol Brilliant Blue decolorization by *Polyporus brumalis* grew on wood chips and tulip tree supports, they found an overexpression of laccase activity and high lignin degradation used in pre-treatment of wood between 15-45 days.

3.2 Pesticide degradation

The pesticide contamination has increased as a consequence from the use and misuse of agricultural practices and excessive use pesticide that lead a negative impact in the environment especially in surface water and soil. The discharge of pesticide into soil or water sources constitutes an important problem of contamination because many pesticides are recalcitrant and are persistent in the environment in long period.

Biological treatments are inexpensive and effective to reduce the toxicity of pesticide however some bacteria and fungi can degrade only specific pesticide. In the literature different authors have been discuss the capacity to degrade different pollutants including pesticide by white-rot fungi (Tortella et al. 2005). The pesticides in contact by white-rot fungi have been degraded by ligninolytic enzyme

activities due are extracellular and non-specific and by an intracellular enzymatic complex. One of the main advantages of white-rot fungi when are used for pesticide degradation in soil are related to the capacity to reach the interspaces in soil by that other microorganism cannot do (Pointing 2001). Gianfreda and Rao (2004) described the ligninolytic enzymes of white-rot fungi involve in pollutant degradation in soil, describing the capacity of these fungi to grow in different supports to obtain different nutrients and increase the oxidation of different pollutants (Figure 2.2). As was mentioned above white-rot fungi have been demonstrated a great ability to reduce the toxicity of high amount of pesticides. Rodriguez-Rodriguez et al. (2013) described the importance of microorganisms specially white-rot fungi for pesticide degradation for the environment decontamination through the fungal bioaugmentation in biopurification system for pesticide-containing waste or soil.

The degradation of pesticide by white-rot fungi in soil is conditional to the production of ligninolytic enzymes and the capacity to obtain nutrients as was described above to improve the fungal growth and reduce the competition with native microorganisms, for this reason is important to select a suitable support to improve the degradation of pesticide. Castillo et al. (2001a) described the isoproturon degradation and ligninolytic enzymes activities produced by immobilized *Phanaerochaete chrysosporium* on wheat straw, after 14 days of incubation the degradation was 91%. Castillo et al. (2001b) evaluated the immobilization of *Phanaerochaete chrysosporium* in wheat straw for bentazon and MCPA (4-chloro-2-methylphenoxyacetic acid) degradation and the ligninolytic enzyme activities in a reactor, achieving 100 % of degradation for both pesticides in 72 days but ligninolytic activities were no related at this time. However in 20 days the degradation was 65-75% for both pesticides and the activity of lignin peroxidase was attributed to the presence of white-rot fungus.

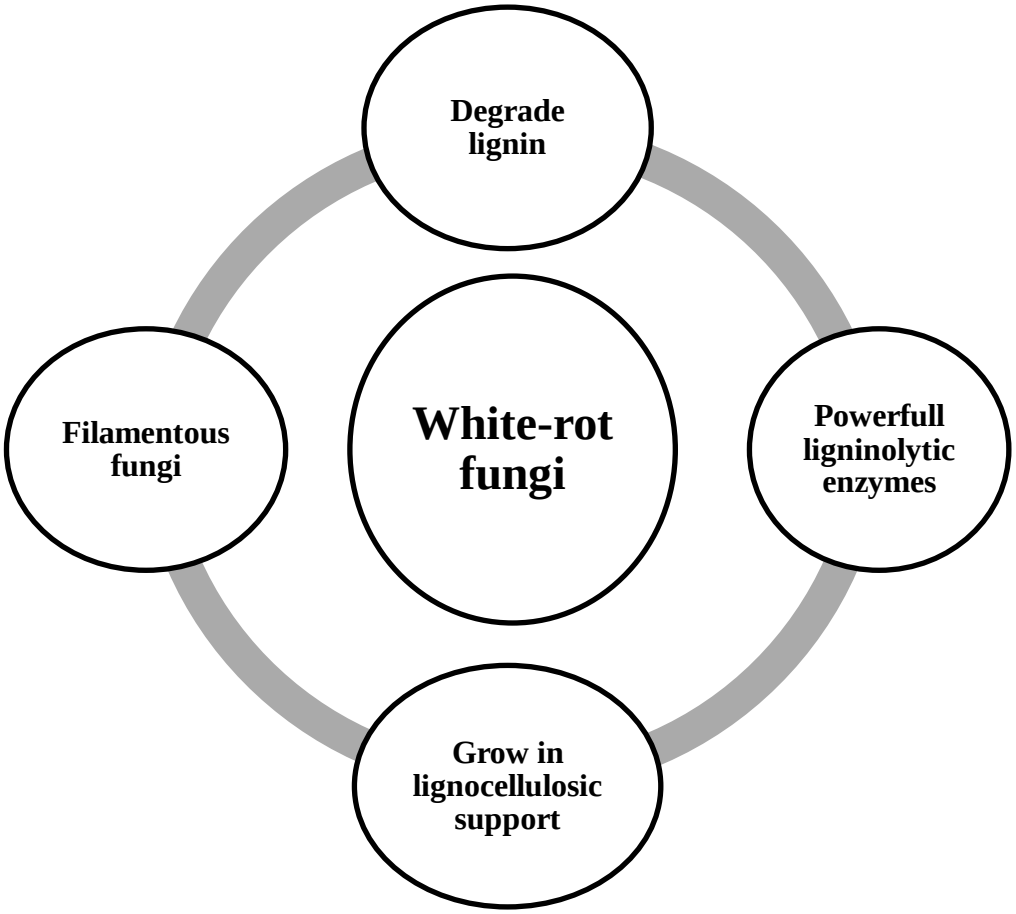

Figure 2.2 Main characteristic of white-rot fungi used for remediation of polluted soils (Gianfreda and Rao 2004).

Bending et al. (2002) studied the degradation of metalaxyl, diuron, atrazine, iprodione, terbuthylazine and chlorpyrifos by white-rot fungi grew in a biomixture formulated with barley straw, soil and compost (50:25:25 %v), after 42 days *Stereum hirsutum* was the fungus with high capacity to degrade contrasting pesticides due their ligninolytic potential. Rigas et al. (2007) evaluated the lindane degradation using *Ganoderma australe* grew in a mixture of soil and wheat straw evaluated through the method of response surface, concluding that the best amount of straw for fungal growth was 45 %, the velocity of propagation 4.25mm/day and biodegradation/biomass 56.9

µg/g, the author consider that these data are important for future applications in lindane bioremediation soil. Fragoeiro and Magan (2008) studied the immobilization of *Trametes versicolor* and *Phanaerochaete chrysosporium* on wood chips for the simazine, trifluralin and dieldrin degradation and ligninolytic enzyme activities and in soil under different condition of humidity, in all the evaluations the degradation were higher when the fungal inoculants were introduced in soil after 12 weeks than control non-inoculated, demonstrating that wood chips were suitable lignocellulosic support for the fungal growth and laccase activity. Bastos and Magan (2009) evaluated the capacity of immobilized *Trametes versicolor* in sawdust for atrazine degradation in a calcareous clay soil under low humidity conditions, finding that the inoculation increased the atrazine degradation compared with a soil only contaminated 96 and 50 % respectively, they evaluated the respiration activity during the incubation obtained values 20% higher than the treatment non-inoculated demonstrating that the inoculation by immobilized *Trametes versicolor* was suitable for the bioremediation of soil under low humidity conditions. Da Silva Coelho et al. (2010) studied the bentazon degradation by *Ganoderma lucidum* grew in corn cob powder and liquid medium, after 10 days bentazon degradation was higher in corn con powder medium (90 %) than liquid medium (55 %) and the highest laccase activity was 1800 U L^{-1} and for manganese peroxidase 262 U L^{-1}. Kara et al. (2011) evaluated the potential of *Phanaerochaete chrysosporium*, *Trametes versicolor* and *Pleurotus ostreatus* grew in a straw medium for pesticide degradation from agro-industrial wastewater, the ligninolytic enzyme were determinated being laccase the high activity detected 5-8 U L^{-1}, all the pesticide were degraded by different fungi but thiabendazole need to more studied further to scale-up. Gonzales Matute et al. (2012) studied the white-rot fungus *Agaricus blazei* immobilized in sunflower seed hulls, wheat straw and wheat bran for de metsulfuron methyl degradation, concluding that the use of compost inoculated with fungus can degrade the metsulfuron methyl in short times due the high capacity to produce laccase activity (490 mU mL^{-1}). Sari et al.

(2013) evaluated the ligninolytic enzymes activities of *Trametes versicolor* immobilized in wood meal and oil palm empty fruit bunch (EFB) for 1, 1, 1-trichloro-2, 2-bis 4-chlorophenyl (DDT) degradation after 30 days, they found that the fungus grew was better in EFB to degrade DDT due the production of manganese peroxidase and lignin peroxidase that were correlated with DTT degradation.

2.5.3 Other pollutants

During bioremediation of soil different factor are important to determine the success of the process, temperature, pH, soil characteristics, and the capacity of microorganisms to resist the presence of different pollutants are determinant to increase the metabolisms of them. In literature the application of immobilized white-rot fungi in suitable supports have been demonstrated as a novel technique to reduce the toxicity of different compounds in soil. Different lignocellulosic supports (Table 2.2) such as wheat straw, sawdust, corn cobs have been demonstrated an improvement in the viability and stability of fungal mycelium during the biodegradation of chlorophenols (Lestan et al., 1996, Walter et al., 2005). The purpose to pre-inoculate in lignocellulosic supports are related to the capacity to decrease the competition with native microorganisms due a fast establishment in soil to obtain the elemental nutrients to active the ligninolytic enzyme activities necessary and oxidize chlorophenols. Pre-inoculated white-rot fungi can produce a large fungal biomass when are introduced in soil but the main factor related with the formulation of immobilized white-rot fungi are the humidity, the biological potential, the ligninolytic enzyme activities, the storage and stability of the inoculants formulated for the process (Lestan and Lamar 1996, Lestan and Lamar 1999). In literature different immobilized white-rot fungi have been evaluated for pollutant degradation in soil. Davis et al. (1993) studied the application of pre-inoculated white-rot fungus *Phanaerochaete sordida* immobilized on

sawdust for bioremediation in soil, after 56 days they found a depletion of creosote of 3 and 4 ring of the molecule compared to the control non-inoculated. Lestan and Lamar, (1996) established the first report of a suitable fungal inocula for bioremediation of contaminated soil, 3 white-rot fungi were immobilized in sawdust, corn meal, and straw. After four weeks *I. lacteus, B. adusta* and *T. versicolor* removed 86, 82 and 90% of pentachlorophenol. Novotny et al. (2004) evaluated de degradation of anthracene and pyrene by immobilized white-rot fungi on wood pine cubes and straw finding that the main enzyme detected was manganese peroxidase 0.65 U g^{-1} and more than 90% degraded of both pollutants. Walter et al. (2005) studied the field-scale bioremediation in soil contaminated with pentachlorophenol by immobilized *Trametes versicolor* using sawdust, corn meal, and starch. The soil inoculations with 40% and 20% of fungal inocula were similar for the PCP degradation. Cea et al. (2010) evaluated the bioremediation of soil contaminated with pentachlorophenol by immobilized *Anthracophyllum discolor* on wheat straw, after 28 days the degradation was 86% and the pick of manganese peroxidase 45 nmol Mn^{+3} $min^{-1}g^{-1}$. Rubilar et al. (2011) used wheat grains for the immobilization of *Anthracophyllum discolor* for the treatment of a soil contaminated with pentachlorophenol PCP (250 and 350 mg kg^{-1}) they found that manganese peroxidase was stimulated due the composition of wheat grains and the application in soil by immobilized fungus allowed a synergistic effect with the strain *P. chrysosporium* increasing the PCP biodegradation (79-85%). Diez et al. (2012) studied bioremediation of PCP by immobilized *Anthracophyllum discolor* on wheat grains in soil columns, the main enzyme detected was manganese peroxidase (up to 70 U L^{-1}) when 2,4 dichlorophenol was degraded and a high correlation was found when trichlorophenol was degraded and manganese peroxidase was produce by fungus. Rosales et al. (2013) evaluated the ability of *Trametes versicolor* and *Pleurotus ostreatus* to grow on orange peels for PHA degradation, after 30 days the degradation of pyrene was 43% and both strains were able to produce high amount of laccase 3000 and 2700 U L^{-1} respectively.

Table 2.2 Immobilized white-rot fungi for bioremediation of soil.

Lignocellulosic supports	White-rot fungi	Pollutant	Reference
Wood chips	*P. chrysosporium*	Pentachlorophenol	Lamar and Dietrich 1990
Grain, sawdust, corn meal	*T. versicolor*	Pentachlorophenol	Lestan et al. 1996
Birch wood	*P. ostreatus* *P. chrysosporium*	Different PHA	Andersson et al. 2000
Sawdust, corn meal, starch and wood chips	*T. versicolor*	Pentachlorophenol	Walter et al. 2005
Flax, straw, pine bark	*T. versicolor*	Anthracene and Benzopyrene	Steffen et al. 2007
Wheat grains	*A. discolor*	Pentachlorophenol	Rubilar et al. 2011

Concluding remarks

The immobilization of white-rot fungi have attracted attention due the advantages that offer for biodegradation and bioremediation of different pollutants in soil or water. The results obtained in different investigation are promise showing the abilities of these microorganisms to grow in many different lignocellulosic supports and the capacity to produce ligninolytic enzymes. The information can be used to improve the knowledge in scale-up applications and decrease the negative impact of different pollutants when are released to the environment. The dye decolorization by white-rot fungi immobilized in lignocellulosic supports is a novel technology, different waste are investigated due their high quality amount of nutrients that are necessary for the fungal metabolism and ligninolytic enzyme production. The main factors related to improve the decolorization are nutrients, temperature, pH and type of dye. However, if all the parameters are studied the success of the process will be better than physical-chemical alternatives and will be possible to replace the current treatment used.

Different technologies have been applied for the immobilization of white-rot fungi for pesticide degradation. The main lignocellulosic supports used for this purpose are straw and sawdust due their easy way to obtain and huge amount of available in many different agro-industries.

The biodegradation of different pollutants in soil using the immobilization of white-rot fungi have been studies since 1990, especially by *Phanaerochaete chrysosporium*, *Trametes versicolor* and *Anthracophyllum discolor* the species have been immobilized in supports that include sawdust, corn meal, starch and wheat grains mainly for pentachlorophenol degradation studies. However, more efforts are necessary to improve the quality of immobilization and more investigations to scale-up to determinate the real capacity of these fungi in the environment.

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CHAPTER 3

3. SELECTION OF WHITE-ROT FUNGI TO FORMULATE COMPLEX AND COATED PELLETS FOR REACTIVE ORANGE 165 DECOLORIZATION

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3. SELECTION OF WHITE-ROT FUNGI TO FORMULATE COMPLEX AND COATED PELLETS FOR REACTIVE ORANGE 165 DECOLORIZATION

Sebastián Elgueta[1, 2], Olga Rubilar[1], Nelson Lima[2], M. Cristina Diez[1, 3]

[1] BIOREN Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Chile.

[2] IBB-Centre of Biological Engineering, Universidade do Minho, Braga, Portugal.

[3] Chemical Engineering Department, Universidad de La Frontera, Chile.

3.1 Abstract

Six strains of white-rot fungi isolated from southern Chile were evaluated for their ergosterol/biomass correlation and ligninolytic potential in solid medium to formulate pellets for reactive orange 165 (RO165) decolorization. The fungus *A. discolor* was selected to formulate complex pellets (fungal mycelium, sawdust, and activated carbon), coated pellets (complex pellet + alginate) and simple pellets (fungal mycelium). The activity of ligninolytic enzymes (laccase, manganese peroxidase, manganese-independent peroxidase, and lignin peroxidase) was evaluated in both the complex and coated pellets in modified Kirk medium, and the morphology of the pellets was studied using scanning electron microscopy (SEM). Complex pellets of *A. discolor* showed a higher enzymatic production mainly MnP (38 U L^{-1} at day 15) compared to coated and simple pellets. Examinations using SEM showed that both pellets produced a black core that was entrapped by a layer of fungal mycelium. Decolorization of RO165 was demonstrated with all the pellets formulated. However, the highest and fastest decolorization was obtained with complex pellets (100% at day 8). Therefore, complex pellets of *A. discolor* can be used for the biological treatment of wastewater contaminated with RO165.

Keywords: White-rot fungi, complex pellets, ligninolytic enzymes, RO165 decolorization.

3.2 Introduction

The increasing use of synthetic dyes in the textile, paper, cosmetics, leather dyeing, pharmaceutical, and food industries has resulted in serious environmental pollution (Tang et al., 2011). Over 10000 dyes and $7x10^5$ ton annual are commercially available worldwide and 5 to 10% of the dye stuff is lost in the industrial effluents. As a result, they generate a considerable amount of colored wastewater (Crini, 2006). The economic removal/degradation of polluting dyes is an important current issue, particularly as new regulations regarding industrial effluent discharge are beginning to be enforced (Bibi et al., 2009). The inefficiency of the dyeing process, poor handling of spent effluents and insufficient treatment of wastes from the dyestuff industries has led to dye contamination in the soil and water. Approximately half of all known dyes are azo dyes, making them the largest group of synthetic colorants used in textile industries. Azo dyes are considered to be toxic to the aquatic biota and are reported to be carcinogenic to humans; some dyes that are non-toxic when they are used but can be transformed into potentially carcinogenic amines if they are released into an aquatic environment (Soares et al., 2002). These compounds can cause problems due to their possible entrance into the food chain of humans and animals, and once they are present in the environment, they begin exerting genotoxic effects in organisms. Therefore, there is a need to remove dyes prior the effluent discharge into water bodies. Azo dyes are in general considered recalcitrant to microbial degradation. However, microbial degradation of azo dyes has been reported by bacteria (Rajaguru et al., 2000) and white-rot fungi (Martins et al., 2003). The advantage of white-rot fungi (WRF) to degrade azo dyes is related with its oxidative mechanism that avoid the formation of anilines formed by reductive cleavage of azo dyes by bacteria (Chung and Stevens, 1993).

Diverse technologies and reactors have demonstrated the capacity of microorganisms, particularly WRF, to decolorize and remove a wide variety of structurally diverse pollutants including synthetic

dyes (Rodriguez-Couto et al., 2003, Rubilar et al., 2012). These fungi possess extracellular ligninolytic enzymes such as laccase and peroxidases and due to the relative lack of specificity have been used to degrade a wide range of organic compounds like dyes (Ramsay et al., 2005, Urra et al., 2006, Eichlerová et al., 2007, Hu et al., 2009, Grassi et al., 2011).

The ability of WRF has opened new prospects for the development of new biological system using microorganisms immobilized in different supports to improve the ligninolytic enzyme production and the capacity to growth and to produce high decolorization. Diverse investigations have shown that different types of organic and inorganic supports allow the fungi pellets formation providing a structural and nutritional function (Walter et al., 2004, Rubilar et al., 2009). In this context, studies developed by Rubilar et al. (2009) reported that pellets of the white-rot fungus *A. discolor* immobilized in a mixture of activated carbon and sawdust demonstrated a high lignin degradation in an airlift reactor (>72%) and 85% of pentachlorophenol degradation when the pellets were applied in soil. Activated carbon has been used for their effective biomass immobilization and pollutant adsorption (Zhang and Yu, 2000, Ortega-Clemente et al., 2007) and lignocellulosic material have been used as support and carbon source for white-rot fungi (Walter et al., 2004, Lechner and Papinutti, 2006). Therefore, the mixture of support can to enhance the enzymatic activity and stability of fungal pellets in a wastewater treatment. However, although the use of support for pellet formation has a key role in degradation process by WRF, no information is available on degradation of highly toxic pollutants such as azo dyes.

Therefore, the aim of this work was to select a strain of WRF that can be utilized to formulate to formulate complex and coated fungal pellets to improve decolorization and degradation of Reactive Orange 165 (azo dye).

3.3 Materials and Methods

3.3.1 Strains and growth media. The WRF *Trametes versicolor* (sp and m-107), *Stereum hirsutum*, *Anthracophyllum discolor, Inonotus* sp2 and *Galerina patagonica* sp3 were isolated in different locations in southern Chile (Tortella et al. 2008). These strains were obtained from the Environmental Biotechnology Laboratory at Universidad de La Frontera, Chile. The fungi were transferred from slant cultures to glucose malt extract agar (GMEA) plates (15 g L^{-1} agar, 10 g L^{-1} glucose, 30 g L^{-1} malt extract, pH 5.2) and incubated at 30 ± 2 ºC for 7 days.

3.3.2 White-rot fungi selection. The WRF were cultivated in Petri dishes (90 mm) containing GMEA supplemented with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) for determination of laccase, activity observed by the green colorization in the growth medium (Pointing, 1999) and Poly R-478 (100 mg L^{-1}) for determination of peroxidase activity observed through the decolorization of dye from purple to yellow (Pointing, 1999). Each strain was inoculated in the center of each Petri dish with a plug (6 mm) of active mycelium from a 7-day-old culture on GMEA medium and incubated in the dark at 25ºC.

The decolorization of Poly R-478 and the oxidation of the medium containing ABTS were measured daily from Petri dishes (mm d^{-1}). The decolorization yield was calculated as the ratio between the diameter of decolourization/oxidation and diameter of fungal growth. To evaluate the ergosterol content similar assay was done in Petri dishes with GMEA medium. Ergosterol content by HPLC and biomass (dry weight) were measured every each 2 days during 10 days at 25ºC. White-rot fungus with the highest ligninolytic potential and ergosterol-biomass correlation was selected for further studies.

3.3.3 Pellets formulation. For the preparation of the inoculum, 100 mL of modified Kirk medium containing 10 g L^{-1} glucose, 2 g L^{-1} peptone, 2 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 0.1 g L^{-1} $CaCl_2$, 2 mg L^{-1} thiamine, and mineral salts (10 mL L^{-1}) were prepared in Erlenmeyer flasks (500 mL), and the pH

was adjusted to 4.5 with 20 mM acetate buffer. After autoclaving, a sterile thiamine stock solution was added to a final concentration of 2 mg L^{-1}. The flasks were inoculated with three glucose malt agar plugs (5 mm diameter) of active mycelium from the fungus *A. discolor* selected in the previous screening assay and incubated at 25°C in the dark. After 7 days, the fungal mycelium was aseptically homogenized in a sterile blender for 1 min and stored at 4 ºC for further pellet formation.

Three types of pellets were prepared: complex (mycelium immobilized on sawdust and powdered activated carbon) and coated (complex pellet coated with calcium alginate) and simple (only mycelium) used as control. All pellets were formulated in Erlenmeyer flasks containing 100 mL of modified Kirk medium. Simple pellets (mycelium-only controls) were prepared by adding 2 mL of fungal inoculum (approximately 30 mg), complex pellets were prepared by mixing 15/15/30 mg of sawdust/powdered activated carbon/mycelium (Ortega-Clemente et al. 2007) and coated pellets were formulated for entrapped of complex pellets with Ca-alginate polymer. Coated pellets were performed using a Na-alginate solution (2%) that was dispensed into a $CaCl_2$ solution (3% w w^{-1}) by shaking. After 30 min, the beads were collected from the solution, washed with distilled water and allowed to harden for 30 min in a solution of $CaCl_2$ (3% w w^{-1}).

Finally, the beads were washed with 0.7% NaCl under sterile conditions. All the solutions used in these experiments were autoclaved previously. All the experiments were performed in triplicate and incubated in a rotary shaker at 100 rpm at 25ºC for 15 days. Microphotographs of pellets were taken using a scanning electron microscope (SEM) to determine the surface structure of fungal pellets. Additionally, a sample of pellets was taken and biomass was determined by dry weight after drying at 60°C to constant weight.

3.3.4 Ligninolytic enzyme activity by pellets of white-rot fungus. Pellets of *A. discolor* (simple, complex and coated) were evaluated for laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) production. Pellets prepared previously (3 g wet pellets) and 100 mL of modified Kirk medium

were added into a 250 mL Erlenmeyer flask. Cultures were incubated in a rotary shaker at 100 rpm at 25ºC, for 15 days. Laccase, MnP and LiP activities were monitored every 2 days in the liquid culture. All assays were run in triplicate.

3.3.5 Decolorization of RO165 by pellets of a white-rot fungus. Complex, coated and simple pellets of *A. discolor* were evaluated for RO165 decolorization. Approximately 3.0 g wet pellets and 100 mL of modified Kirk medium with 100 mg L^{-1} reactive orange 165 (RO165) were added into a 250 mL flask. The flasks were incubated at 25ºC by shaking (100 rpm) for 11 days. Samples were collected periodically and centrifuged at 5,000 rpm for 15 min at 4ºC. Sepctrophotometric method was used to monitor the optical density at 493 nm by Spectronic Genesys 2PC UV-Visible spectrophotometer. The residual RO165 in the culture medium was quantified by standard curve (R^2=0.998) and the percentage of decolorization of dye was calculated with the following formula: % dye removal = $\frac{A- B}{A}$

Where A is the absorbance value after 1h of incubation and B is the absorbance value at time *t.*

Analyses

3.3.6 Ergosterol in solid medium. A method described by Marin et al. (2006) was used. The calculated recovery of ergosterol was approximately 80% for the concentrations found in this study. The fungal mycelium from Petri dishes was extracted with 40 mL of 10% KOH in methanol by magnetic stirring for 30 min. A 10 mL aliquot was transferred to a screw-cap tube and placed in a hot water bath (60 ºC) for 20 min. The tubes were transferred to room temperature and allowed to cool. Three milliliters of water and 2 mL hexane were added to the tubes, which were then vortexed for 2 min. After separation of the upper hexane layer, the sample was transferred to a 10 mL vial. The hexane extraction was repeated twice using 2 mL each time. The extracts were combined and evaporated to dryness under a stream of nitrogen. The dry extracts were dissolved in 2 mL of

methanol, filtered through 0.45 µm cellulose acetate membrane and finally transferred to a 5 mL vial. The high-performance liquid chromatography equipment consisted of a Merck Hitachi L-7100 pump, a Rheodyne 7725 injector with a 20-µL loop and a Merck Hitachi L-7455 diode array spectrum detector. The detector was set at 282 nm, and the column was a RP-18 (Superspher RP-C18, 5 µm, 4.6 x 150 mm). The mobile phase was methanol, with a flow rate of 1 mL min^{-1}. Ergosterol standard for the calibration line (R^2=0.99) was purchased from Sigma (St Louis, MO). Finally, the biomass dry weight was determined after drying at 60 °C to constant weight.

3.3.7 Scanning electron microscopy (SEM). Photomicrographs of complex pellets were taken using a scanning electron microscope (SEM) in Centre of Biological Engineering, Universidade do Minho. To prepare samples for testing, the core of each sample was cut into pieces of the same length using a flame-sterilized knife. Fixation of the sample was performed with glutaraldehyde (2.5%) at 4 °C for 1.5 hours, cacodylate salt (0.1 M, pH 7.0) for 30 min, osmium tetroxide (1%), and dehydration with acetone, followed by drying and metallization with gold.

3.3.8 Ligninolytic enzyme activities in liquid medium. Ligninolytic enzymes were analyzed in the culture medium after centrifugation at 5000 *g* for 10 min. The MnP activity was monitored by the peroxide-dependent degradation of 2,6-dimethoxyphenol (DMP) at pH 4.5 (deJong et al., 1994). The reaction mixture contained 50 mM sodium malonate (pH 4.5) 1 mM DMP, 1 mM $MnSO_4$, and up to 600 µL supernatant in a total volume of 1 mL. The reaction was initiated by the addition of 0.4 mM H_2O_2. Laccase was assayed by the peroxide-independent degradation of 2,6- dimethoxyphenol (deJong et al., 1994). The reaction mixture contained 50 mM sodium malonate at pH 4.5. For both enzymatic reactions, the products were measured at 468 nm in a spectrophotometer (Spectronic Genesys 2PC). 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. LiP activity was determined with veratrylic alcohol as a substrate (Tien and Kirk, 1988).

One unit of enzymatic activity was defined as the quantity of enzyme needed to produce 1 μmol of oxidized product and was expressed in U L^{-1}. The daily values of different enzyme activities (Lac, MnP, MiP and LiP) were used to determine the accumulated enzyme activity.

3.4. Results and Discussion.

3.4.1 White-rot fungi selection. Fungi isolated from various geographic sites in southern Chile were evaluated to select an efficient strain with high ligninolytic activity and high ergosterol/biomass correlation in solid medium. The strains evaluated were selected using the standard decolorization method with Poly R-478 (Rigas and Dritsa, 2006) that is associated with the ability to produce peroxidases (MnP and LiP) and, ABTS oxidation that is associated with the ability to produce laccase (Pointing, 1999). We analyzed the relationship between fungal growth and the ability to produce ligninolytic enzymes (Figure 3.1) and we calculated the decolorization (Poly R-478) and colorization (ABTS) yields.

In our study, the six evaluated strains produced Poly R-478 decolorization at different level, and four strains presented ABTS colorization (Figure 3.1). With respect to the decolorization of Poly R-478, the strains that showed the highest decolorization were *A. discolor, T. versicolor* sp and *Inonotus* sp with 13.6, 11 and 10 mm d^{-1}, respectively. However, the major difference observed between these strains was the yield. The yield is the ratio between the decolorization of Poly R-478 or oxidation of ABTS and radial fungal growth, indicating that when yield is near 1 the fungal growth is associated with high secretion of enzymes. Therefore, the results demonstrated that *A. discolor* was the strain that showed the highest ability to produce peroxidase (MnP) with a yield value of 1 (Figure 3.1).

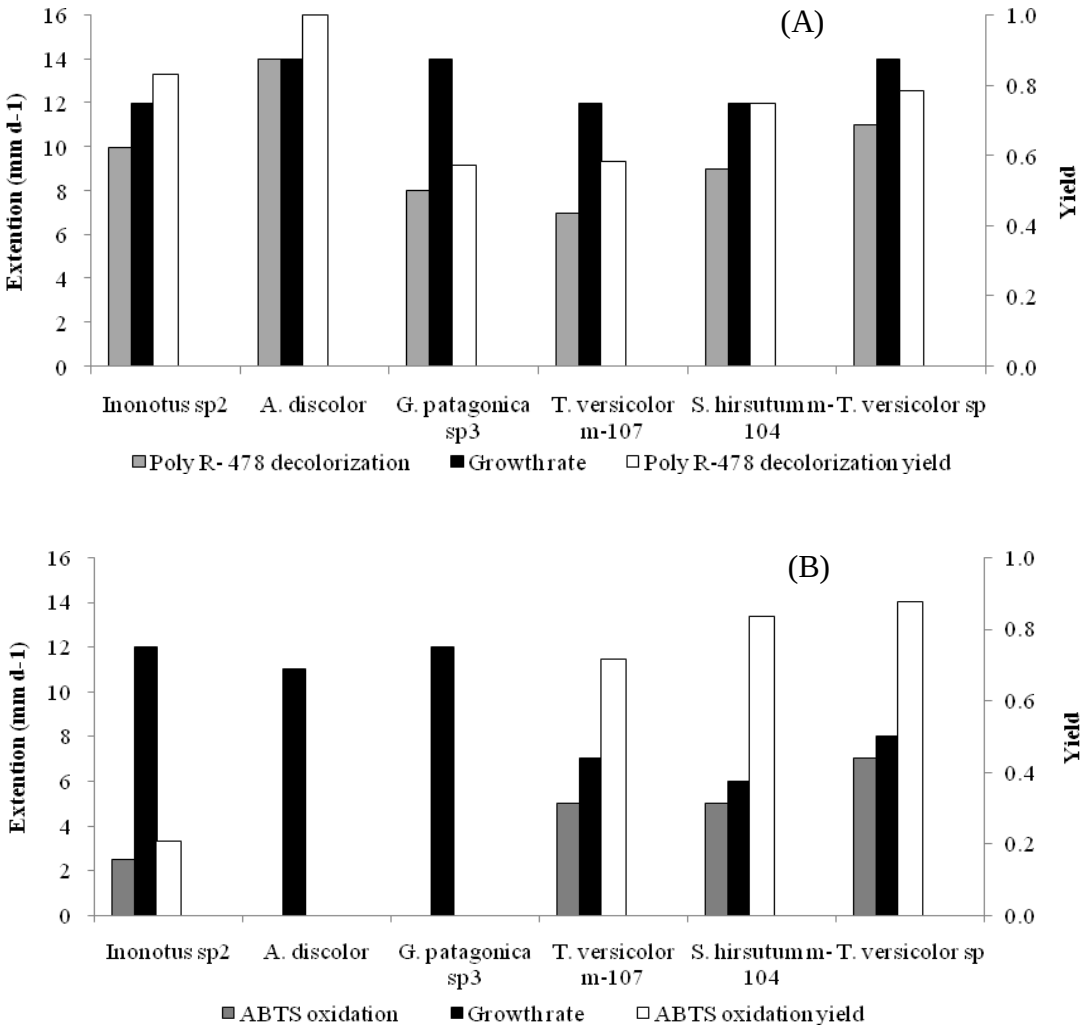

Figure 3.1 Decolorization of Poly R-478, growth rate and yield (A), oxidation of ABTS, growth rate and yield (B) of white-rot fungi in GMEA medium after 10 days of incubation at 25 ºC.

In relation to the ABTS oxidation, the strains that showed highest ability to produce laccase were *T. versicolor* sp, *T. versicolor* m-107 and *S. hirsutum* m-104 with 7, 5 and 5 mm d^{-1}, respectively. The yield for these strains was between 0.7 and 0.8, indicating that the ABTS oxidation is less associated with fungal growth. The strains *A. discolor* and *G. patagonica* showed no visible ABTS oxidation. Different results were reported by Tortella et al. (2008) for the same strains but using different culture composition. This difference could be due to that enzyme production in solid medium

depends on different factors such as nutrient limitation, substrate composition and nitrogen source (Leonowicz and Grzywnowicz, 1981, Machado et al., 2005, Kachlishvili et al., 2006).

Similar studies have been reported for white-rot fungi selection (De Koker et al., 2000, Martins et al., 2003, Mendonça et al., 2008). Mendonça et al. (2008) evaluated comparatively *Ganoderma australe*and *Ceriporiopsis subvermispora* in the biodegradation of ABTS and Poly R-478 in liquid medium, and in the pretreatment of *Eucalyptus globulus* wood chips. Laccase was detected in liquid and wood cultures with *G. australe* and *C. subvermispora* produce Lac and MnP when grown in liquid medium and only MnP was detected during wood decay. ABTS was totally depleted by all strains after 8 days of incubation while Poly R-478 was degraded up to 40% with *G. australe* strain and up to 62% by *C. subvermispora* after 22 days of incubation (Mendonça et al., 2008).

Another parameter used to select fungal strains was ergosterol/biomass correlation (Figure 3.2). This indicator is appropriate to select a strain with a high capacity for fungal biomass production in order to obtain a strain for the production of pellets that can be used in dye decolorization. Ergosterol is a sterol found in cell membranes of fungi and microalgae with the advantage of indicating only viable biomass, since it is quickly degraded after the cell´s death (Gutarowska and Zakowska, 2009). Ergosterol content has been used to estimate fungal biomass in various environments because there is a strong correlation between ergosterol content and fungal dry mass (Newell et al., 1988, Bermingham et al., 1995, Montgomery et al., 2000, Barajas-Aceves et al., 2002). However, the amount of ergosterol in fungal tissue is not constant and depends on several factors, such as age of the culture, the developmental stage and the growth conditions (type of growth media, pH and temperature) (Suberkropp et al., 1993).

In our study total mycelia ergosterol ranged from 48 to 104 mg g^{-1}, and total dry biomass ranged from 223 to 526 mg in the six strains evaluated (Figure 3.2). The ergosterol content was closely correlated with dry biomass production during the growth of each of the fungal species and r^2 was > 0.92 in all fungal strains (Table 3.1). Similar results were obtained by Montgomery et al. (2000) in pure cultures of six fungal species originating from soil and plant materials.

Therefore, the results obtained in solid medium demonstrated that *A. discolor* has the metabolic and growth capacity to formulate pellets containing activated carbon and sawdust that are coated with alginate. This agrees with the results of Tortella et al. (2008), who described the strain *A. discolor* as very promising for its use in future studies of the degradation of pollutants and indicates its potential for use in biotechnological applications.

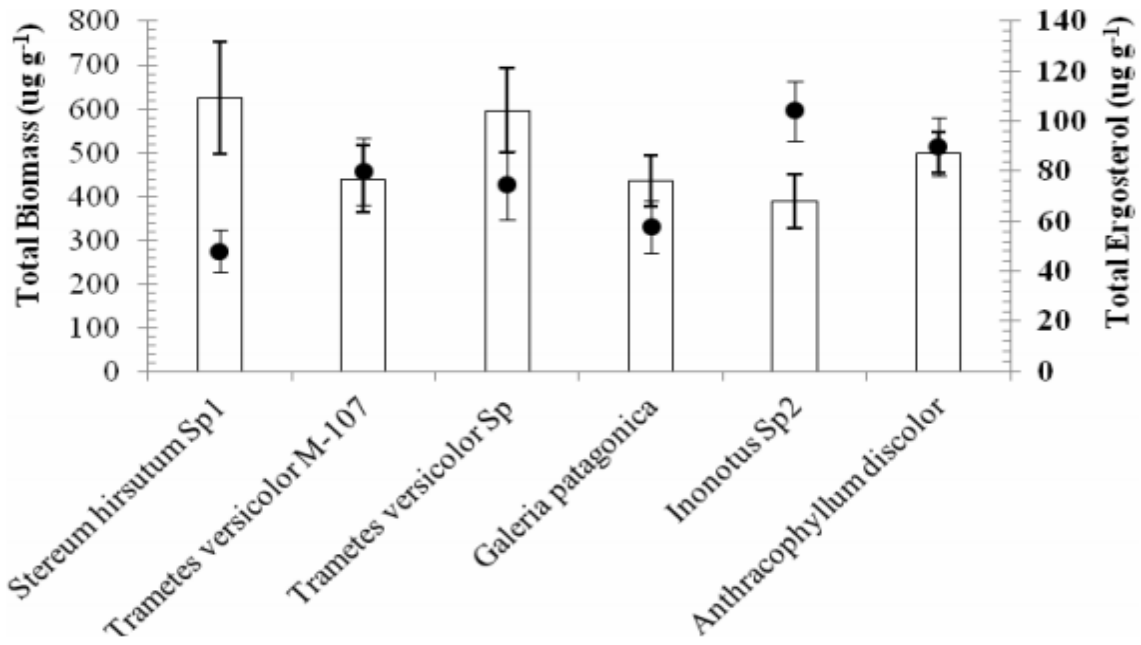

Figure 3.2 Total amount of biomass (bars) and total amount of ergosterol of white-rot fungi in GMEA medium after 10 days of incubation at 25 ºC.

Table 3.1 Linear regression for ergosterol content versus dry biomass and r^2 values of pure cultures of six fungal strains.

Fungal strains	Lineal regression	r^2
Stereum hirsutum	y=118.52x – 2.6267	0.9395
Trametes versicolor m-107	y=173.56x + 0.5565	0.9968
Trametes versicolor sp	y=135.94x – 1.3185	0.9656
Galerina patagonica sp3	y=194.5x – 0.9711	0.9228
Inonotus sp2	y=293.35x – 1.9521	0.9615
Anthracophyllum discolor	y=228.5x + 0.1994	0.9872

3.4.2 Pellets morphology evaluation. Complex and coated pellets of *A. discolor* were evaluated after 7 days of incubation at 25 ºC in Kirk medium to determine the size and shape of fungal pellets (Figure 3.3). The pellets were approximately 4 and 5 mm in diameter for the complex and coated pellets, respectively. Complex pellets showed a stable sphere of irregular surface composed of hyphal agglomeration formed by the effect of agitation during cultivation (Figure 3.3A) whereas coated pellets were sphere of homogeneous surface due to calcium alginate coating (Figure 3.3B). The biomass of complex and coated pellets was determined to examine the capacity of coated pellets to grow inside alginate beads after 10 days of incubation. After 7 days of incubation, complex pellets produced 20% more biomass than coated pellets, and at the end of the incubation, the difference decreased by 15% (data non-shown).

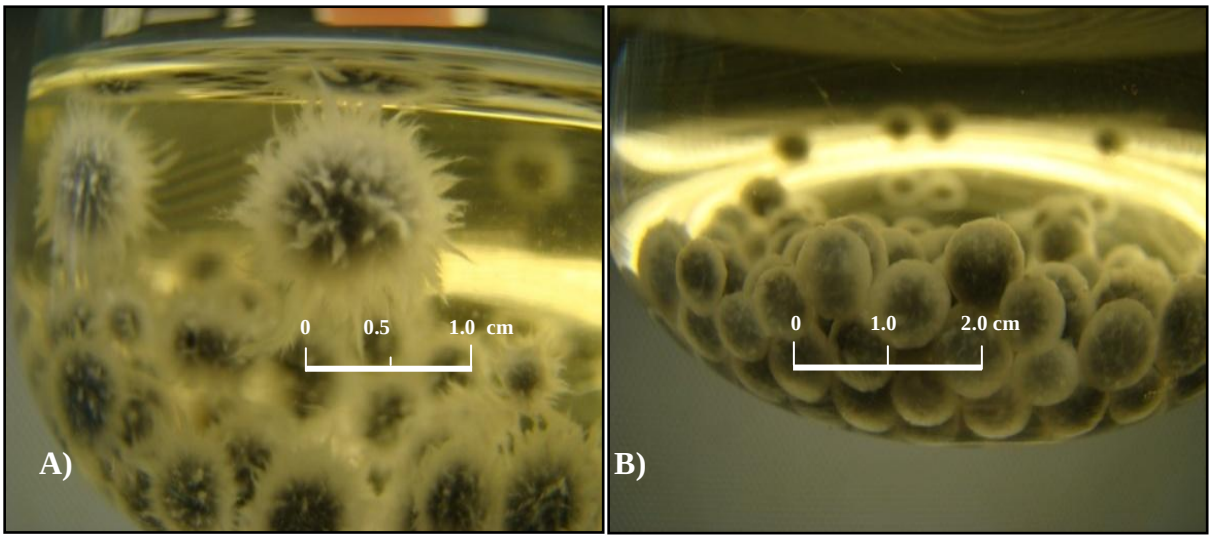

Figure 3.3 Complex (A) and coated pellets (B) of *A. discolor* formed after 7 days of incubation at 25 ºC.

SEM analysis was developed in outside and inside of both pellets (complex and coated) in order to analyze the surface of pellets and presence of activated carbon and sawdust as supports. Results of SEM analysis showed that both pellets contained a black core of activated carbon and sawdust that was entrapped inside pellets. Fungal hyphae were not detected in the core of pellets due to the low transference of oxygen (Figure 3.4A). Fungal mycelia on the surface of complex pellets showed hyphae agglomeration (Figure 3.4B) whereas the SEM of surface of coated pellets shows a smooth surface without mycelia, demonstrating the efficiency of the encapsulation process (Figure 3.4C). Calcium alginate completely surrounded the pellets, however, fungal mycelia under of surface of coated pellets was observed (Figure 3.4D). Eichlerová et al. (2007) presented SEM micrographs after 14 days of cultivation of *Dichomitus squalens* and showed substantial morphological changes in mycelia growing in a media containing Orange G. The hyphae deformations were more intensively manifested in solid media than in liquid culture.

The use of activated carbon has been investigated as an adsorbent of several organic compounds (Lin et al. 2005). It also acts to stimulate secondary metabolism and produces more ligninolytic enzymes

due to the physical properties of activated carbon and nutrient extraction from sawdust (Zhan and Yu, 2000). The presence of sawdust provides different compounds such as hemicelluloses, lignin and cellulose. Therefore, it is not surprising that immobilized fungi can produce extracellular secondary metabolites. One the disadvantage of complex pellets is that the biomass due to hyphae agglomerations surrounding the pellets could cause a breakdown of complex pellet when are used for prolonged incubation time (Rubilar et al., 2009).

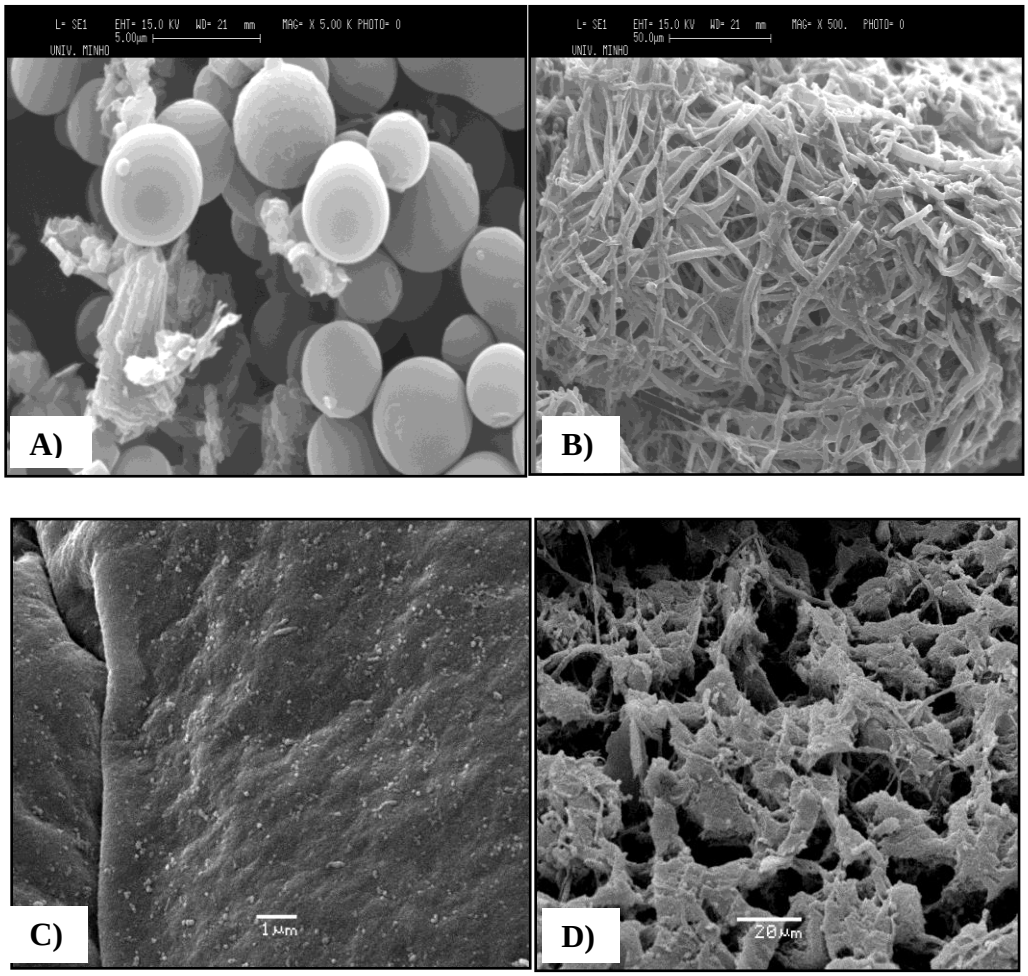

Figure 3.4 Scanning electron microscopy of complex pellets of *A. discolor*, after 7 days of incubation. A) Black core inside both pellets (5 µm); B) fungal mycelia on the surface of complex pellets (50 µm); C) surface of coated pellets (1µm); D) fungal mycelia under the surface of coated pellets (20 µm).

These morphological changes of complex pellets could be diminished by alginates coating due to that this polymer present various advantages such as biodegradability, hydrophilicity, low density and mechanical stability, all of which make them highly suitable for producing alginate beads. The properties of the beads can be affected by the immobilization process, by the effects of alginate viscosity, by the length of time in the $CaCl_2$ bath, and by the $CaCl_2$ concentration (Yakup et al., 2004). Some reports have demonstrated that re-coating of pellets reduces the viability of pellets entrapped in Ca-alginate beads (Ramsay et al., 2005).

3.4.3 Ligninolytic enzyme activities of *A. discolor* pellets. Ligninolytic activity and accumulated ligninolytic activity during the incubation of complex, coated and simple pellets of *A. discolor* in liquid medium for 15 days are shown in Figure 3.5A, 3.5B and 3.5C. The main enzyme produced by the three types of pellets was MnP followed by MiP. Lacasse and LiP were produced in a lesser extent. Accumulated Lac activity was 18 U L^{-1}, 32 U L^{-1} and 0.4 U L^{-1} in the complex, coated and simple pellets, respectively. Accumulated MnP activity was 147 U L^{-1}, 98 U L^{-1} and 93 U L^{-1} for the complex, coated and simple pellets, respectively. Accumulated MiP peroxidase activity was 69 U L^{-1}, 34 U L^{-1} and 27 U L^{-1} for the complex, coated and simple pellets, respectively.

Finally, the accumulated LiP activity was 4 U L^{-1}, 11 U L^{-1} and 18 U L^{-1} for the complex, coated and simple pellets, respectively. Based on these results, we observed that the accumulated activity of Lac and MiP increased significantly due to the introduction of sawdust and activated carbon during pellet formulation (complex and coated) in comparison with simple pellets; on the other hand, accumulated MnP activity was the highest in complex pellets (Figure 3.5A). The maximum peak of enzyme activity for the complex pellet was observed on day 15 (38 U L^{-1} for MnP). Similar results were obtained by Rubilar et al. (2009). The authors demonstrated that complex pellets of *A. discolor* had a high stability with a high MnP production in comparison with simple pellets, during lignin degradation in an airlift reactor. Ortega-Clemente et al. (2007) demonstrated that pellets of *T.*

versicolor formulated with sawdust and activated carbon (triple pellets) showed MnP and Lac average values 30% higher than in double pellets (only sawdust). Our results suggest that increasing MnP activity with complex pellet could be associated with highest dyes degradation.

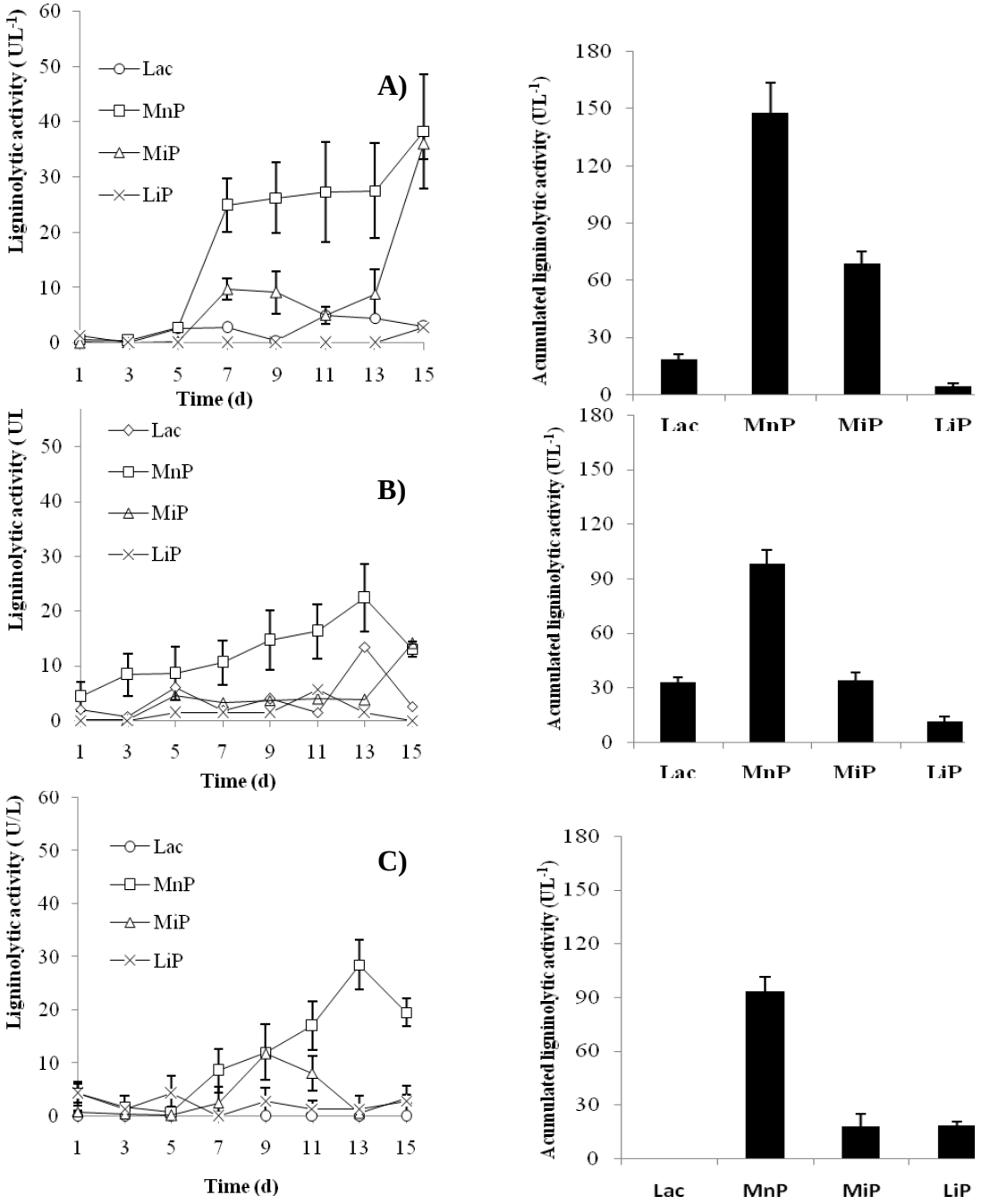

Figure 3.5 Ligninolytic enzyme activities (left) and accumulated ligninolytic activity (right) of *A. discolor* pellets in liquid medium after 15 days of incubation at 25 °C. A) Complex pellets; B) Coated pellets; C) Simple pellets.

3.4.4 Decolorization of RO 165 by pellets of *A. discolor*. RO165 decolorization by complex, coated and simple pellets of *A. discolor* was evaluated during 11 days of incubation and the results are shown in Figure 6. We can observe that the dye RO165 was degraded by the three types of pellets; however complex pellets showed a faster decolorization compared to coated and simple pellets. At day 4 complex pellets achieved 87% of dye decolorization while simple and coated pellets obtained 64 and 34%, respectively. At day 8 complex pellets achieved 100% of decolorization while simple and coated pellets achieved 100% of decolorization after 11 days of incubation. During the first 6 days, the RO165 decolorization by coated pellets was slower compared to complex and simple pellets. It is due to that in coated pellets the penetration of fungal mycelium in layer of alginate is slower. The highest and fastest RO165 decolorization by complex pellets could be related to MnP production (Figure 3.6). In this context, studied developed by Urra et al. (2006) showed that the decolorization of 100 mg L^{-1} of RO165 by *P. chrysosporium* was 91% after 15 days, and they suggested that RO165 was degraded by the oxidative action of MnP present in the medium concordant with our result. Baldrian and Snajdr (2006) evaluated the decolorization of azo dyes by different white-rot fungi, demonstrating that the fastest degradation of Poly B-411 was performed by the strains with high levels of Lac and MnP. In relation to complex pellets Rubilar et al. (2009) reported that complex pellets of *A. discolor* containing sawdust and activated carbon exhibited higher MnP activity level than simple pellets; therefore, this led to a higher level of pollutant degradation. This effect can be explained by the presence of a microenvironment inside fungal pellets that changes in the presence of supports, thus improving the enzymatic activity and hence the decolorization (Zhang and Yu, 2000). The absorbance spectrum of RO165 during the incubation with complex pellets changed drastically, showing a peak at 493 nm at 0 day and no peak at 11 days. This reduction corresponded to a shift in the coloration of the medium from orange to yellow,

demonstrating the dye degradation (Figure 3.7). Similar behavior was observed with simple and coated pellets of *A. discolor* (data non-shown).

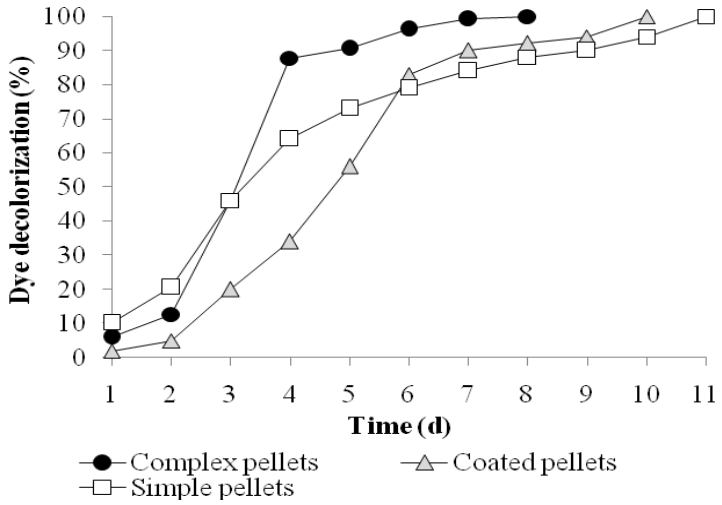

Figure 3.6 Decolorization of RO165 by complex, coated and simple pellets of *A. discolor* after 11 days at 25 °C.

At the end of decolorization, all the pellets were transferred to flasks containing ethanol for 24 hours in order to identify the dye adsorbed in the mycelium. In our study, no dye was detected adsorbed onto mycelium.

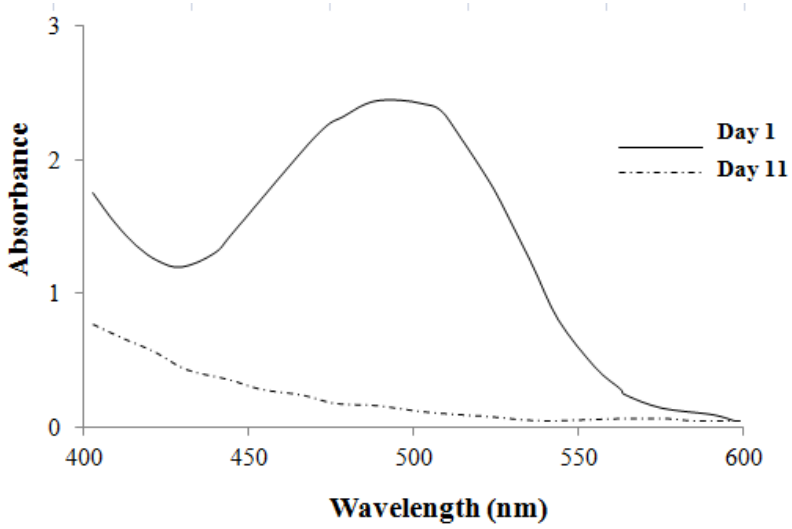

Figure 3.7 Absorbance spectrum of RO165 at time 0 and after 11 days of incubation with complex pellets of *A. discolor* at 25 °C.

3.5. Concluding Remarks

Some general, promising conclusions may be derived for the results here obtained:

- Chilean white-rot fungus *A. discolor* showed high level of peroxidases and high biomass/ergosterol correlation demonstrating a great potential for pellets formulation to dyes decolorization.
- Complex pellets of *A. discolor* showed a higher enzymatic production mainly MnP compared to coated and simple pellets.
- Decolorization of RO165 was demonstrated with all the pellets formulated. However, the highest and fastest decolorization was obtained with complex pellets.
- The highest and faster RO165 decolorization by complex pellets was correlated with enzymatic activity of MnP and the change in the absorbance spectrum demonstrated the degradation of the dye.

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CHAPTER 4

4. FORMULATION OF SUPPORTS FOR IMMOBILIZATION OF *ANTHRACOPHYLLUM DISCOLOR* TO INOCULATE A BIOPURIFICATION SYSTEM FOR ATRAZINE DEGRADATION

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4. FORMULATION OF SUPPORTS FOR IMMOBILIZATION OF *ANTHRACOPHYLLUM DISCOLOR* TO INOCULATE A BIOPURIFICATION SYSTEM FOR ATRAZINE DEGRADATION

Sebastián Elgueta[1], Nelson Lima[2], Cledir Santos[2], Maria Rao[3], M. Cristina Diez*[4]

[1] BIOREN Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Chile.

[2] IBB-Centre of Biological Engineering, Universidade do Minho, Braga, Portugal.

[3]Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Naples , Italy.

[4] Chemical Engineering Department, Universidad de La Frontera, Chile.

4.1Abstract

Three formulations of supports F1 (sawdust 74%, starch 6%, corn meal 2% and flaxseed 15%); F2 (sawdust 74%, starch 8%, corn meal 5% and flaxseed 10%) and F3 (sawdust 74%, starch 10%, corn meal and 8% flaxseed 5%) were evaluated to immobilize *Anthracophyllum discolor*. Supports were formulated with a carrier, nutrient source, binder and lubricant, and then pelletized. Fungal growth, storage conditions and ligninolytic enzymes activities (laccase (Lac), manganese peroxidase (MnP), manganese-independent peroxidase (MiP) and lignin peroxidase (LiP) in coated pelletized support (CPS) and uncoated pelletized support (UPS) for the three formulations were evaluated. After all evaluations, the uncoated pelletized with the formulation F1 (UPS-F1) was selected to inoculate a biomixture of a biopurification system to evaluate atrazine degradation, phenoloxidase activity and PCR-DGGE analyses. The results showed that the highest enzymatic activity was MnP for F1, F2 and, F3 supports and the lowest was Lac. The atrazine degradation and phenoloxidase activity were higher in the biomixture inoculated with UPS-F1 than in non inoculated biomixture. Finally, the DGGE analysis showed that no negative effect on native microbial population was observed when UPS-F1 was inoculated in the biomixture, especially after 30 days of atrazine degradation.

4.2 Introduction

Worldwide, the generation of lignocellulosic residue results in pollution of the environment every year and leads to a loss of valuable materials that can be bioconverted to several value-added products (Rodríguez-Couto et al., 2001). Lignocellulosic residues are mainly composed by polysaccharides (cellulose and hemicellulose) and lignin and are present in a wide range of wastes from the agricultural and forestry industries. The degradation of plant residues by microorganisms specially white-rot fungi depends on chemical and physical characteristics such as porosity, compound content, type of components and structure (Sanchez, 2009).

Different studies have demonstrated the importance of lignocellulosic support for enhancing of ligninolytic enzymes produced by white-rot fungi (Rodríguez-Couto et al., 2001, Lorenzo et al., 2002, Kapich et al., 2004, Rodríguez-Couto and Sanromán, 2005). The main factors, affecting the production of ligninolityc enzymes are the availability and chemical form of nutrients, temperature, and the presence of inhibitors or inducers (Baldrian, 2008). However, not all white-rot fungi produce these enzymes simultaneously, and it is difficult to find a correlation between pollutant degradation and production of enzymes even if they are detected (Gianfreda et al., 2006). White-rot fungi present an effective system that is able to degrade a range of different pollutants, the native Chilean white-rot fungus *Anthracophyllum discolor* can produce ligninolytic enzymes activities in presence of chlorophenols (Tortella et al., 2008, Rubilar et al., 2011) polycyclic aromatic hydrocarbons (Acevedo et al., 2011) and dyes (Elgueta et al., 2012).

Immobilized white-rot fungi have been historically applied to soil as live microorganisms in liquid culture or attached to a carrier material (Pepper et al., 2002). The immobilization on different supports increases the viability and provides sources of nutrients to increase the degradation of pollutants. Lignocellulosic supports provide an environment similar to the natural habitat of white-rot fungi and can stimulate the secretion of ligninolytic enzymes (Elgueta and Diez, 2010).

Additionally, carbon compounds can be added to confer an advantage to immobilized fungi and resist contamination by indigenous soil microorganisms. The growth of white-rot fungi in most soils is limited due to the low amount of available carbon and nitrogen, although soils rich in organic (humic) compounds contain enough nutrients to support the growth, external substrate addition is usually required (Baldrian, 2008). Frequently, immobilized fungal biomass grows faster and is more successful during the establishment of fungi on soil. The use of a natural support for fungal immobilization conducting a satisfactory pollution removal process becomes more feasible (Lestan et al., 1996). However, there are diverse factors to produce immobilized fungi as the production, the storage, the viability and later utilization in the bioremediation process, the influence of these factors has mainly been studied by several authors Lestan and Lamar (1996), Lestan et al. (1998), Walter et al. (2004), Schmidt et al. (2005) and Ford et al. (2007).

Several researchers have been predominantly making studies focused on the capacity to degrade different pesticides and selection of different alternatives for biopurification systems denominated biobed (Castillo and Tortensson, 2007). The principal component of biobed is the biomixture composed by straw: peat: soil in the proportions 1:1:1 %v, their efficiency based on the ability to retain and degrade pesticides through microorganisms especially white-rot fungi (Castillo et al., 2008).

Meanwhile, the importance of microbial community involves degradation of pesticides in biomixture of biobed is unknown. Few studies are related to. Coppola et al. (2011) described the impact of pesticides on microbial communities; they found an evident modification of microbial diversity after the addition of several applications of fungicides, demonstrating that yeast flora and ascomycete filamentous fungi seemed to be involved in the degradation activity. Therefore, there is no evidence reported for the use suitable immobilized white-rot fungi to improve the biological activities and pesticide degradation in biomixture of a biobed. The aim of this research was formulate suitable

support for immobilization of *Anthracophyllum discolor* and evaluate their effect on biological activities during atrazine degradation in a biomixture of a biopurification system.

4.3 Materials and Methods

4.3.1 Microorganism: The fungal strain *Anthracophyllum discolor* was isolated in the south of Chile. This strain was obtained from the Environmental Biotechnology Laboratory at Universidad de La Frontera, Chile. The fungus was transferred from slant cultures to glucose malt extract agar (GMEA) plates (15 g L^{-1} agar, 10 g L^{-1} glucose, 30 g L^{-1} malt extract, pH 5.2) and incubated at 25 ± 1 ºC for 7 days.

4.3.2 Support formulation for *A. discolor* immobilization: A preliminary study was done to evaluate different materials to be used to formulate different supports for *A. discolor* immobilization. Ten grams (g) of each sawdust, corn meal, potato starch flaxseed meal and a mixture of sawdust, corn meal and starch, were placed in a 250 mL Erlenmeyer flask, adjusted to 50% gravimetric water content and autoclaved (15 min, 121 ± 2 °C). The flasks were centrally inoculated with a plug (5 mm) of active mycelia overgrown on GMEA and incubated at 25 ± 1 ºC. Flasks were visually assessed every 3 days to examine fungal growth using a growth index "++" to indicate sparse growth, "+++" to indicate average growth and "++++" to indicate abundant growth (Walter et al., 2004). In parallel 10 g of each single material and mixture were inoculated with *A. discolor* (three plugs) to evaluate the ligninolytic enzyme activities: Laccase (Lac), manganese peroxidase (MnP), manganese-independent peroxidase (MiP) and lignin peroxidase (LiP) during 18 days of incubation. The results were plotted as the total amount of each ligninolytic enzyme during the incubation.

Supports formulations: Three formulations including the materials previously tested were formulated in different proportions (g): support F1 (sawdust 74%, starch 6%, corn meal 2% and flaxseed 15%), support F2 (sawdust 74%, starch 8%, corn meal 5% and flaxseed 10%) and support F3 (sawdust

74%, starch 10%, corn meal and 8% flaxseed 5%); besides, lignosulphonate 3% was added to each formulation. Each formulation (1000 g) was mixed, moistened to aprox 10% with distilled water and pressed to obtain pelletized supports (PS) of 8 mm using a pellet mill ZLSP300B R-Type.

The fungus immobilization on supports was done as described by Lestan and Lamar (1996), with some modifications. First, homogenized fungus mycelia was produced transferring 3 plugs of active mycelium of *A. discolor* to 250 mL liquid Kirk medium, incubated at 25 ± 1 ºC and after 7 days they were homogenized in a sterilized blender for 1 min. This homogenized mycelium (250 mL) was mixed with 100 mL of alginate solution (2%) and was used to prepare the immobilized fungus. For coated pelletized support (CPS), 10 g of PS of each formulation previously described were transferred in a mixture of alginate-mycelium and shaken for 1 min. After that, supports were collected from the solution and allowed to harden for 3 min in a solution of $CaCl_2$ (3%). All the solutions used in these experiments were autoclaved previously. For uncoated pelletized support (UPS), 10 g of PS were transferred to plastic bags moistened with 4 mL of GMEA and inoculated with 5 plugs of active mycelium of *A. discolor* previously grown in GMEA; then, bags were incubated at 25 ±1 °C during 30 days.

To evaluate the growth capacity and ligninolytic enzymes activities of *A. discolor* in CPS and UPS supports for F1, F2, F3 formulations, 50 g of each formulation were put in a plastic bag and incubated for 15 days at 25 ± 1 °C. The growth was evaluated by the index described above and samples of CPS and UPS were taken to be analyzed.

To demonstrate the storage stability of *A. discolor* in CPS and UPS supports for F1, F2, F3 formulations, 30 g of samples were transferred to plastic bags and stored at two temperatures (25 and 4 °C). The fungal growth after the storage was analyzed by SEM analysis.

4.3.3 Biomixture preparation and inoculation: A biomixture was prepared mixing soil, wheat straw, and peat in a proportion of 1:2:1 %v. The soil (Andisol) was collected between 0 to 15 cm

deep and sieved (< 3 mm). Wheat straw was cut into 2 cm pieces using a food processor and the peat was obtained in a commercial market. The soil has pH of 5.4, 18.6 mg kg^{-1} of available nitrogen, 17.1 mg kg^{-1} of available phosphorous and 12% organic matter. The commercial peat has 33.4% cellulose and 21.9 % lignin. Wheat straw has 9.9% lignin, 41.8% cellulose and 66.1% organic matter, pH 5.9 and 0.56% total nitrogen. The biomix was stored in a polypropylene bag at 4 ºC until use. The resulting biomix has pH 4.8, 30.8% organic carbon and 0.54% total nitrogen (C/N=57) (Diez et al., 2013). The constituents were mixed vigorously to obtain a homogeneous biomixture; then, the biomixture with 60 % of water holding capacity (WHC) was pre-incubated for 30 days at 20 ± 1 °C in a polypropylene bag until use (Fernández et al., 2012).

The biomixture (1000 g) was transferred to glass pots and inoculated with 10% (w w^{-1}) of previously selected immobilized *A. discolor* (UPS-F1). The fungal inoculum was incorporated in the first 5 cm of the biomixture. Then, the biomixture was contaminated with 60 mg kg^{-1} of atrazine through sprinkling. Four treatments were prepared: Biomixture + atrazine (B+A), Biomixture + fungal inoculum (B+F), Biomixture +atrazine + fungal inoculum (B+A+F) and Biomixture (B) as control. Each experiment was carried out in triplicate under destructive sampling mode. The biomixture were incubated at 20 ± 1 °C and 60% WHC for 30 days. To evaluate the microbial communities an analysis by PCR-DGGE was done, and samples were extracted after 0, 20 and 30 days of incubation for DNA extraction. Phenoloxidase activity and residual atrazine were measured during 30 days. In parallel, respiration activity (CO_2) was evaluated during 30 days. All the samples were in triplicate.

4.3.4 Ligninolytic enzyme activities: Ligninolytic enzymes were extracted from 1 g (w w^{-1}) of each samples with 5 mL of 50 mM sodium malonate (pH 4.5) (Tien and Kirk, 1988). The samples were centrifuged at 10000 rpm and stored up to 24 hours at 4 ºC prior to analysis. The Lac activity was determined with 2,6-dimethoxyphenol (DMP) as the substrate in sodium malonate (pH 4.5). 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 $MnSO_4H_2O$, 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 laccase activity (DeJong et al. 1994). The extinction coefficient was 49600 $M^{-1}cm^{-1}$. One enzymatic activity was defined as the amount of enzyme transforming 1 µmol DMP per minute. The LiP activity is based on the oxidation of veratryl alcohol. The reaction mixture contained 1420 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) and was monitored at 310 nm for 2 min. The extinction coefficient was 93000 $M^{-1}cm^{-1}$. One enzymatic activity unit was defined as the amount of enzyme transforming 1 µmol DMP per minute (Moreira et al. 1997).

4.3.5 Scanning electron microscopy (SEM): Photomicrographs were taken using a scanning electron microscope (SEM). Samples were prepared by cutting immobilized fungus into small pieces, and these were cut from the surface and center for SEM analysis. To prepare the samples, the core was cut at same length for all formulations at 2 cm using a sterilized knife. The samples were fixed with glutaraldehyde (2.5%) for 1.5 hours at 4 °C followed by cacodylate salt (0.1 M, pH 7.0) for 30 min. The samples were post-fixed with osmium tetroxide (1%), dehydrated with acetone, dried and metalized with gold.

4.3.6 Pesticide extraction and analytical procedures: To extract atrazine from the samples, 20 mL of methanol were added to 10 g biomixture and incubated for 1 hour at 25 °C with shaking (350 rpm). Then, the samples were sonicated at full power for 30 min. The samples were subsequently centrifuged at 10000 rpm and filtered through 0.2 µm PTFS membrane filters. The extraction process

was performed twice to obtain the maximum amount of atrazine from the biomixture, and the supernatants were measured by injecting 20 µL into a Merck Hitachi L-2130 pump, a Rheadhyne 7725 injector with µL loop and a Merck Hitachi L-2455 diode array detector. Separation was achieved using a C18 column (Chromolit RP-18e, µmx4.6 mm x 100 mm). Eluent A was ammonium acetate (1 mM) and eluent B was acetonitrile. The flow rate was set at 1.0 mL min^{-1}, with 0-10 min in an isocratic mode. The column temperature was maintained at 30 °C. The detector was set at 222 nm for the data acquisition. Instrument calibrations and quantificactions against pure ATZ reference standars (0.1- mg L^{-1}) (Tortella et al 2013). The calculation of Half-life value of atrazine was described using the first-order kinetic equation as $C=C_o e^{-kt}$, and from the equation, we obtained (Eq. [1]): $t_{1/2}= Ln(2) / k$ [1] k= 0,027

4.3.7 Biological activities in biomixture: *Phenoloxidase activity* was determined in all degradation assays and was performed using MBTH/DMAB (Castillo et al., 1994). Briefly, samples (10 g) of the biomixture were agitated (150 rpm, 2 hours) with 25 mL of a 100 mM succinate–lactate buffer (pH 4.5). Samples were centrifuged (4000 rpm, 20 min). The supernatant of each sample was collected, filtered through 0.45 µm membrane and measured immediately. The reaction mixture contained 300 µL of 6.6 mM DMAB, 100 µL of 1.4 mM MBTH, 30 µL of 20 mM $MnSO_4$, 1560 µL of the filtered sample and, the reaction is initiated with the addition of 10 µL of 10 mM H_2O_2. The reaction is followed in a spectrophotometer Spectronic Genesis 2PC at 590 nm ($\varepsilon = 0.053\ \mu M^{-1}\ cm^{-1}$). Because no correction was made for the possible presence of lignin peroxidase (LiP) and laccase (Lac) activity, this measurement may represent the sum of manganese peroxidase, LiP and Lac (Castillo and Torstensson 2007) and is expressed as phenoloxidase activity.

4.3.8 Microbial community analyses: DNA was isolated from 4 treatments at 0, 20 and 30 days, using the FastDNA® Spin Kit for soil. Isolation was performed on 0.5 g of biomixture following the

manufacturer´s instructions 1% agarose gel electrophoresis in 0.5x TBE was used to analyze the quality and quantity of extracted DNA.

PCR conditions for bacteria were determinate following the protocol described by (Ercolini et al. (2002). Bacterial DNA was amplified with primers V3f-GC and V3r spanning the variable V3 region of the 16S rDNA. The first primer (V3F-GC) was 5´ CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3´. The second primer (V3R) was 5´-ATTACCGCGGCTGCTGG-3´. Combination of both primers generates a PCR fragment of about 200 bp suitable for a subsequent DGGE analysis. Each mixture (final volume, 25 µL) contained 20 ng of template DNA, each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 2.5 µL of 10x PCR buffer and 2.5 U of *Taq*-DNA polymerase (Invitrogen). Template DNA was denatured for 5 min at 94 °C. A "touchdown" PCR was performed (Muyzer et al., 1993), to increase the specificity of amplification and to reduce the formation of spurious by-products. The initial annealing temperature used was 10 °C above the expected annealing temperature (65 °C), and the temperature was decreased by 1 °C every second cycle until the touchdown temperature (55 °C) was reached; then additional 10 cycles were carried out at 55 °C. Primer extension was carried out at 72 °C for 3 min. Finally, the samples were incubated for 10 min at 72 °C (final extension). The presence of PCR products was ascertained by agarose (1.5% w/v) gel electrophoresis, at 100 V for 1 hour.

PCR conditions for fungi were determinate following the protocol described by (Iacumin et al. 2009). Fungal DNA was amplified by PCR using the primers NL1-GC 5´-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCCATATCAATAAGCGGA GGAAAAG-3´ and LS2 (5´-ATTCCCAAACAACTCGACTC-3´), combination of NL1 and LS2 generates a PCR fragment of about 250 bp of 5- end region of the 26S rDNA. Each mixture (final volume, 25 µL) contained 20 ng of template DNA, each primer at a concentration of 0.4 µM, each deoxynucleoside

triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$, 2.5 µl of 10x PCR buffer and 1.25 U of *Taq*-DNA polymerase (Invitrogen).The reactions were run for 30 cycles: denaturation was at 95 ℃ for 60 s, annealing at 52 ℃ for 45 s and extension at 72 ℃ for 60 s. An initial 5 min denaturation at 95 ℃ and a final 7 min extension at 72 ℃ were used.

DGGE analysis was determinate following the protocol described by Ercolini et al. (2002). PCR products were analyzed by DGGE by using a Bio-Rad Dcode apparatus and the procedure first described by Muyzer et al. (1993). Samples were applied to 8% (w v^{-1}) acrylamide-bisacrylamide (37:5:1) gels in 1x TAE buffer. Parallel electrophoresis experiments were performed at 60 ℃ by using gels containing a 30 to 60% urea-formamide denaturing gradient (100% corresponded to 7 M urea and 40% (w v^{-1}) formamide) increasing in the direction of electrophoresis. The gels were analyzed by gel electrophoresis for 10 min at 50 V and for 4 hours at 200 V, stained with ethidium bromide for 5 min and rinsed for 20 min in distilled water. The dendrogram of DGGE profiles was constructed using Phoretix 1D analysis software.

4.3.9 Statistical analysis of data. Experiments were conducted using three independent replicates. Data were subjected to a one-way analysis of variance (ANOVA) and the averages were compared by Tukey's range tests.

4.4 Results and discussion

4.4.1 Support formulation for *A. discolor* immobilization. A preliminary study was done to evaluate different materials to be used to formulate different supports for *A. discolor* immobilization. *A. discolor* grown on all materials tested at different visual growth estimations (Table 4.1) Flaxseed meal showed the highest visual fungal growth estimation and low growth was observed with starch and sawdust, and sparse growth was observed with corn meal. An average growth (++) was observed when a mixture of materials (sawdust, corn meal and potato starch) was evaluated.

Table 4.1. Growth estimation of *A. discolor* after 12 days at 25 ºC.

Support	**Visual growth estimate**
Sawdust	+
Mixture (sawdust+ ground corn + starch)	+++
Corn meal	++
Starch (from potato)	+
Flaxseed	++++

++++ (100%) Abundant growth; +++ (75%) medium growth; ++ (50%), sparse growth; + (25%), low growth.

The ligninolytic enzyme activities (Lac, MnP, MiP and LiP) in all materials used were evaluated. The MnP activity was the highest and Lac activity was the lowest in all materials used (Figure 4.1). The highest cumulative enzymatic activities were obtained when flaxseed meal was used. In this material MnP activity was 518 µmol $min^{-1}g^{-1}$, MiP activity was 158 µmol $min^{-1}g^{-1}$ and LiP activity was 222 µmol $min^{-1}g^{-1}$. When a mixture of materials without flaxseed was tested an increment in the

enzymatic activities was observed respect to starch and corn meal added individually and was similar respect to sawdust. On the other hand, the activities of MiP, MnP and LiP were lower than those obtained when flaxseed was used for *A. discolor* growth (Figure 4.1).

The capacity to grow and produce ligninolytic enzymes of *A.discolor* can be explained through the natural inclination of filamentous fungi to adhere to a surface and colonize the materials used in this study. Flaxseed meal contains approximately 28% lignocellulose, 23% lignin, 25% hemicellulose, and 47% cellulose (Coskuner and Karababa, 2007). The amount of cellulose present in flaxseed represents a source of hydrolysable oligomer that is easy to degrade; for this reason, it is more susceptible to enzymatic degradation by fungi (Sanchez, 2009). Currently, in the literature, different types of lignocellulosic supports or materials are used for immobilization in bioremediation processes. White-rot fungi growth have been studied in various inexpensive agricultural and wood industrial materials including wood chips (Lamar and Dietrich, 1990), pine bark, peat, and wheat straw (Morgan et al., 1993), grains, sawdust, starch, corn meal (Lestan et al., 1996), birch wood (Andersson et al., 2000) sawdust, corn meal, starch, and wood chips (Walter et al., 2005) and flax, straw, and pine bark (Steffen et al. 2007) wheat grains, wood chips and sawdust (Rubilar et al., 2011). Three supports (F1, F2 and F3) including the materials previously tested were formulated for *A. discolor* immobilization. One set of supports was coated with alginate (CPS) and another was uncoated (UPS). During incubation *A. discolor* immobilized in all supports grown and produced a layer of dense mycelia on the surface and interspaces inside of the supports (Figure 4.2).

Formulation F2, both CPS and UPS showed the most abundant growth, whereas formulations F1 (CPS) showed medium growth and F1 (UPS) showed abundant growth. Instead of F3 for both CPS and UPS showed medium growth (Table 4.2). However, *A. discolor* growth in uncoated supports was faster than in coated supports during the overgrowth period (visual observation).

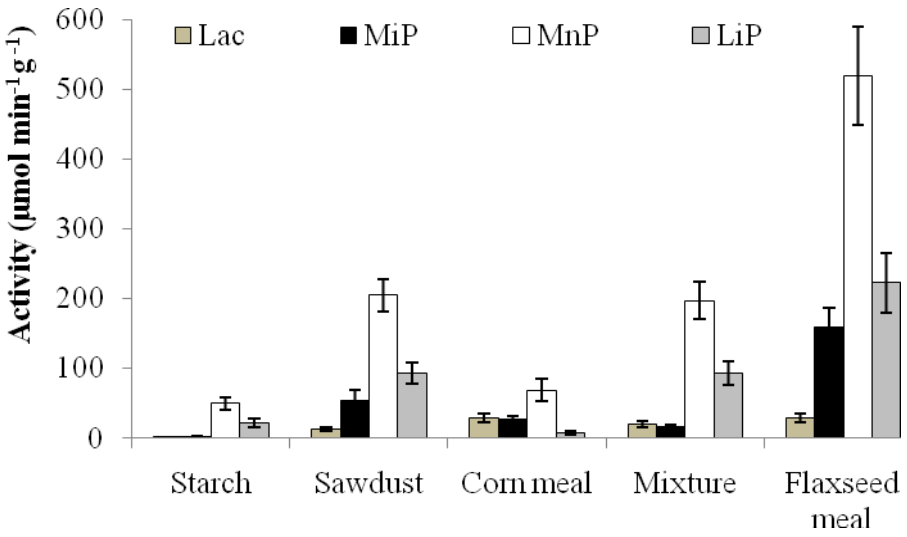
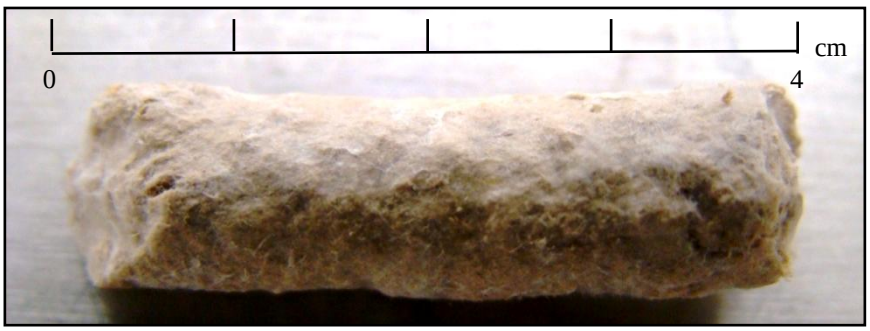


Figure 4.1. Cumulated ligninolytic activities of *A. discolor* on different materials.

A



B

Figure 4.2. Growth capacity *A. discolor* in UPS (A), and CPS (B), supports for F1 formulation.

Table 4.2. Growth estimation of *A. discolor* on formulations after 15 days incubation at 25 ºC.

Formulation	CPS	Visual growth	UPS	Visual growth
F1	+++	Medium growth	++++	Abundant growth
F2	++++	Abundant growth	++++	Abundant growth
F3	+++	Medium growth	+++	Medium growth

++++ (100%) +++ (75%) ++ (50%).

The Figure. 4.3, illustrates the ligninolytic enzymes activities of F1, F2 and F3-CPS (Figure. 4.3A) and F1, F2 and UPS-F3 (Figure. 4.3B). The activity of MnP was the highest and Lac activity was the lowest, and LiP was not detected in both CPS and UPS supports.

The production of Lac was low and constant through the incubation period with CPS-F3 and UPS-F3 supports (15 µmol $min^{-1}g^{-1}$ and 10 µmol $min^{-1}g^{-1}$, respectively). The Lac production in CPS-F2 was higher than in UPS-F2 support, with a pick at day 9 of 32 and 18 µmol $min^{-1}g^{-1}$, respectively. However after 15 days the incubation the activity decreased al level below 5 µmol $min^{-1}g^{-1}$ in both supports. Respect to CPS-F1 support the pick of Lac activity was 22 µmol $min^{-1}g^{-1}$ after 9 days of incubation. For UPS-F1 support no pick at the same time was observed; however, the activity increased since day 9 reaching 24 µmol $min^{-1}g^{-1}$ at day 15 of incubation.

The production of MnP activity increased through the incubation period for both CPS and UPS supports. The activity for CPS-F1 and UPS-F1 was the highest, achieving 155 and 220 µmol $min^{-1}g^{-1}$, respectively, at day 15. However, for CPS-F2 and UPS-F2 the activity was below 63 and 100 µmol $min^{-1}g^{-1}$ in both supports, at the same time of incubation. Respect to UPS-F3 support, the pick of MnP activity was 73 µmol $min^{-1}g^{-1}$ after 13 days of incubation. For UPS-F2 support no pick at the

same time was observed; however, the activity increased since day 11 reaching 100 µmol $min^{-1}g^{-1}$ at day 15 of incubation.

The production of MiP was constant through the incubation period with CPS-F1 and UPS-F3 supports (15 µmol $min^{-1}g^{-1}$ and 25 µmol $min^{-1}g^{-1}$, respectively). The MiP production in CPS-F2 was similar for UPS-F2 support, with a pick for both at day 13 of 38 and 36 µmol $min^{-1}g^{-1}$, respectively. However after 15 days the incubation the activity decreased al level below 25 µmol $min^{-1}g^{-1}$ in both supports. Respect to CPS-F3 support, the pick of MiP activity was 27 µmol $min^{-1}g^{-1}$ after 11 days of incubation. For UPS-F1 support the activity was increasing through the time reaching 59 µmol $min^{-1}g^{-1}$ at day 15 of incubation.

Similarly to other studies done with *A. discolor*, MnP activity is the main ligninolytic enzymes produced by this fungus (Acevedo et al., 2011, Bustamante et al., 2011, Rubilar et al., 2011) and the highest concentration was obtained with *A. discolor* immobilized in UPS-F1support with 15% flaxseed meal Walter et al. (2004) produced immobilized *Trametes versicolor* isolated in New Zealand; the main enzyme detected in this case was laccase, and the pick of this enzyme was 730 µmol $min^{-1}g^{-1}$ at day 19. Lestan et al. (1996) formulated immobilized *Trametes versicolor* isolated in the USA, which mainly produced MnP with a pick of approx, 300 µmol $min^{-1}g^{-1}$. They established that the immobilized fungus is more resistant when is used for bioremediation processes in soil.

The formulation UPS-F1 include the highest amount of flaxseed meal (15%), this material has poly unsaturated fatty acids (PUFAs), compounds that provide oxygen species that when they are peroxidized by MnP, the enzyme requires organic acids such as oxalate or malonate to chelate Mn^{+3}, this in turn are capable of oxidizing PUFAs and their hydroperoxides to generate acyl radicals and peroxyl radicals respectively (Srebotnik and Boisson, 2005). It means that lipid peroxidation (LPO), may be involved in lignin degradation by *A. discolor*. Therefore, the use materials that include PUFAs in the formulation, may increase the rate of LPO indicating that free radicals capable of

degrading lignin can be produced for a combination of enzymes, metal-complex and lipid hydroperoxides from the PS formulated. Kapich et al. (1999) showed that MnP can initiate the LPO, thereby generating lipid peroxyl radicals, in turn, may be the primary agents of fungal attack on prevailing non-phenolic lignin in wood. Kapich et al. (2007) indicates that MnP mediated LPO may play an important role in lignin degradation even in the presence of the phenolic antioxidant compounds, and supports the possibility of the involvement of LPO in the degradation of lignin in wood.

The growth of *A. discolor* immobilized in formulations F1, F2 and F3 was evaluated during the storage at 4 and 25 ºC for 30 days. In general, we observed that *A. discolor* totally colonized both surface and core of the supports, and no differences were observed at 4 and 25 ºC of storage temperature. Representative SEM micrographs for the immobilization of *A. discolor* on F1 support (CPS and UPS) is presented in Figure. 4.4A uniform fungal growth inside the supports was observed indicating that mycelium becomes entrapped and colonized the core of the supports (CPS and UPS) even at temperature of 4°C (Figure. 4.4A and 4.4C). At the same time, the Figure. 4.4B and 4.4D shown the fungal colonization to surfaces and interspaces of the pelletized supports (CPS and UPS-F1) stored at 25 ºC. The high colonization of both supports is due to its high porosity and nutrient contents of the supports. It is necessary to highlight that no contamination by other microorganisms was observed during the storage at both temperatures (4 and 25 ºC).

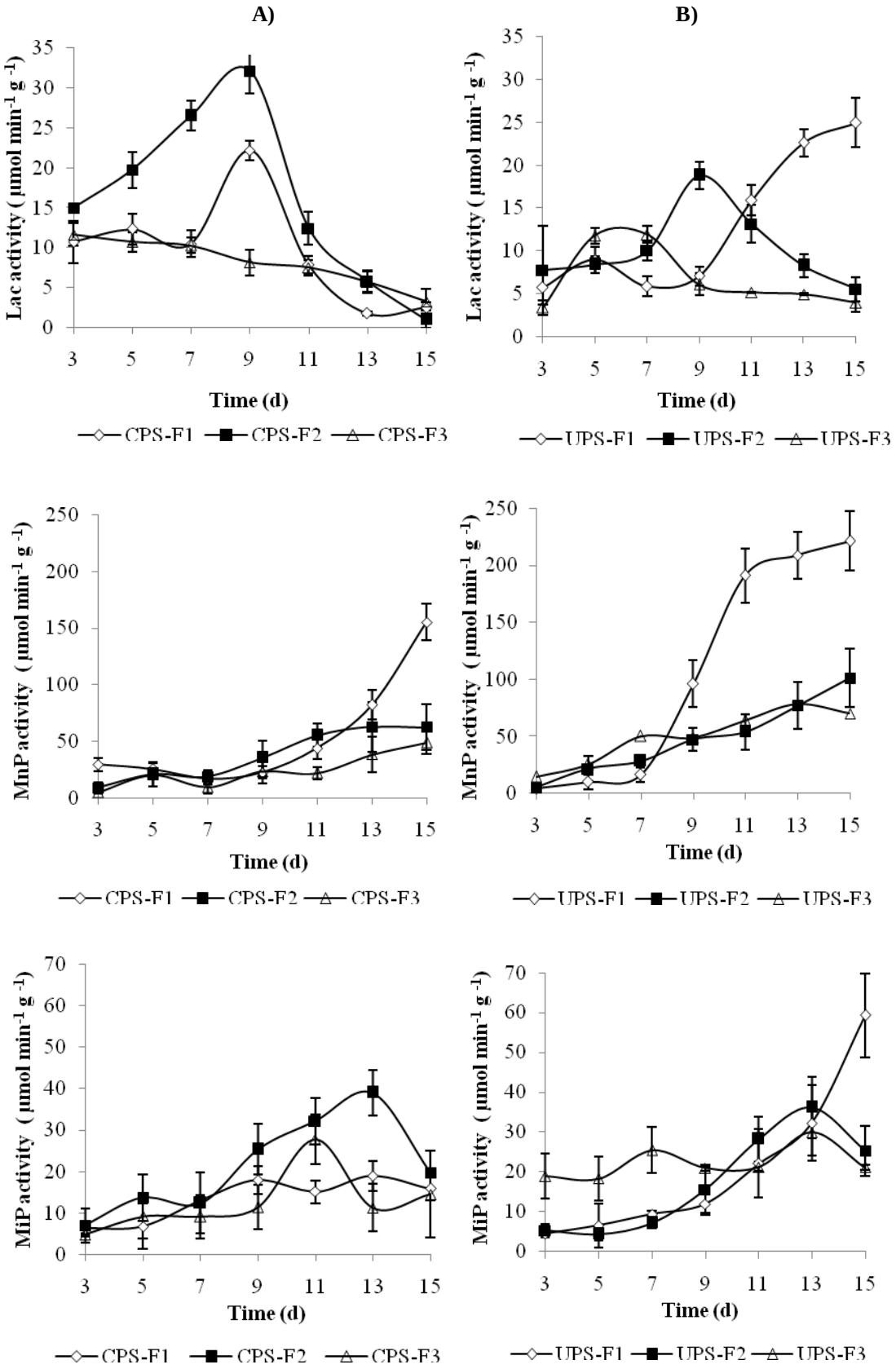


Figure 4.3 Ligninolytic activities of *A. discolor* after 15 days of incubation at 25 °C, A)CPS; B)UPS**.**

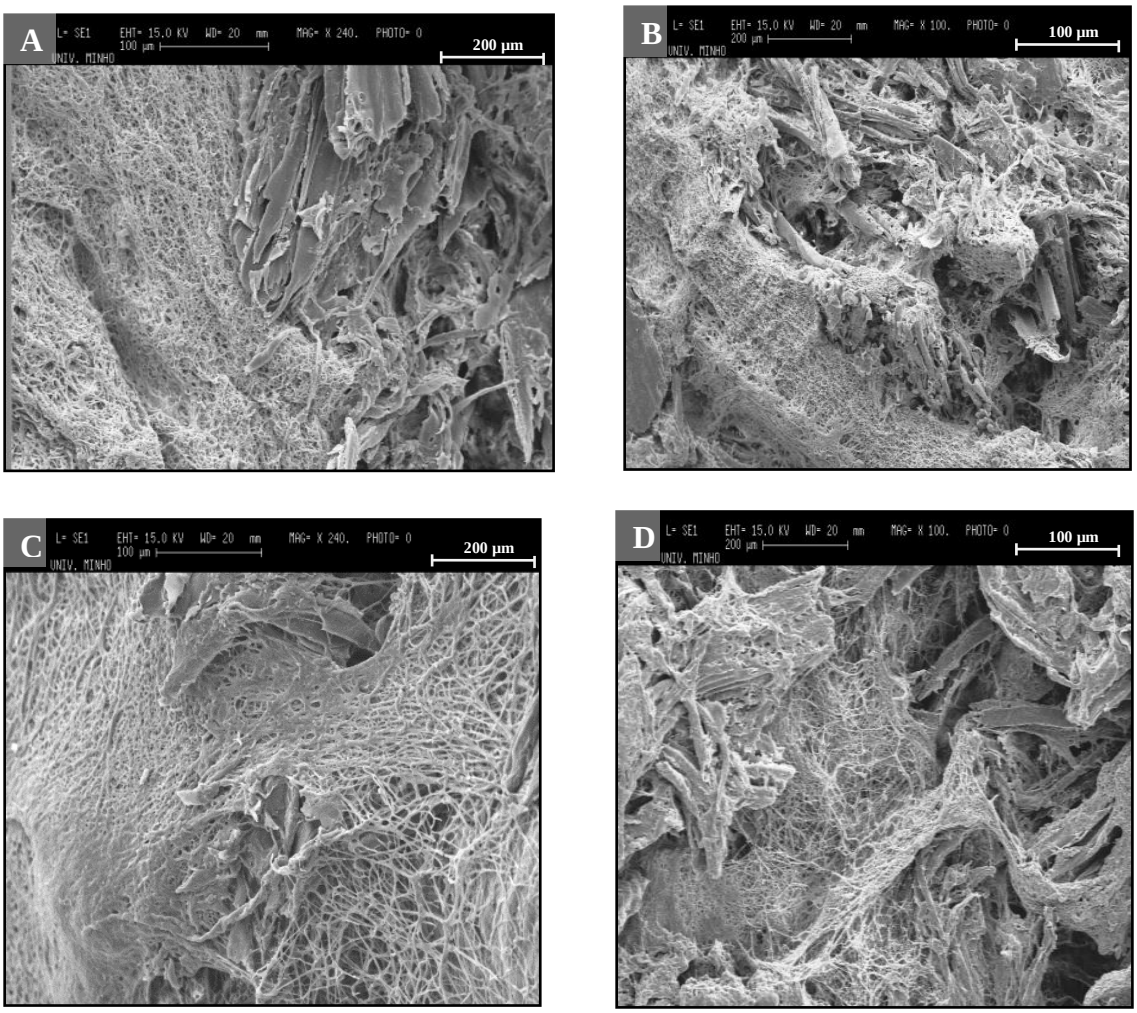


Figure 4.4 Scanning electron microscopy of *A. discolor* overgrown in the formulation F1 after 30 days of storage. Core of F1-CPS at 4 ºC (A), surface F1-CPS at 25 ºC (B), core F1-UPS at 4 ºC (C), surface F1-UPS at 25 ºC (D).

4.4.2 Effect of inoculation of immobilized *A. discolor* in the biomixture After the selection of a suitable support and immobilization system of *A. discolor* (UPS-F1), we evaluated its inoculation in a biomixture contaminated with atrazine. Significant difference ($P \leq 0.05$) in atrazine degradation rate was observed between inoculated and non-inoculated biomixture. Atrazine degradation was 95% in the biomixture inoculated with *A. discolor* immobilized in UPS-F1 after 30 days of incubation,

while, 75% of atrazine was degraded in the biomixture non-inoculated at the same time of incubation (Figure 4.5). The inoculation of the biomixture with *A. discolor* increased significantly the degradation of atrazine, even considering that we added a high dose of atrazine (30 times the field dose) in the biomixture, in order to simulate a pesticide spillage.

The half-life ($t_{1/2}$) of atrazine decreased from 6 days in the biomixture inoculated (B+A+F) to 14.5 days for the biomixture non-inoculated (B+A). This result demonstrate that stimulation of microbial activity in the biomixture by adding white-rot fungus *A. discolor* immobilized in UPS-F1 support decreased significantly the half-life ($t_{1/2}$) of atrazine. The microbial activity of the biomixture may be also stimulated by adding terpenes as has been reported by Tortella et al. (2013). The authors found that the half-lives ($t_{1/2}$) of atrazine in a similar biomixture (non-inoculated) in the presence of 50 µg kg^{-1} of limonene or eucalyptol (9.4 and 9.5 days, respectively) were significantly lower than the half-life in the control (13.2 days) without terpens.

The increment in the degradation of other compounds using white-rot fungi had been also reported. Lestan et al. (1996) described the pentachlorophenol (PCP) degradation by immobilized white-rot fungi. They inoculated (3%) of immobilized fungi in artificially contaminated non-sterile soil and found that *I. lacteus*, *B. adusta*, and *T. versicolor* removed 86, 82, and 90%, respectively, of pentachlorophenol in 4 weeks. Walter et al. (2004) found that wheat straw and SCS (sawdust–cornmeal–starch-mix) could be suitable carriers for native N. Zealand *T. versicolor* for bioremediation of PCP in soil. Ford et al. (2007) evaluated PCP bioremediation by white-rot fungi (31 to 175 g kg^{-1} inocula) in highly contaminated field soils (100 to 2137 mg kg^{-1} PCP) and found that bioavailability and extractability of PCP in the contaminated field soil may significantly increase after bioaugmentation. Schmidt et al. (2005) found a strong correlation between the amount of fungal inoculum used and fungal colonization in a soil bioaugmented for bioremediation. Rubilar et al. (2011) described a series of studies on the ability of *A. discolor* and *Phanerochaete chrysosporium*

immobilized in wheat straw for the treatment of soil contaminated with pentachlorophenol (PCP). They reported high growth and colonization of the soil by fungi, and high manganese peroxidase (MnP) activity. As compared to free mycelia, immobilization of *A. discolor* and *P. chrysosporium* in wheat grains favored the spread of fungi in the soil and consequently also the removal of the pollutant (93 and 79%, respectively).

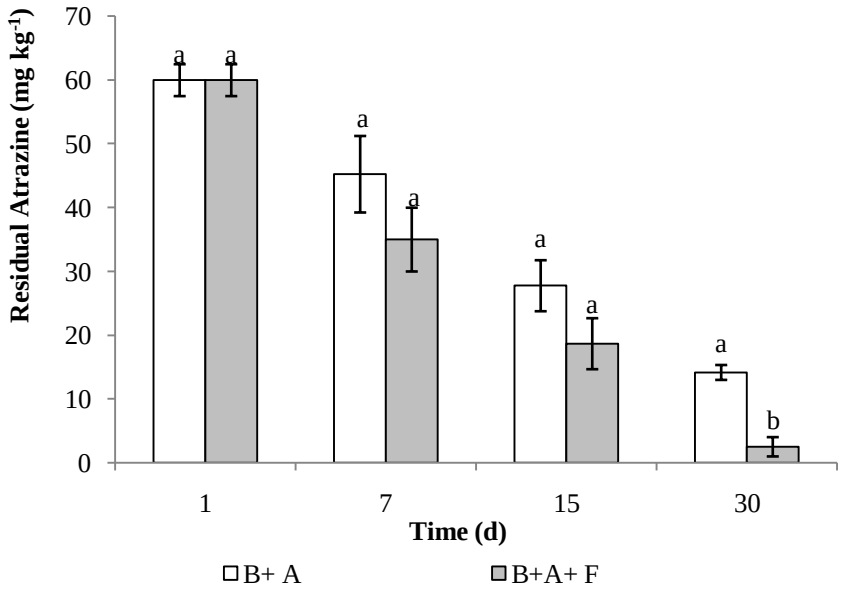


Figure 4.5. Residual atrazine (mg kg^{-1}) during 30 days of incubation at 20 °C in a biomixture contaminated with atrazine non-inoculated (B+A) and inoculated with UPS-F1 (B+A+F).

The phenoloxidase activity in the biomixture contaminated with atrazine was improved by the inoculation of A. *discolor* immobilized in UPS-F1 support (Figure 4.6). The biomixture contaminated and inoculated (B+A+F) showed the highest phenoloxidase activity, while the biomixture non-contaminated and non-inoculated (B) showed the lowest phenoloxidase activity during all incubation periods. Similar and low phenoloxidase activities (up to 0.75 U kg^{-1}), were found during the first 7 days in the biomixtures inoculated non-contaminated (B+F), biomixture contaminated non-inoculated (B+A) and in biomixture non-contaminated non-inoculated (B). After

15 days of incubation, the phenoloxidase activity in biomixtures B+A+F and B+F were doubled respect to the values obtained after 7 days of incubation (2.8 and 1.9 U kg^{-1}, respectively). At the end of the incubation period (30 days) the phenoloxidase activities were 3.2, 2.8, 1.7 and 1.2 U kg^{-1} in the biomixtures B+A+F, B+F, B+A and B, respectively. The presence of the inoculum in the biomixtures (B+A+F and B+F) clearly increased the phenoloxidase activity in these biomixtures due to the high content of lignocellulosic compounds that are degraded by white-rot fungi.

Many researchers have assumed that degradation of pesticides by white-rot fungi is mediated by peroxidases, and these enzymes are involved in lignin degradation. The main material of biomixture is wheat straw that contains readily available carbon sources that are linked to lignin degradation, which probably explains why straw is correlated with phenoloxidase activity (Castillo et al., 2001). The ability to degrade lignin efficiently only in the presence of an alternate energy source (such as cellulose, hemicellulose or simple carbohydrates) through fungal growth allows the transport of nutrients as carbon source (Hammel 1993). Karas et al. (2011) described *Trametes versicolor* as an efficient degrader of pesticides in wastewaters from fruit packaging industry; however, they found contrasting results for ligninolytic enzymes and were not associated with degradation of three pesticides showing that other enzymes were also involved in the degradation.

Castillo and Tortensson (2007) established that straw in the biomixture produced the main activity for the dissipation of pesticides containing readily available carbon sources promoting white-rot fungi growth that can contribute to respiration activity. In our study, respiration activity was measured during the 30 days of incubation and the highest respiration activity was obtained in biomixture contaminated and inoculated (B+A+F) and the lowest was obtained in the biomixture non-inoculated non-contaminated (B) (data non-shown)

During transformation in biomixture, humic substances are formed from both lignocellulose and structural components of microbial decomposers. It is important to note that, while polysaccharides

are sources of both carbon and energy-acquisition by soil microorganisms, the degradation of lignin and humic substances does not provide enough energy to maintain decomposition, and does not play the primary nutritional role.

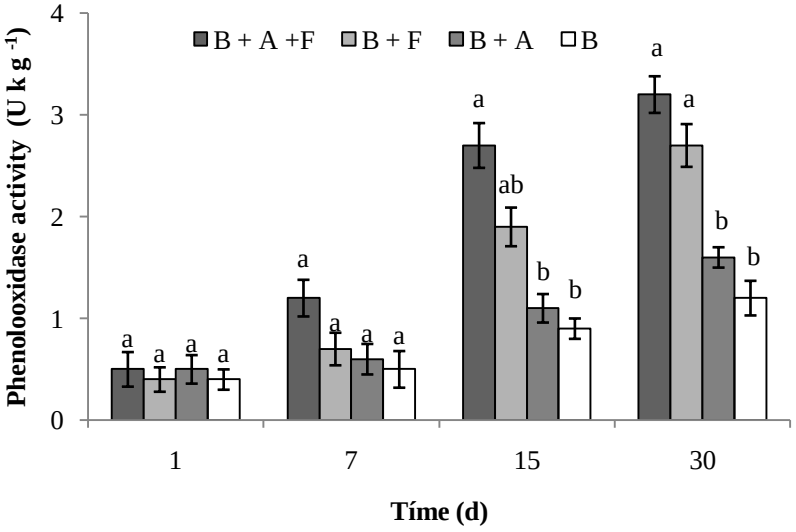


Figure 4.6. Phenoloxidase activity (U kg^{-1}) in a biomixture contaminated with atrazine non-inoculated (B+A), inoculated with UPS-F1 (B+A+F) and biomixture non- contaminated (B) and inoculated (B+F).

The effect of *A. discolor* UPS-F1 in biomixture contaminated with atrazine on microbial communities (bacteria and fungi) was evaluated after 0, 20 and 30 days assessed with PCR-DGGE (Figure 4.7). The DGGE patterns representing the bacterial communities in the biomixture samples had high similarity indices (>80%). DGGE profiles for bacteria strains are shown in the Figure 4.7A. The treatment, that included the biomixture, atrazine and *A. discolor* UPS-F1 (B+A+F) showed stimulation for bacteria and fungi during the evaluation especially at the end of experiment. The band pattern in DGGE showed a decreasing number of bands the first days of degradation compared to control, which can be explained by the effect of atrazine on the bacterial community. Our results indicated that there was little change at day 30 mainly for B+F this process can be explains for the

normal stabilization of the biomixture inoculated. However, these differences in our results dose not demonstrated that *A. discolor* UPS-F1 affect the microbial communities in the biomixture. Tortella et al., (2013) described the impact bacterial communities in a biomixture (1:2:1 %v) contaminated with atrazine. They found the existence of two major groups with a similarity of 78%. The analysis revealed that the chronologic applications of atrazine produce shortly changes on bacterial communities with respect to Ctrl (80% similarity) in the same application period. The DGGE patterns representing the fungal communities in the biomixture samples had high similarity indices (>70%). DGGE profiles for fungi are shown in the Figure 4.7B, the treatment B+A+F, showed stimulation for some fungi mainly at day 30. The DNA of *A. Discolor* was used as a control during the experiment to follow through the time, DGGE analysis showed differences of microbial community between treated and control samples for fungi mainly in the treatment B+A+F. Our results indicated the presence of *A. discolor* DNA in all treatment through the time this can be explained due the similarities of native basidiomycetes present in the biomixture. Therefore, the addition of *A. discolor* UPS-F1, determined certain change in the structure of microbial community, making a differentiation between treated and control samples. Tortella et al. (2013) described DGGE patterns for fungal communities in a biomixture (1:2:1) contaminated with atrazine. They found high similarity indices (>85%) in DGGE patterns of fungal communities in the biomixture and no difference between treated and untreated samples of communities structure, however they observed differences associated with the pesticide application times. Coppola et al. (2012) showed in DGGE analysis an evident modification of microbial diversity in a biomixture contaminated with fungicides. The band pattern in DGGE gel showed a drastic change over the application of fungicide. At these sampling times the lowest similarity compared with the controls in both bacterial (48% and 45% respectively) and fungal (81% and 60% respectively).

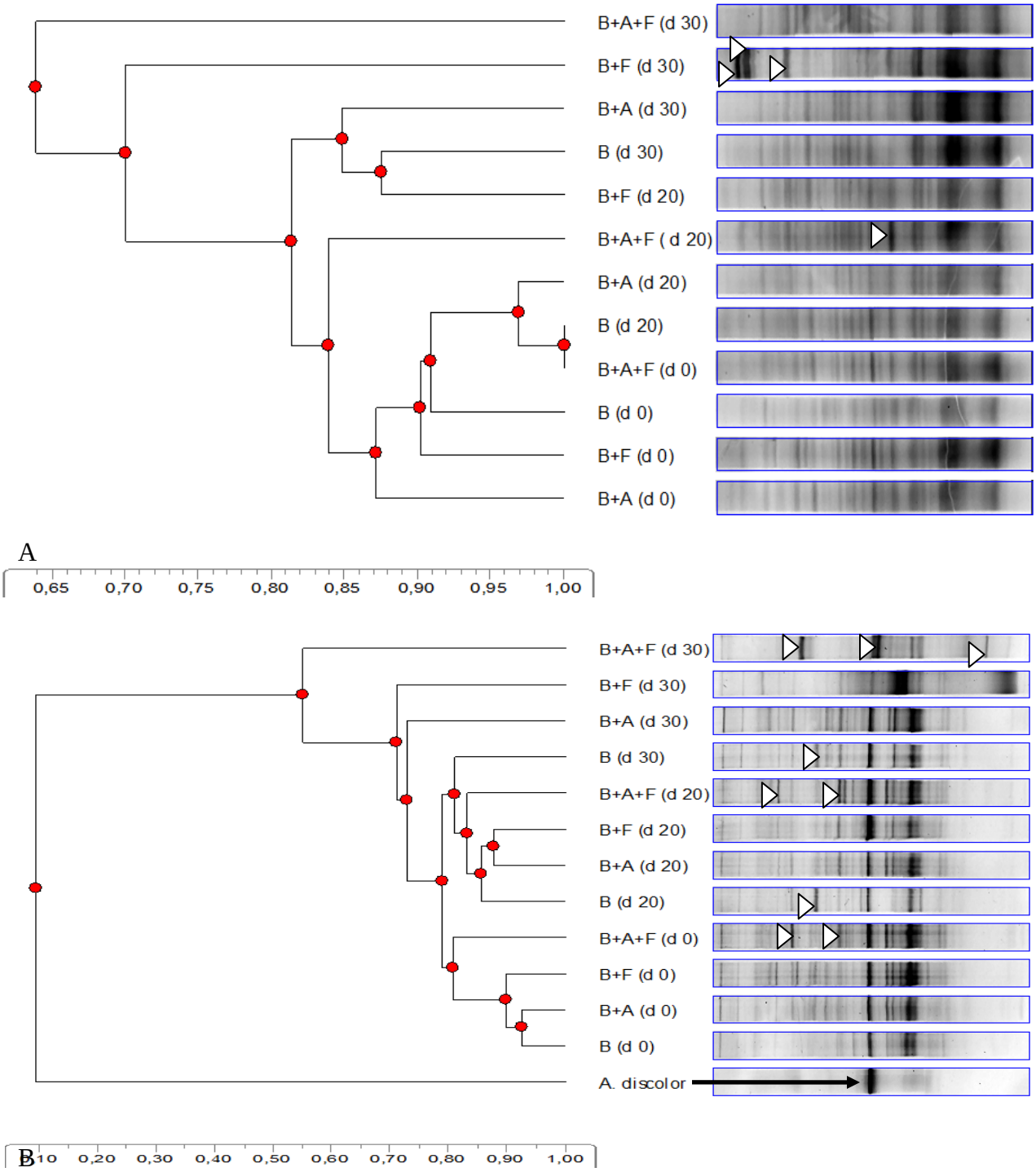


Figure 4.7. Microbial communities by PCR-DGGE during 30 days. A) Bacterial DNA amplified with primers V3f-GC and V3r; B) Fungal DNA was amplified by PCR using the primers NL1-GC and LS2. A. biomixture (B), biomixture +atrazine (B+A), biomixture + fungus (B+F), biomixture +atrazine + fungus (B+A+F).

4.5 Concluding remarks

Some general, promising conclusions may be derived for the results here obtained:

- Flaxseed meal used as material for *A. discolor* immobilization allowed a greater growth and peroxidase activities (MnP, MiP, LiP) compared to other materials evaluated (starch, corn meal and sawdust).
- The formulation (F1), with the high proportion of flaxseed meal (15%) presented the best condition for growing and ligninolytic enzymes productions by *A. discolor*. On the other hand, the uncoated pelletized support (UPS-F1) demonstrated better condition to immobilize the fungus than coated pelletized support (CPS-F1). Nevertheless, no differences were observed in both types of support during the storage period at 4 and 25 ºC.
- The inoculation of the biomixture with *A. discolor* immobilized in UPS-F1 support was a positive strategy to improve atrazine degradation. The atrazine degradation was higher (95%) in biomixture inoculated than in biomixture non-inoculated (75%), and the half-life ($t_{1/2}$) of atrazine decreased from 14.5 in biomixture non-inoculated to 6 days in biomixture inoculated. Besides, the phenoloxidase activity was highest at day 30 (3.2 U kg^{-1}) in the biomixture inoculated.
- The DGGE patterns of bacterial and fungal communities in the biomixture in all treatments had high similarity indices (80 and 70% respectively). The inoculation with *A. discolor* in the biomixture contaminated with atrazine produced a stimulation in the fungal communities at the end of the experiment.
- In conclusion, *A. discolor* UPS-F1 improved the atrazine degradation in a biomixture of biopurification system.

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CHAPTER 5

5. ATRAZINE DEGRADATION IN A BIOPURIFICATION SYSTEM INOCULATED WITH IMMOBILIZED WHITE-ROT FUNGI

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5. ATRAZINE DEGRADATION IN A BIOPURIFICATION SYSTEM INOCULATED WITH IMMOBILIZED WHITE-ROT FUNGI

Sebastián Elgueta[1], Olga Rubilar[1], M. Cristina Diez [2]

[1] BIOREN Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Chile.

[2] Chemical Engineering Department, Universidad de La Frontera, Chile.

5.1 Abstract

In this study immobilized white-rot fungi in uncoated pelletized support (UPS) (*Stereum hirsutum* Ru-104, *Inonotus* sp2, and *Trametes versicolor)* were inoculated in a biomixture contaminated with atrazine (80 mg kg^{-1}). The effect of inoculation was evaluated through atrazine degradation and biological activities such as: phenoloxidase activity, fluorescein diacetate activity (FDA) and respiratory activity. The biomixtures inoculated with fungi immobilized in UPS support increased the degradation of atrazine and biological activities compared with the biomixture non-inoculated. However, not all strains evaluated showed the same behavior during atrazine degradation. The strain *S. hirsutum* Ru-104 showed high capacity to degrade atrazine (92%) and grow in a biomixture. However, *T. versicolor* showed highest phenoloxidase activity (0.8 U kg^{-1}) at day 30 and the highest FDA activity (53 µg FDA $g^{-1}h^{-1}$) at day 45, and *S. hirsutum* Ru-104 showed the highest cumulative respiration activity (155 mg CO_2 g^{-1}). In general, the three white-rot fungi immobilized in UPS support can be used to improve the atrazine degradation in biomixture of a biopurification system.

Keywords: white-rot fungi, biopurification system, atrazine, biological activities.

5.2 Introduction

The widespread contamination of soil with pesticides every year is a major concern because they can potentially pose a threat against human health as well as against the quality of water and soil (Bastos and Magan, 2009). Atrazine is a chlorinated aromatic herbicide that is used worldwide for the control of broad-leaved weed in agricultural production (Ralebisto et al. 2002) as well as in urban and recreational areas. Inadequate management practices on farms, especially related to the handling of pesticides, appear to be the most important source of contamination. Also, spillage often occurs, particularly during filling spraying equipment. The contamination at these sites is described as a point source of contamination. Due to the environment concerns associated with the accumulation of pesticides in food products and water supplies, there is a need to develop safe and economical methods for pesticide degradation (Zhang and Chiao, 2002).

The biobed is a biopurification system and represent a low-cost technology for degradation of pesticides. They provide a matrix composed of straw, peat and soil that absorbs and facilitates biodegradation of pesticides due to microbial activity, especially white-rot fungi (Castillo et al., 2008).

White-rot fungi have demonstrated high capacity to degrade a wide range of pesticides through their ligninolytic enzymes that are extracellular and nonspecific (Pizzul et al., 2009). Although the precise rol of enzymes in pesticide degradation by white-rot fungi has not been established, evidence suggests that lignin degradation enzyme complex including LiP, MnP and Lac, are responsible at least in part for the degradative capabilities of these fungi (Tortella et al., 2008). Different strains *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* have showed great promise for bioremediation of pesticide polluted matrices (Mougin et al., 1997, Castillo et al., 2001, Bending et al., 2002, Fragoeiro and Magan, 2008).

The application of white-rot fungi in bioaugmentation process has been used mainly through the addition of pre-grown fungal cultures on lignocellulosic supports, due to that these cultures can perform specific biodegradation functions and have demonstrated the ability to survive in presence of other microorganisms in soil (Walter et al., 2005, D´Annibale et al., 2006). Several types of lignocellulosic supports such as sawdust and wheat grains (Lestan and Lamar, 1996, Walter et al., 2005, Rubilar et al., 2011) have been successfully used for the introduction of pre-inoculated white-rot fungi into soil to access and consume specific nutrients, to produce the fungal growth and ligninolytic activities. However, there is no evidence for the use of immobilized white-rot fungi formulated with native strains used on the biomixture. In this context, it is necessary to address this need developing methods to improve the growth and ligninolytic activity of white-rot fungi in a biomixture and thus the pesticide degradation. Therefore, the main objective of this study was to evaluate the use of immobilized white-rot fungi to inoculate a biomixture contaminated with atrazine.

5.3 Materials and Methods

5.3.1 Microorganisms: The fungal strains; *Stereum hirsutum* Ru-104, *Inonotus* sp2, and *Trametes versicolor* were isolated in the south of Chile. The strains were obtained from the Environmental Biotechnology Laboratory at Universidad de La Frontera, Chile. The fungi were transferred from slant cultures to glucose malt extract agar (GMEA) plates (15 g L^{-1} agar, 10 g L^{-1} glucose, 30 g L^{-1} malt extract, pH 5.2) and incubated at 25 ± 1 ºC for 7 days.

5.3.2 Supports formulation: A support formulation including: 74% sawdust, 6% starch, 2% corn meal, 15% flaxseed and 3% lignosulphonate was prepared for fungi immobilization. The components were mixed, moistened to aprox 10% humidity with distilled water and pressed to obtain a pelletized support (PS) of 8 mm using a pellet mill ZLSP300B R-Type.

The fungal immobilization on supports was done as described by Lestan and Lamar (1996), with some modifications. The uncoated pelletized support (UPS) was produced with 10 g of PS transferred to plastic bags moistened with 4 mL of GMEA and inoculated separately with 5 plugs of active mycelium of *S. hirsutum* Ru-104, *T. versicolor* and *Inonotus* sp2 previously grew in GMEA; then, bags were incubated at 25 ±1°C during 30 days.

5.3.3 Biomixture preparation and inoculation: A biomixture was prepared mixing soil, wheat straw, and peat in a proportion of 1:2:1 (% v). The soil (Andisol) was collected between 0 to 15 cm deep and sieved (< 3 mm). Wheat straw was cut into 2 cm pieces and the peat was obtained in a commercial market. The soil has pH of 5.4, 18.6 mg kg^{-1} of available nitrogen, 17.1 mg kg^{-1} of available phosphorous and 12% organic matter. The commercial peat has 33.4% cellulose and 21.9 % lignin. Wheat straw has 9.9% lignin, 41.8% cellulose and 66.1% organic matter, pH 5.9 and 0.56% total nitrogen. The biomixture was stored in a polypropylene bag at 4 ºC until use. The resulting biomixture has pH 4.8, 30% organic carbon and 0.54% total nitrogen (C/N=57) (Diez et al., 2013). The constituents were mixed vigorously to obtain a homogeneous biomixture; then, the

biomixture with 60 % of water holding capacity (WHC) was pre-incubated for 30 days at 20 ± 1°C in a polypropylene bag until use (Fernández et al., 2102). The biomixture (1000 g) was transferred to glass pots and inoculated superficially with 10% (w w^{-1}) of immobilized white-rot fungi (UPS) and were contaminated with 80 mg kg^{-1} of atrazine through sprinkling. Two treatments were prepared: Biomixture + atrazine (B+A); Biomixture + atrazine + UPS inoculum (B+A+F). Each experiment was carried out in triplicate under destructive sampling mode. The biomixtures were incubated at 20 ± 1°C and 60% WHC for 60 days. Phenoloxidase activity, FDA activity and residual atrazine were measured during 60 days. In parallel, respiration activity (CO_2) was evaluated during 60 days.

5.3.4 Pesticide extraction and analytical procedures: To extract atrazine from the samples, 20 mL of methanol were added to 10 g biomixture and incubated for 1 hour at 25°C with shaking (350 rpm). Then, the samples were sonicated at full power for 30 min. The samples were subsequently centrifuged at 10000 rpm and filtered through 0.2 µm PTFS membrane filters. The extraction process was performed twice to obtain the maximum amount of atrazine from the biomixture, and the supernatants were measured by injecting 20 µL into a Merck Hitachi L-2130 pump, a Rheadhyne 7725 injector with µL loop and a Merck Hitachi L-2455 diode array detector. Separation was achieved using a C18 column (Chromolit RP-18e, µmx4.6 mm x 100 mm). Eluent A was ammonium acetate (1 mM) and eluent B was acetonitrile. The flow rate was set at 1.0 mL min^{-1}, with 0-10 min in an isocratic mode. The column temperature was maintained at 30 °C. The detector was set at 222 nm for the data acquisition. Instrument calibrations and quantificactions against pure ATZ reference standars (0.1- mg L^{-1}) (Tortella et al. 2013a). The calculation of Half-life value of atrazine was described using the first-order kinetic equation as $C= C_0 e^{-kt}$, and from the equation, we obtained (Eq. [1]): $t_{1/2}= Ln(2) / k=0.03$

5.3.5 Biological activities: ***Phenoloxidase activity*** was determined in all degradation assays and was performed using MBTH/DMAB (Castillo et al., 1994). Briefly, samples (10 g) of the biomixture

were agitated (150 rpm, 2 hours) with 25 mL of a 100 mM succinate–lactate buffer (pH 4.5). Samples were centrifuged (4000 rpm, 20 min). The supernatant of each sample was collected, filtered through 0.45 μm membrane and measured immediately. The reaction mixture contained 300 μL of 6.6 mM DMAB, 100 μL of 1.4 mM MBTH, 30 μL of 20 mM $MnSO_4$, 1560 μL of the filtered sample and the reaction is initiated with the addition of 10 μL of 10 mM H_2O_2. The reaction is followed in a spectrophotometer Spectronic Genesis 2PC at 590 nm ($\varepsilon = 0.053\ \mu M^{-1}\ cm^{-1}$). Because no correction was made for the possible presence of lignin peroxidase (LiP) and laccase (Lac) activity, this measurement may represent the sum of manganese peroxidase, LiP and Lac (Castillo and Torstensson 2007) and is expressed as phenoloxidase activity. ***Fluorescein diacetate hydrolysis*** (FDA), was performed according to Schnurer and Rosswall (1982). Briefly, 1 g of incubated biomixture was transferred to a flask with 9.9 mL of 0.1 mM sodium phosphate buffer (pH 7.8) and FDA solution (2.0 mg mL^{-1}). After 1 hour of incubation at 25 °C, the reaction was stopped with 10 mL of acetone, and measurements were taken at 490 nm. The results were compared with a calibration curve with standard quantities and expressed in μg FDA $g^{-1}h^{-1}$. ***Respiration activity*** was measured according to of Iannotti et al. (1994). The respiration of biomixture was measured as CO_2 produced and absorbed in a 0.2 M NaOH solution at 20 °C and plotted against time. The values are expressed in mg $CO_2\ g^{-1}$ dry biomixture. All the samples were in triplicate.

5.3.6 Statistical analysis of data: Experiments were conducted using three independent replicates. Data were subjected to a one-way analysis of variance (ANOVA) and the averages were compared by Tukey's range tests.

5.4 Results and Discussion

5.4.1 Fungal growth and biological activities in the biomixture. All immobilized white-rot fungi (UPS) were capable to growth and colonize the biomixture, and the type of colonization was different depending on the fungi *S. Hirsutum* Ru-104 grew over the support as a dense layer and expanded its mycelium over the biomixture. On the other hand, *T. versicolor and Inonotus* sp2 grew over the support similarly without superficial colonization of the biomixture (Figure 5.1). The fungal immobilization allows hyphal to penetrate inside of biomixture reaching contaminants in ways that other microorganisms cannot do. This capacity can increase the phenoloxidase activity in the surrounding environment. Certainly, better colonization could help introduced fungi to overcome competition from indigenous microorganism and enhance degradation of atrazine. This is critical, due microorganisms may occupy components of biomixture and restrain growth and activity of UPS strains inhibiting lignino-cellulose decomposition and reducing enzymes released (Lang et al., 2000). Immobilized white-rot fungi have been historically applied to soil as live microorganisms attached to carrier supports (Baldrian, 2008) to increase the degradation and to protect the fungi from native microflora. Therefore, there is great interest in development of different types of carriers for use in fungal inoculums for biodegradation protocols, and the main carriers are sawdust, corncobs, bark and corn products (Lestan and Lamar, 1996, Pepper et al., 2002, Walter et al., 2004, Rubilar et al., 2011). Another reason to immobilize white-rot fungi is to solve the problems of low nutrient in soil to overcome a large amount of biomass improving colonization, tolerance of environmental stresses and microflora competition.

Figure 5.1. Fungal colonization of UPS in a biomixture contaminated with atrazine after 7 days A) *Inonotus* sp2, B) *T. versicolor*, C) *S. hirsutum* Ru-104.

The atrazine degradation by immobilized fungi in inoculated biomixture was higher than the control non-inoculated after 60 days of incubation (Figure 5.2). In general, the highest degradation of atrazine was obtained when biomixture was inoculated by *S hirsutum* Ru-104 (93%) and the lowest (78%) was in non-inoculated biomixture, after 60 days of incubation.

Bending et al. (2002) investigated the capacity of white-rot fungi to degrade atrazine (20 µg g^{-1}) in a sterile biomixture. The atrazine degradation was; *Coriolus versicolor* 48%, *Hypholoma fasciculare* 38% and *S. hirsutum* 42%, after 42 days of incubation. They concluded that white-rot fungi showed contrasting abilities to access poorly available substrates and the mechanisms involve in degradation are not clearly related to ligninolytic potential compared to the control. Tortella et al., 2013b described the effects of repeated atrazine application (40 mg kg^{-1}) during 90 days on its degradation, microbial communities and enzyme activities in a similar biomixture non-inoculated. They found atrazine removal efficiency was high (96%, 78% and 96%) after each atrazine application respectively and did not found a lag phase.

The half-life ($t_{1/2}$) of atrazine in biomixture inoculated with *S. hirsutum* was higher (20 days) than in control non-inoculated biomixture (28 days); while when *T. versicolor* and *Inonotus* sp2 were inoculated in biomixture the half-life ($t_{1/2}$) was 27 days. However, when *Inonotus* sp2 was inoculated, the degradation of atrazine was slow probably due to the low intensity of colonization and by microbial competition

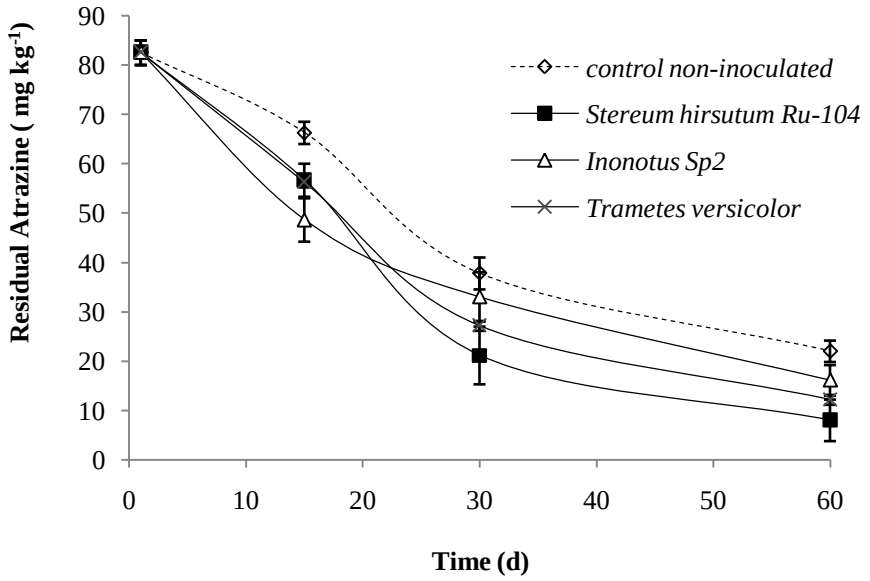

Figure 5.2. Residual atrazine (mg kg^{-1}) after 60 days at 20 °C, in a biomixture inoculated with UPS white-rot fungi.

Our results of $t_{1/2}$ are concordant with Urrutia et al. (2013). The authors studied the degradation of atrazine (100 mg kg^{-1}) in a biomixture non-inoculated composed for soil, oat husk and peat (1:2:1 %v) and found that $t_{1/2}$ of atrazine was 28 days. They concluded that wheat straw can be partial or totally replaced by another lignocellulosic material such as oat husk the biomixture allowing efficient degradation. Tortella et al. (2013a) found that the half-lives ($t_{1/2}$) of atrazine in a similar biomixture (non-inoculated) in the presence of 50 µg kg^{-1} of limonene or eucalyptol (9 and 9 days, respectively)

were significantly lower than the half-life in the control (13 days) without terpens. Our results of half-life ($t_{1/2}$) of atrazine (between 20 to 28 days) were low compared to the behavior of atrazine in soil, this pesticide is moderately persistent in soil and the values reported ranged from 35 to 60 days, depending largely on soil environmental conditions (Pointing, 2001, Rhine et al., 2003).

5.4.2 Determination of biological activities in the biomixtures. The phenoloxidase activity in the biomixture contaminated with atrazine was improved during the first 30 days of incubation by the inoculation of immobilized white-rot fungi with the exception when *Inonotus* sp2 was inoculated (Figure 5.3). The phenoloxidase activities in the control was almost constant (around 0.2 to 0.25 U kg^{-1}) during 30 days decreasing with the incubation time. Phenoloxidase activity was in the range of 0.3 to 0.4 U kg^{-1} during first 30 days when the biomixture was inoculated with *S. hirsutum* Ru-104 and decreasing later with the incubation time The biomixture inoculated with *T. versicolor* increased during the incubation time and the highest phenoloxidase activity (0.8 U kg^{-1}) was obtained at day 30, while the biomixture non-inoculated showed the lowest phenoloxidase activity during all incubation periods. After 30 days of incubation, the phenoloxidase activity in biomixtures inoculated with *T. versicolor* was almost four times respect to the biomixture non-inoculated (0.82 and 0.24 U kg^{-1}, respectively). After 60 days, the phenoloxidase activity in the biomixture decreased in all treatment evaluated. The decrease in phenoloxidase activity in biomixture might be explained by production of phenolic compounds, which occurs during decay of straw (Welch et al., 1990, Urrutia et al., 2013). Tortella et al. (2013b) found similar phenoloxidase activity in the same biomixture (1:2:1 %v) contaminated with atrazine in presence of limonene and eucalyptol, the results were similar in all treatments after 30 days of incubation. They found high values of phenoloxidase activity (between 0.5 and 0.6 U kg^{-1}) when the biomixture was biostimulated with 100 µg kg^{-1} of individual terpenes.

Many researchers have assumed that degradation of pesticides by white-rot fungi is mediated by peroxidases, enzymes that are involved in degradation of lignin. The biomixture used include 50% of wheat straw and this support contains readily available carbon sources and those that are linked to lignin degradation which probably explains why straw is correlated with phenoloxidase and respiration activity (Castillo and Torstensson, 2007). In our study we found a high correlation between phenoloxidase activity and atrazine degradation only when biomixture was inoculated with *T. versicolor*. Castillo et al. (2000) described the enzymatic production and degradation of the herbicide bentazon by *Phanerochaete chrysosporium* growing on straw using a small bioreactor obtaining 0.58 U g^{-1} of manganese peroxidase (MnP) after 40 days of incubation.

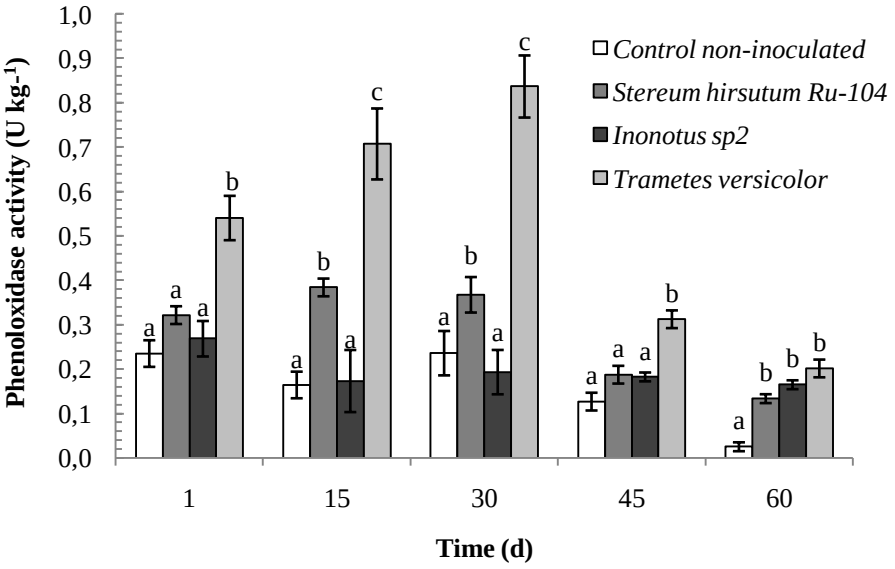

Figure 5.3. Phenoloxidase activity (U kg^{-1}) after 60 days at 20 °C, in a biomixture inoculated with UPS white-rot fungi. Upper letter indicate significance difference (P≤0.05) between immobilized white-rot fungi.

FDA is a test for the rapid estimation of biological potential provides a measure of microbial biomass growth rate by measuring the activity of a number of enzymes (lipases, proteases, esterases), that are produced during fungal growth (Lestan and Lamar, 1996). The fluorescein diacetate activity (FDA) in all biomixture was in the range of 30 and 55 μg $g^{-1}h^{-1}$ during the incubation period indicating that biomixtures inoculated and non-inoculated were biologically active during the incubation time (Figure 5.4). The biomixture inoculated with *T. versicolor* showed the highest FDA activity (53 μg $g^{-1}h^{-1}$) at day 45, while the biomixture non-inoculated showed the lowest FDA activity in all periods demonstrating that inoculation increased the metabolic activity of microorganisms in biomixture. After 45 days, FDA activity in biomixtures inoculated with *T. versicolor, Inonotus* sp2 and *S. hirsutum* Ru-104 showed significant differences ($P \leq 0.05$) compared with the biomixture non-inoculated. After 60 days, FDA activity in the biomixtures decreased for all treatment with values between of 30 and 40 μg $g^{-1}h^{-1}$. Urrutia et al. (2013) found similar values for FDA activity in a biomixture non-inoculated (1:2:1 %v) contaminated with atrazine, where the wheat straw was replaced with barley husk, demonstrating that high microbial activity was obtained by this biomixture. However, they mentioned that a high biological activity is not always associated with pesticide degradation because the microorganisms in the biomixture may not have all the capacity to degrade pesticides. Tortella et al. (2013b) found in a similar biomixture non-inoculated (1:2:1 %v) contaminated with atrazine that FDA activity decreased noticeably in all treatments after 45 days of incubation. They described that this decreasing in FDA activity could be caused by depletion of the readily available carbon sources, including the added terpenes, in the biomixture, based on the behavior of phenoloxidase and FDA activities evaluated.

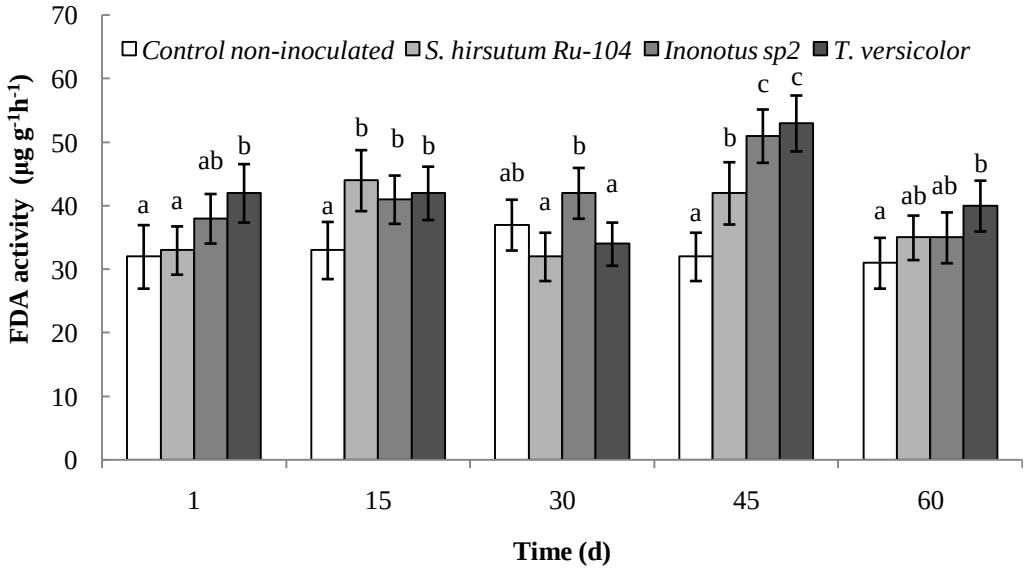

Figure 5.4. FDA activity (µg FDA g^{-1} h^{-1}) after 60 days at 20 °C, in a biomixture inoculated with UPS white-rot fungi. Upper letter indicate significance difference (P≤0.05) between white-rot fungi immobilized.

In this study the respiration activity was used as an indicator of potential microbial activity. This activity was higher in biomixtures inoculated than in non-inoculated biomixture (Figure 5.5). Castillo and Torstensson (2007) established that straw in the biomixture produce the main activity for the dissipation of pesticides containing readily available carbon sources that can contribute to respiration activity but do not necessarily depend on phenoloxidase activity. A high respiration rate (19 mg CO_2 g^{-1} d^{-1}) was observed in biomixture inoculated with *S. hirsutum* Ru-104, followed by biomixture inoculated with *Inonotus* sp2 (17 mg CO_2 g^{-1} d^{-1}). The high respiration rate of biomixture inoculated with *S. hirsutum* Ru-104 could be related with the high atrazine degradation in this biomixture (Figure 5.2) and can be attributed to the presence of easily degradable carbon source for this microorganism. However, low respiration rates were observed for biomixture non-inoculated (16 mg CO_2 $g^{1}d^{-1}$). The cumulative respiration activities in biomixtures inoculated were higher than non-

inoculated biomixtures (Figure 5.5). The cumulative respiration activity during 60 days was the highest (155 mg CO_2 g^{-1}) in the biomixture inoculated with *S. hirsutum*, while the lowest was in the biomixture non-inoculated (99 mg CO_2 g^{-1}). For biomixtures inoculated with *Inonotus* sp2 and *T. versicolor* the cumulative respiration activity was 126 and 108 mg CO_2 g^{-1}, respectively. Urrutia et al. (2013) found similar respiration levels in the biomixtures non-inoculated contaminated with atrazine (100 mg kg^{-1}) where wheat straw was replaced by biomixtures with barley husk and sawdust, where the accumulated respirations after 120 days of incubation were 47 and 42 mg CO_2 g^{-1}, respectively. Their results demonstrated that a high pesticide degradation rate does not always correlate with a high respiration rate likely because an appropriate microbial activity is required to obtain a high degradation efficiency as has been reported by Castillo et al. (2008).

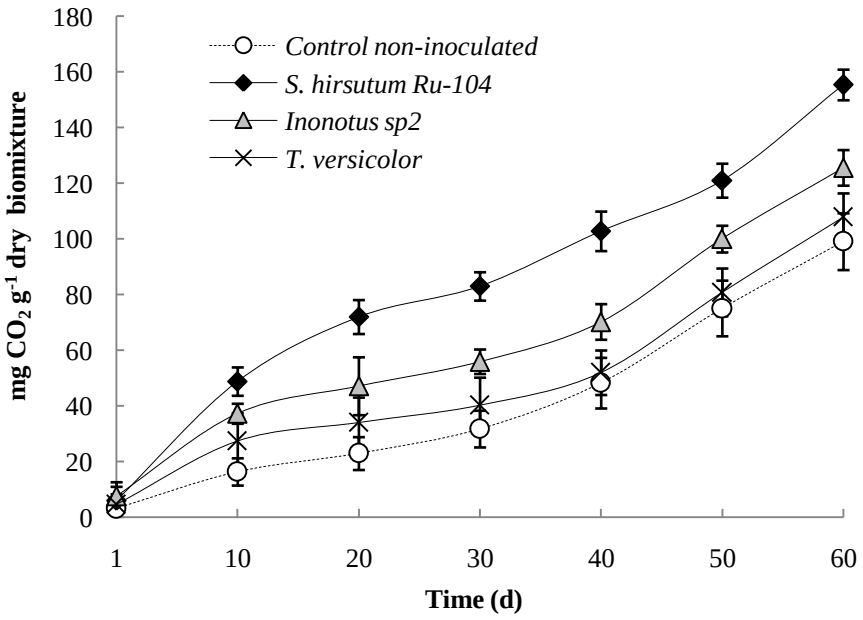

Figure 5.5. Cumulative respiration activity (mg CO_2 g^{-1} biomixture) after 60 days at 20 °C, in a biomixture inoculated with immobilized white-rot fungi. Each value is the mean of three replicates, and the error bars show the standard deviation of the mean.

5.5 Concluding remarks

Some general, promising conclusions may be derived for the results here obtained:

The biomixtures inoculated with fungi immobilized in UPS support increased the degradation of atrazine and biological activities compared with the biomixture non-inoculated. However not all strains evaluated showed the same behavior during atrazine degradation. The strain *S. hirsutum* Ru-104 showed high capacity to degrade atrazine and grow in a biomixture. However, *Trametes versicolor* showed highest phenoloxidase activity (0.8 U kg^{-1}) at day 30 and *S. hirsutum* Ru-104 showed the highest cumulative respiration activity (155 mg CO_2 g^{-1}).

Therefore, UPS white-rot fungi appear as a suitable biological system for the immobilization of microorganisms to be used in a biomixture of a biopurification system.

Acknowledgments: The work was supported by FONDEF VIU 110060 and FONDECYT 1120963 grants.

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CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS AND PROJECTIONS

6. GENERAL DISCUSSION, CONCLUSIONS AND PROJECTIONS

The last years, mechanisms of action of white-rot fungi related to organic pollutants have been studied and have been demonstrated that offer great potential for the use of dye and pesticide degradation in bioremediation processes. The contribution of the highly oxidation potential of a non-specific ligninolytic enzymatic system characteristics of these fungi can be used in the removal of complex mixtures of dye and pesticides.

Different studies have demonstrated the importance of lignocellulosic support for enhancing of ligninolytic enzymes produced by white-rot fungi, Immobilized white-rot fungi have been historically applied to soil as live microorganisms in liquid culture or attached to a carrier material. The immobilization on different supports increases the viability and provides sources of nutrients to increase the degradation of pollutants. Lignocellulosic supports provide an environment similar to the natural habitat of white-rot fungi and can stimulate the secretion of ligninolytic enzymes. Therefore, materials such as sawdust, flaxseed meal, and wheat straw have been used as economical source of nutrients to formulate supports for fungi immobilization.

Textile wastewater contains a mixture of different aromatic dyes which are difficult to degrade. Several synthetic dyes are resistant to microbial degradation and cannot be removed easily in conventional wastewater treatment systems. Shortcomings of both physicochemical and biological methods limit their large-scale application in dye decolorization. Great interest is shown by white-rot fungi for biodegradation of these recalcitrant compounds. The isolation of new strains from the rainforest of South of Chile such as *Anthracophyllum discolor*, *Stereum hirsutum* Ru-104 or *Inonotus* sp will probably increase the efficacy of dye degradation in the near future. There is a need for

including new methodologies for immobilization of white-rot fungi in elucidating the chemical structures of metabolites produced by the ligninolytic enzymes. We studied different form for fungal immobilization and we demonstrated that *A. discolor* was able to degrade dyes efficiently when immobilized as complex and coated pellets. Besides, the production of manganese peroxidase increased when the fungus was immobilized.

Currently the knowledge on white-rot fungi is derived primarily from studies on individual microorganism degrading individual pesticides. It is important to describe that the metabolisms of microorganism during the degradation of a pesticide leads to the formation of metabolic products, and these products are broken down by other microorganism in the biopurification system, thus leading to partial or complete transformation of the contaminant.

The immobilization on different supports increased the viability of fungal mycelium through the time to improve the competition with native microorganisms during the colonization in the biomixture. One of the main support tested was flaxseed meal used as material for *A. discolor* immobilization this material allowed a greater growth and peroxidase activities (MnP, MiP, LiP) compared to other materials evaluated (starch, corn meal and sawdust). Considering the proportion of constituents of the support influenced the ligninolytic potential, we formulated 3 supports to immobilize *A. discolor* and othrer fungi. The formulation (F1), including a high proportion of flaxseed meal (15%) presented the best condition for growing and ligninolytic enzymes productions by *A. discolor*. On the other hand, the uncoated pelletized support (UPS-F1) demonstrated better condition to immobilized fungus than coated pelletized support (CPS-F1). Nevertheless, the other strains immobilized showed different behavior respect to de atrazine degradation and biological activities in the biomixture.

Therefore, adding a suitable fungus as *A. discolor through* the biomixture enrichment techniques as immobilization on suitable supports has resulted in the degradation of pesticides such as atrazine.

The conclusions of this study:

- The use of immobilized white-rot fungi to remediate polluted environments is a promising technology, demonstrating an important reduction in bioavailability and toxicity of organic pollutants. In literature, several types of support for immobilization have been used successfully for degradation of persistent organic pollutants and dyes.
- Six strains of white-rot fungi isolated from southern Chile were evaluated for their ergosterol/biomass correlation and ligninolytic potential in solid medium to formulate pellets for reactive orange 165 (RO165) decolorization. The fungus *A. discolor* was selected to formulate complex pellets (fungal mycelium, sawdust, and activated carbon), coated pellets (complex pellet + alginate) and simple pellets (fungal mycelium).
- Complex pellets of *A. discolor* showed a higher enzymatic production mainly MnP (38 U L^{-1} at day 15) compared to coated and simple pellets in liquid medium. Examinations using SEM showed that both pellets produced a black core that was entrapped by a layer of fungal mycelium. Decolorization of RO165 was demonstrated with all the pellets formulated. However, the highest and fastest decolorization was obtained with complex pellets (100% at day 8).
- The white-rot fungus *A. discolor* showed high fungal growth and peroxidases activities (MnP, MiP) in lignocellulosic supports tested especially for flaxseed meal. After all evaluations the formulation UPS-F1 was selected to inoculate a biomixture. The results showed that the highest enzymatic activity was MnP for F1, F2 and, F3 supports and the lowest was Lac. The atrazine degradation and phenoloxidase activity were higher in the biomixture inoculated with UPS-F1 than

in non inoculated biomixture. Finally, the DGGE analysis showed that no negative effect on native microbial population was observed when UPS-F1 was inoculated in the biomixture, especially after 30 days of atrazine degradation.

- The biomixtures inoculated with fungi immobilized in UPS support increased the degradation of atrazine and biological activities compared with the biomixture non-inoculated. However not all strains evaluated showed the same behavior during atrazine degradation. The strain *S. hirsutum* Ru-104 showed high capacity to degrade atrazine and grow in a biomixture. However, *T. versicolor* showed highest phenoloxidase activity (0.8 U kg^{-1}) at day 30 and *S. hirsutum* Ru-104 showed the highest cumulative respiration activity (155 mg CO_2 g^{-1}).
- Therefore, UPS white-rot fungi appear as a suitable biological system for the immobilization of microorganisms to be used in a biomixture of a biopurification system.

PROJECTIONS

The time is ripe to improve the scientific knowledge to design new strategies to scale-up commercially and viable fungal bioremediation technology. White-rot fungi can degrade a wide range of pollutants including pesticides their capacity can be used to decrase the environmental contamination in soil or water. Currently, little is known on the isolation of specific white-rot fungi has the capacity to degrade different types of pesticides and continuous efforts are required in this direction. Due to the versatility of this biological system, possibilities exist for the commercial use of immobilized fungi for the degradation of pesticide.

This research was applied to the national council of research, FONDEF VIU 2012, to obtain sources to develop a company BIOLUTION Ltda for environmental solutions, using the results obtained in this doctoral thesis. The total project was estimated in US$ 50.000. The results obtained in this

doctoral thesis, were protected through a patent and a scientific brand. Finally, is necessary to look for new opportunities to improve our knowledge and we are under the support of CORFO and INCUBATEC-UFRO.

Annex I

1ER CONCURSO VIU FONDEF

INFORME DE EVALUACIÓN DE LA ETAPA 1

Código VIU110060	**Título del Proyecto**: Estudio de pre-factibilidad para el establecimiento de Biolution, una planta productora de inóculos fúngicos aplicables a procesos de bioremediación de sitios contaminados.
Alumno(a) Jefe de Proyecto:	Sebastian Andrés Elqueta Palma
Profesor(a) Guía:	María Cristina Diez Jeréz
Institución:	Universidad de La Frontera
Carrera o Programa:	Doctorado en ciencias de recursos naturales
Grado:	Doctorado
Monto Solicitado a FONDEF:	$20.000.000
Monto Aportado 3eros:	$ 4.000.000
Costo Total del Proyecto:	$24.000.000
Duración del Proyecto:	10 Meses

PLAN DE NEGOCIOS:

El producto es un inoculo fúngico aplicables a procesos de bioremediación de sitios contaminados. El formato corresponde a un pellet cilíndrico. Permite la remediación y descontaminación derivados de actividades industriales. El modelo de negocios considera desde la fabricación, hasta la venta de los inóculos. El mercado potencial son todas las plantas industriales que tienen desechos contaminantes para el medio ambiente entre ellas la industria; minería, química, pinturas, celulosa y petróleo que representan un 66,5% de los residuos peligrosos generados en Chile. El valor estimado del emprendimiento es bajo. Los emprendedores consideraron parámetros de estimación conservadores. Existe una adecuada estimación de los ingresos y egresos. Las ventajas del producto han sido descritas. Sin embargo, será fundamental validar las ventajas de los inóculos en condiciones de desempeño real, en particular, deben considerar el tipo de suelo y/o cultivos en los cuales se pretendan llevar adelante las validaciones y pruebas.

PLAN DE TRABAJO:

Plan de trabajo ambicioso. Se recomienda focalizar las actividades hacia la formación de la empresa y la validación de las ventajas tecnológicas de los inóculos para atraer a nuevos inversionistas y clientes. El estudio de PI, lo podría aportar la Universidad.

CONVENIO:

Si bien se establece una licencia exclusiva por 10 años, el tema de la titularidad de la Propiedad Industrial, en particular de los resultados de patentes que se obtengan durante la etapa 2 esta debe estar destinada exclusivamente a la empresa que se cree a partir de este proyecto.

PUNTAJE DEL PROYECTO: 3,6 RECOMENDACIÓN: APROBAR

RECOMENDACIONES A ABORDAR DURANTE LA EJECUCIÓN DEL PLAN DE TRABAJO:

El valor estimado del negocio por los emprendedores es bajo, el modelo de negocios no refleja la manera cómo lograr implementar el negocio en una primera etapa. Esto implica, además que la estrategia comercial debe ser reformulada y con su foco puesto en un mercado de prueba atractivo.

Final resolution FONDEF VIU 2012, the project was approved to create BIOLUTION Ltda., and for the protection of doctoral thesis results.

Annex I

DIARIO OFICIAL
DE LA REPUBLICA DE CHILE

Núm. 40.404 | Jueves, 08 de noviembre de 2012 | E-132 Pág. 1 de 1

SECCIÓN SOCIEDADES

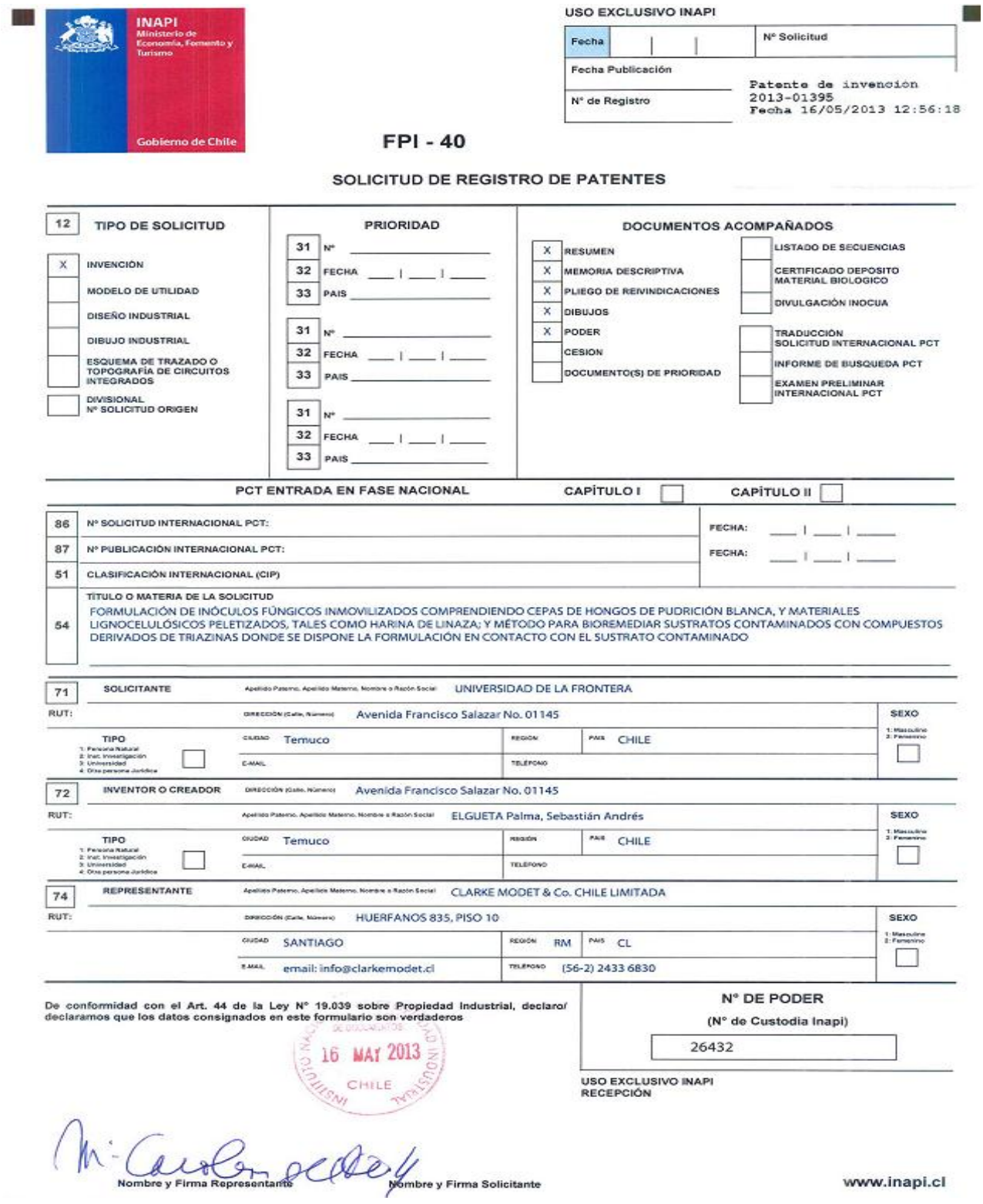
CONSTITUCIONES SOCIEDADES DE RESPONSABILIDAD LIMITADA

559116 *ELGUETA Y DIEZ SERVICIOS PROFESIONALES AMBIENTALES LIMITADA.*

JORGE ELIAS TADRES HALES, Notario Temuco, con oficio en esta ciudad calle A. Varas 976, CERTIFICA: Por escritura de fecha 24 de Octubre de 2012, ante mí, doña MARÍA CRISTINA DIEZ JEREZ, domiciliada en kilómetro doce camino Temuco a Labranza; y don SEBASTIAN ANDRÉS ELGUETA PALMA, con domicilio en calle La Estancia veintinueve, Altos de Maipo, de la ciudad de Temuco; constituyeron una sociedad de responsabilidad limitada. RAZON SOCIAL: **ELGUETA Y DIEZ SERVICIOS PROFESIONALES AMBIENTALES LIMITADA**, nombre fantasía: "BIOLUTION LTDA.". OBJETO: La prestación de servicios profesionales, de investigación y análisis científica y tecnológica en áreas de competencias profesionales medioambientales, tales como la venta de servicios, capacitaciones, evaluaciones de riesgo, monitoreos, supervisión, evaluaciones, modelaciones, estudios, mediciones, recolección de datos, instalaciones, ejecución de acuerdos, seguimientos financieros, administrativos y legales, inversiones, exploraciones, explotación y comercialización, pudiendo en el desempeño del giro participar en toda clase de concursos públicos o licitaciones, además de constituir filiales, constituir y formar parte de otras instituciones, sean de derecho público o privado, corporaciones, fundaciones y sociedades civiles y comerciales y tener participación en ellas como accionista o prestador de servicios, en cualquier proporción o cantidad. ADMINISTRACIÓN Y USO RAZON SOCIAL: Corresponderá a ambos socios, quienes actuando por la sociedad y anteponiendo a su firma la obligarán y representarán en todos los actos relativos a su objeto o giro ordinario, y en especial y además, en la celebración de los actos y contratos necesarios para el funcionamiento de su actividades, tanto comerciales como civiles, no siendo necesario acreditar frente a terceros si ellos corresponden o no al giro social, asumiendo obligaciones que afecten a la sociedad frente instituciones privadas o públicas, siendo don SEBASTIAN ANDRÉS ELGUETA PALMA el socio que represente a la sociedad frente a terceros, sin perjuicio de la comparecencia de todos los socios para obligar a la sociedad. CAPITAL: QUINIENTOS MIL PESOS, que se entera y paga aportando cada socio la suma de doscientos cincuenta mil pesos, en dinero efectivo y ya ingresado a la caja social. DURACIÓN: Empezará a regir desde fecha escritura y tendrá una duración de cinco años, contado de la fecha de escritura, renovable tácita y sucesivamente por periodos iguales forma estipulada en escritura. DOMICILIO: Temuco, sin perjuicio de las agencias o sucursales que puedan establecerse en el resto del país. Demás estipulaciones constan en escritura extractada. Temuco, 06 de Noviembre de 2012.

Biolution Ltda.

Annex I



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Ministerio de Economía, Fomento y Turismo
Gobierno de Chile

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Fecha Publicación

Nº de Registro

Patente de invencion
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SOLICITUD DE REGISTRO DE PATENTES

12 TIPO DE SOLICITUD

- [X] INVENCIÓN
- [ ] MODELO DE UTILIDAD
- [ ] DISEÑO INDUSTRIAL
- [ ] DIBUJO INDUSTRIAL
- [ ] ESQUEMA DE TRAZADO O TOPOGRAFÍA DE CIRCUITOS INTEGRADOS
- [ ] DIVISIONAL Nº SOLICITUD ORIGEN

PRIORIDAD

31 Nº
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DOCUMENTOS ACOMPAÑADOS

- [X] RESUMEN
- [X] MEMORIA DESCRIPTIVA
- [X] PLIEGO DE REIVINDICACIONES
- [X] DIBUJOS
- [X] PODER
- [ ] CESION
- [ ] DOCUMENTO(S) DE PRIORIDAD
- [ ] LISTADO DE SECUENCIAS
- [ ] CERTIFICADO DEPOSITO MATERIAL BIOLOGICO
- [ ] DIVULGACIÓN INOCUA
- [ ] TRADUCCIÓN SOLICITUD INTERNACIONAL PCT
- [ ] INFORME DE BUSQUEDA PCT
- [ ] EXAMEN PRELIMINAR INTERNACIONAL PCT

PCT ENTRADA EN FASE NACIONAL CAPÍTULO I [ ] CAPÍTULO II [ ]

86 Nº SOLICITUD INTERNACIONAL PCT: FECHA: ___ | ___ | ____
87 Nº PUBLICACIÓN INTERNACIONAL PCT: FECHA: ___ | ___ | ____
51 CLASIFICACIÓN INTERNACIONAL (CIP)

54 TITULO O MATERIA DE LA SOLICITUD
FORMULACIÓN DE INÓCULOS FÚNGICOS INMOVILIZADOS COMPRENDIENDO CEPAS DE HONGOS DE PUDRICIÓN BLANCA, Y MATERIALES LIGNOCELULÓSICOS PELETIZADOS, TALES COMO HARINA DE LINAZA; Y MÉTODO PARA BIOREMEDIAR SUSTRATOS CONTAMINADOS CON COMPUESTOS DERIVADOS DE TRIAZINAS DONDE SE DISPONE LA FORMULACIÓN EN CONTACTO CON EL SUSTRATO CONTAMINADO

71 SOLICITANTE — Apellido Paterno, Apellido Materno, Nombre o Razón Social: UNIVERSIDAD DE LA FRONTERA
RUT:
DIRECCIÓN (Calle, Número): Avenida Francisco Salazar No. 01145
CIUDAD: Temuco | REGION | PAIS: CHILE
E-MAIL | TELÉFONO
TIPO (1: Persona Natural, 2: Inst. Investigación, 3: Universidad, 4: Otra persona Jurídica) [ ]
SEXO (1: Masculino, 2: Femenino) [ ]

72 INVENTOR O CREADOR — DIRECCIÓN (Calle, Número): Avenida Francisco Salazar No. 01145
RUT:
Apellido Paterno, Apellido Materno, Nombre o Razón Social: ELGUETA Palma, Sebastián Andrés
CIUDAD: Temuco | REGIÓN | PAIS: CHILE
E-MAIL | TELÉFONO
TIPO (1: Persona Natural, 2: Inst. Investigación, 3: Universidad, 4: Otra persona Jurídica) [ ]
SEXO (1: Masculino, 2: Femenino) [ ]

74 REPRESENTANTE — Apellido Paterno, Apellido Materno, Nombre o Razón Social: CLARKE MODET & Co. CHILE LIMITADA
RUT:
DIRECCIÓN (Calle, Número): HUERFANOS 835, PISO 10
CIUDAD: SANTIAGO | REGIÓN: RM | PAIS: CL
E-MAIL: email: info@clarkemodet.cl | TELÉFONO: (56-2) 2433 6830
SEXO (1: Masculino, 2: Femenino) [ ]

De conformidad con el Art. 44 de la Ley N° 19.039 sobre Propiedad Industrial, declaro/ declaramos que los datos consignados en este formulario son verdaderos

INSTITUTO NACIONAL DE PROPIEDAD INDUSTRIAL DE CHILE
16 MAY 2013

Nº DE PODER
(Nº de Custodia Inapi)
26432

USO EXCLUSIVO INAPI
RECEPCIÓN

Nombre y Firma Representante

Nombre y Firma Solicitante

www.inapi.cl

Patent request 2013-1395, INAPI, Chile.

Annex I

INAPI
Ministerio de Economía, Fomento y Turismo
Gobierno de Chile

Clarke, Modet & Co
CHILE

USO EXCLUSIVO INAPI

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SOLICITUD DE REGISTRO O RENOVACIÓN DE MARCAS SERVICIOS

SOLICITANTE

Apellidos y Nombres o Razón Social		RUT	
UNIVERSIDAD DE LA FRONTERA		RUT	87.912.900-1
		RUT	
Domicilio	AVDA. FRANCISCO SALAZAR 01145	Fono	
Ciudad: TEMUCO	Comuna:	País: CHILE	E-Mail:

REPRESENTANTE

Apellidos y Nombres	CLARKE MODET & CO. CHILE	RUT	78.773.320-4
Domicilio	HUÉRFANOS 835, PISO 10		
Ciudad: SANTIAGO	Comuna: SANTIAGO	País: CHILE	E-Mail: INFO@CLARKEMODET.CL

MARCA / DENOMINACIÓN SOLICITADA Palabra o Denominación [X] Etiqueta [ ] Mixta [ ] Sonora [ ]

BIOLUTION

DESCRIPCIÓN DE ETIQUETA (OBLIGATORIO)

PEGAR ETIQUETA (Máx. 7x7 Cm)

La imagen debe inscribirse en el área definida.

PUBLICAR DESCRIPCIÓN (Marque opción) SI [ ] NO [ ]

EN ESTE CUADRO INDICAR CON (X) LA/S CLASE/S DE SERVICIO/S

35	36	37	38	39	40	41	42	43	44	45
							X			

DE LA CLASE 42 **ESPECIFICAR SERVICIO/S**

SERVICIOS CIENTÍFICOS Y TECNOLÓGICOS, ASÍ COMO SERVICIOS DE INVESTIGACIÓN Y DISEÑO EN ESTOS ÁMBITOS; SERVICIOS DE ANÁLISIS E INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA EN EL ÁREA DE MEDIOAMBIENTAL.

CANTIDAD DE ANEXOS QUE SE ADJUNTAN PARA LA ENUMERACIÓN DE PRODUCTOS A PROTEGER [ ]

SI SE SOLICITA RENOVACIÓN
N° de Registro a Renovar

N° DE PODER
(N° de Custodia Inapi)

PRIORIDAD

País	
Fecha	
N°	

Brand BIOLUTION.

Annex I

Temuco, 21 Junio de 2013

Sr. Sebastián Andrés Elgueta Palma
Representante Biolution Ltda.
Presente.-

El motivo de la presente el comunicar a usted los resultados de su postulación al programa "**LEVANTAMIENTO DE OPORTUNIDADES DE INVERSIÓN Y FINANCIAMIENTO, DEL PROGRAMA MULTISECTORIAL DE LA PROMOCION Y ATRACCIÓN DE INVERSIONES PARA LA REGIÓN DE LA ARAUCANIA"**, llamado realizado entre el 22 de Marzo al 17 de Mayo de 2013.

De un universo de 140 proyectos postulados al programa los que fueron evaluados con una nota entre 1 a 5 (siendo 1 nota mínima), acorde a los criterios establecidos con sus respectivas ponderaciones, esto es:

Criterio de Evaluación	Ponderador
Oportunidad de Mercado	30%
Modelo de Negocios	25%
Equipo Emprendedor/ de Gestión	30%
Línea de Ingresos (Plan comercial)	15%
	100%

El resultado del puntaje del proyecto "Escalamiento venta de lechos biológicos para degradar peticidas" fue de 3,12. Por lo que usted ha sido SELECCIONADO para participar de una asesoría para la preparación de book de negocio, capacitación para presentar ante inversionistas y participación de una ronda de inversión.

Para continuar con el proceso un ejecutivo de proyectos asignado se pondrá en contacto con usted en un plazo de 7 días hábiles o deberá ponerse en contacto con el ASESOR al teléfono 2746242 o en la dirección Antonio Varas 854 Of. 1001, Temuco.

Sin otro particular, se despide atte.
Equipo evaluador Incubatec-Ufro

UNIVERSIDAD DE LA FRONTERA incubatecufro TEMUCO

Bulnes 837 Oficina 14, Temuco, Chile | 045 94 48 50 | **www.corfo.cl**

IMAGINACHILE 2013 Año de la Innovación

Support of CORFO Chile and INCUBATEC UFRO.

ANNEX II

COMPLEX PELLETS

Decolorization of RO165 by complex and coated pellets of *A. discolor* after 11 days at 25 °C.

Annex III

Pelletization of supports

Pelletization of supports in pellet mill ZLSP300B R-Type

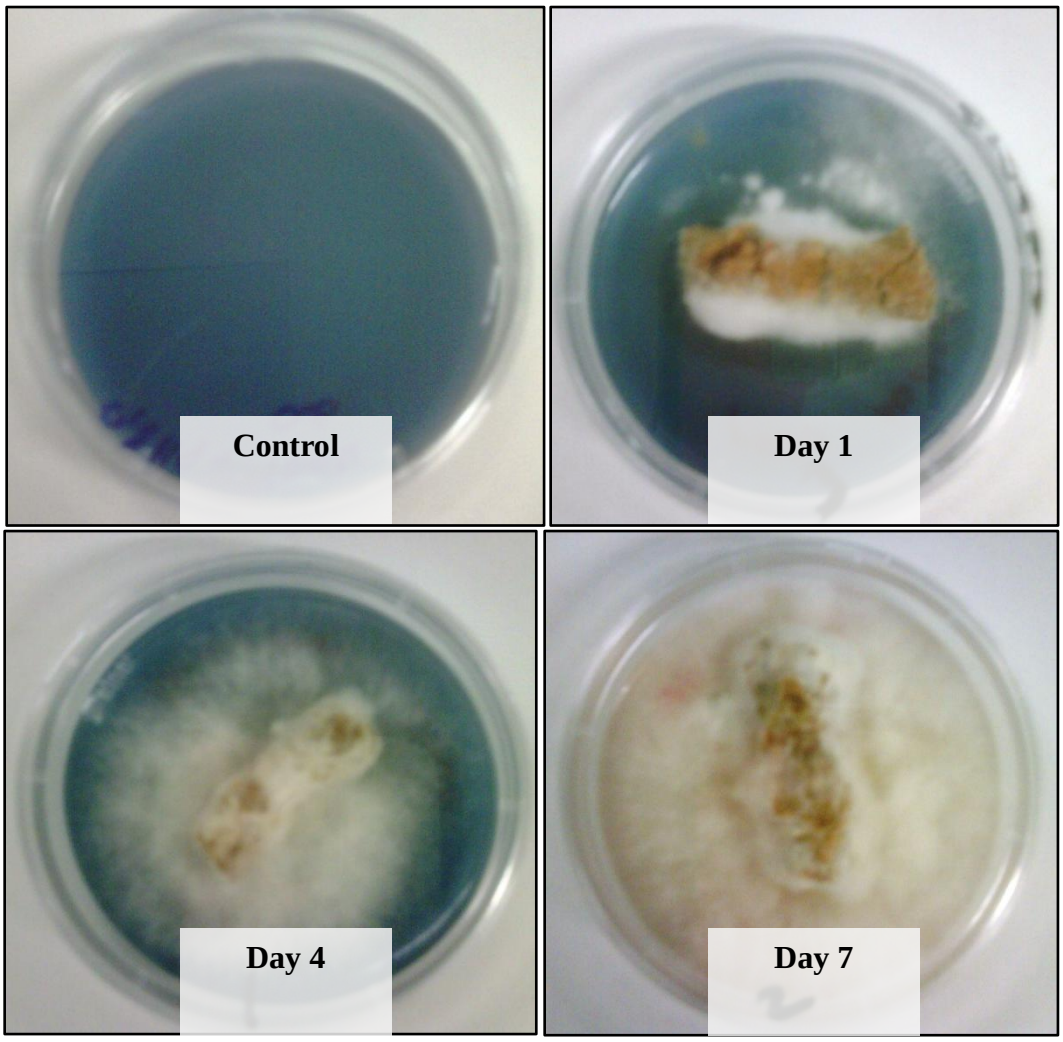
Annex III

Uncoated Pelletized Support UPS

Fungal growth UPS *A. discolor* in plastic bags during 30 days of incubation at 25°C.

Annex III

UPS *A. discolor* used for dye degradation



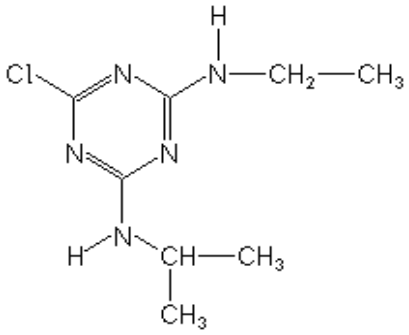
Decolorization of 100 mg L^{-1} by UPS-F1 *A. discolor* after 7 days of incubation.

Annex III

Inoculation in the biomixture

Fungal colonization of UPS *A. discolor* after 7 days in a biomixture contaminated with atrazine.

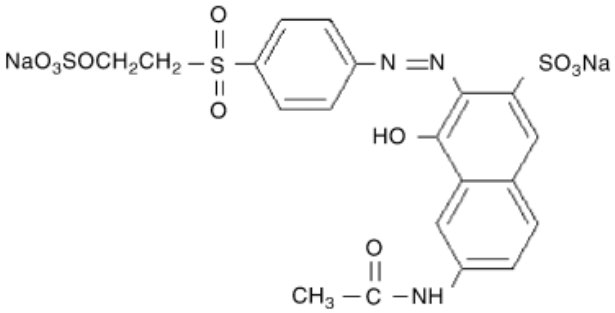
Fungal colonization of CPS *A. discolor* after 7 days in a biomixture contaminated with atrazine.



Atrazine

Compuesto	Peso molecular (g mol^{-1})	pKa	Solubilidad (mg L^{-1})	Log K_{ow}
Atrazina	215	1.6-1.7	33	2.2
Hidroxiatrazina	197	4.9-5.2	5.9	1.4
Deetilatrazina	187	1.3-1.7	3200	1.5
Deisopropilatrazina	173	1.3-1.5	670	1.1

Fuente: Tomlin, 1995 obtained from doctoral thesis Gabriela Briceño, 2009.



Reactive Orange 16

Chemical formula $C_{20}H_{17}N_3Na_2O_{11}S_3$ Molar mass 617.54

Color index number 17757 λ max (nm) 494

Use for cotton or viscose fiber dyeing. http://www.worlddyevariety.com