Potential bioremediation role of manglicolous fungi associated with the mangrove tree, *Rhizophora mangle*, in Puerto Rico

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ABSTRACT

Mangroves are tropical ecosystems that are constantly exposed to unknown amounts of different pollutants from motorboats such as gasoline or diesel. They harbor a group of fungi called "manglicolous fungi", able to synthesize all the necessary enzymes to degrade lignin. Lignolytic fungi produce extracellular enzymes with very low substrate specificity, being fit for degradation of different compounds. Several lignolytic fungi are known to degrade PAHs due to the irregular structure of lignin. Since lignolytic enzymes have low substrate specificity, compounds with similar structure to lignin might induce the production of such enzymes. The main objective of this study was to establish the cability of some fungal isolates from *Rhizophora mangrove* wood to use other carbon and energy sources, especially xenobiotics such as Congo Red or naphthalene through the lignin degradation pathway. For this study two sampling areas were selected: Bahía Salinas, Cabo Rojo and La Parguera, Lajas. A total of 20 fungi were isolated. Three isolates from each site were picked for experiments using Congo Red and naphthalene as their only carbon and energy source. Samples *Penicillium citrinum* (RmBS 2-1-2) and *Aspergillus caelatus* (IRmPL 5) showed a better ability of using Congo Red, while *Fusarium solani* (RmBS 3) and *Purpureocillium lilacinum* (RmPL 5-1e-2) showed a better ability of using naphthalene. These the three isolates were tested for laccase, Mn peroxidase and Li peroxidase activity in the supernatant. All of the isolates showed activity when grown in basal media with Congo Red or naphthalene. This study supports the hypothesis of fungal isolates from *Rhizophora mangle* wood using Congo Red and naphthalene as carbon and energy source other than lignin.

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RESUMEN

Los mangles son ecosistemas tropicales que están constantemente expuestos a cantidades desconocidas de distintos contaminantes de botes de motor como lo son la gasolina o el diésel. Albergan un grupo de hongos llamados "hongos manglicolos", capaces de sintetizar las enzimas necesarias para degradar lignina. Los hongos lignolíticos producen enzimas extracelulares con baja especificidad de sustrato, adecuados para la degradación de diferentes compuestos. Varios hongos lignolíticos se conocen por ser capaces de degradar hidrocarburos policíclicos aromáticos debido a la estructura irregular de la lignina. Debido a que las enzimas lignolíticas tienen baja especificidad los compuestos con estructuras similares a la de lignina podrían inducir la producción de esas enzimas. El objetivo principal de este estudio era establecer la habilidad de algunos hongos aislados de la madera del mangle rojo, *Rhizophora mangle*, de utilizar otra fuente de carbono y energía, en especial xenobióticos como el colorante Congo Red o naftaleno por medio del mecanismo de degradación de lignina. Para este estudio dos áreas de muestreo fueron seleccionados: Bahía Salinas en Cabo Rojo y La Parguera en Lajas. Un total de 20 hongos fueron aislados. De cada área de muestreo se seleccionaron 3 muestras para experimentos usando Congo Red y naftaleno como su única fuente de carbón y energía. Las muestras *Penicillium citrinum* (RmBS 2-1-2) y *Aspergillus caelatus* (IRmPL 5) mostraron mejor habilidad de utilizar el Congo Red, mientras que *Fusarium solani* (RmBS 3) y *Purpureocillium lilacinum* (RmPL 5-1e-2) mostraron mejor habilidad de utilizar el naftaleno. A los cuatro hongos se les realizó una prueba de actividad enzimática para las enzimas lacasa, manganeso peroxidasa y lignina peroxidasa. Los cuatro hongos mostraron actividad enzimática. Este estudio apoya la hipótesis sobre los hongos aislados de la madera del mangle rojo, *Rhizophora mangle*, y su capacidad de utilizar Congo Red o naftaleno como fuentes alternas de carbono y energía a la lignina.

I dedicate this work to the ones that believed in me and witness all this process. To my love ones, my mother Emilia Castro and my father Carlos Kelly for guiding me. As well to Carlos J. Bernal, my love, for always believing in me and giving me the strength and motivation to complete this journey.

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1 General Overview

1.1 Introduction

Mangroves are complex ecosystems found in tropical and subtropical coastlines around the world in the same range of latitudes near the equator. There are 80 different species of mangrove trees growing in areas of low-oxygen soil, where slow-moving waters allow fine sediments to accumulate (US Department of Commerce 2014). Mangrove ecosystems in Puerto Rico play a large ecological role by stabilizing the coastline, reducing erosion from storm surges, currents (US Department of Commerce 2014), waves, and tides (Pérez Rivera 2006). In addition, these trees provide shelter for vertebrate and invertebrate marine species and harbor a wide range of microorganisms such as bacteria and fungi (US EPA 2015). Microorganisms have different ecological roles, which include pathogenic and protector species of mangrove trees (US Department of Commerce 2014). On the down side, mangrove forests are highly vulnerable to pollution and can be further damaged by many types of cleanup activities (US Department of Commerce 2014). Conventional cleanup methods include removal, alteration or isolation of the pollutant, but these techniques can be expensive and in many cases transfer the pollutant from one phase to another (Haritash and Kaushik 2009). Oil spills are an example of pollution that have been occurring for decades around the world and have a negative effect on mangrove ecosystems. Unfortunately, the south coast of Puerto Rico has been affected by two major oil tanker spills of crude oil: the Argea Prima in 1962, which released 69,000 bbl, the Zoe Colocotronis in 1973, which released 24,000 bbl (Corredor et al. 1990, Hoff et al. 2002), and the Mystery Spill in August 29, 2007, were approximately 45,000 gallons of crude oil released (Fallis 2013). Another

source of contamination was the petrochemical complex, thermoelectric power plant and transshipment port abandoned 20 years ago located in Guayanilla Bay.

Mangrove forests are constantly exposed to unknown amounts of different pollutants from motorboats such as gasoline or diesel. The incomplete combustion of diesel results in polycyclic aromatic hydrocarbons (PAHs) compounds (Szewczyńska et al. 2013), which are toxic, mutagenic and carcinogenic ubiquitous contaminants (International Agency for Research on Cancer 1983, Haritash and Kaushik 2009). These compounds accumulate in the sediments of the aquatic environments because of their low solubility and hydrophobic nature (Ranjan et al. 2012) and due to their persistence it is difficult to eliminate them (US EPA, OSWER 2008). As the molecular weight increases so does the persistence of the PAHs (Haritash and Kaushik 2009).

In a study conducted by Klekowski et al. (1994), they documented that mangrove trees, such as *Rhizophora mangle*, undergo tissue mutagenesis after being chronically expose to xenobiotics, which are foreign chemical substances, such as PAHs. Exposure to PAHs causes them to produce unfertile propagules (Klekowski et al. 1994), which are seed-like structures that detach from the plant and give rise to a new one. Unfertile propagules could then be a problem for the restoration or regeneration of mangrove forests.

The process of degradation of PAHs can take weeks to months. Investigations of the stability of PAHs in soil or sewage found that half-lives of PAHs were in the range of 3-3,111 days (Włodarczyk-Makuła 2012). They can undergo chemical oxidation, photolysis, bioaccumulation, volatilization and absorption, but microbial degradation is the primary process (US Department of Health and Human Services 1995, Li et al. 2008). To solve the problem of polluted marine ecosystems, a variety of physical techniques emerged, but there

is evidence that these techniques can also cause damage to the associated biota and prevent the restoration of certain habitats, including mangrove ecosystems (Olguín et al. 2007). For example, pressure washing can drive oil deeper into the beach killing the organisms that live there (US EPA 1993). This is why bioremediation techniques such as biostimulation, bioaugmentation and bioattenuation emerge to avoid more damage and accelerate natural processes. In **biostimulation** nutrients are added to the environment to stimulate *in-situ* microorganisms capable of degrading pollutants whereas in **bioaugmentation** microorganisms are added to the environment to serve as degraders. **Bioattenuation** is a method which relies in natural processes to dissipate the contaminants through biological transformation (Mrozik and Piotrowska-Seget 2010).

To take advantage of the microorganisms in mangrove ecosystems we must first know who they are and what role or functions they have. Mangrove ecosystems harbor a group of fungi called "manglicolous fungi", able to synthesize all the necessary enzymes to degrade lignin, cellulose and other plant components (Sahoo and Dhal 2009). Lignocellulosic substrates in the marine environment, particularly mangrove wood, support a diverse mycobiota (Sarma and Hyde 2001). Lignolytic enzymes produced by manglicolous fungi have a low specificity for substrates, meaning that they are suitable for degradation of different compounds (Haritash and Kaushik 2009). Since lignin and pollutants such as PAHs have similar chemical structures these enzymes can degrade both. Studies have shown that ligninolytic fungi represent a promising alternative for biodegradation of various aromatic pollutants (Čvančarová et al. 2012).

Most available reports on manglicolous fungi are from the Pacific and Indian Oceans (Sarma and Hyde 2001), and little is known about them in the Atlantic Ocean or the

Caribbean, especially for mangroves in Puerto Rico. Although, similar manglicolous fungal community is expected due to equivalent tropical environmental conditions between Puerto Rico and the Pacific and Indian oceans. The purpose of this project was to identify manglicolous fungi associated with *Rhizophora mangle* wood in Puerto Rico that may be able to produce lignolytic enzymes. Since PAHs are persistent in the environment these enzymes could be applied to aid in the degradation process of these compounds. Various areas in Puerto Rico exposed to PAHs contamination could benefit from bioremediation using lignolytic enzymes secreted by manglicolous fungi. This information could be used to design projects of biostimulation in mangrove ecosystems of Puerto Rico that have been exposed to these contaminants.

1.2. Literature Review

1.2.1. Manglicolous Fungi

Mangrove ecosystems are forests composed of specialized trees called mangroves with particular adaptations to survive in high salinity environments(Department of Natural and Environmental Resources 2009). Those adaptations give trees the advantage of resisting high concentrations of salt. Mangroves are found around the shores of tropical and subtropical regions serving as barriers to protect the coastlines and also as home to diverse groups of vertebrates, invertebrates and microorganisms. Mangroves are of great ecological and economical value all around the world (Lacerda 1993, Lugo 2002, Martinuzzi et al. 2009). There are 80 species of mangrove trees around the world, but the Caribbean only has four (Department of Natural and Environmental Resources 2009): *Rhizophora mangle, Avicennia germinans, Languncularia racemosa* and *Conocarpus erectus*. Puerto Rico has 97 sites were mangroves have been documented between 1977–2002 representing an increase in mangrove cover of 12% around the island (Martinuzzi et al. 2009). Research on mangrove ecosystems in Puerto Rico range from mangrove forest distribution to the role of certain invertebrates inhabiting mangrove trees. For example, Martinuzzi et al. (2009) conducted a study were they explore the influences of human activities over mangrove ecosystems and how they have been conserved/recovered over a period of 200 years. Other studies centered on the importance of mangrove forests on the input/output of the organic cycles. For example, Corredor et al. (1990) observed that fluxes of nitrous oxide in mangrove sediments vary in response to nitrogen substrate availability (Corredor et al. 1999). Most studies about invertebrates that live in mangrove

ecosystems deal with oysters and clams (Dubilier et al. 2008, Laurent et al. 2009), especially, *Phacoides pectinatus,* a clam that lives buried in the marine sludge surrounding red mangroves (Dubilier et al. 2008).

Microorganisms have an important role in the recycling of organic matter. Tree and subsurface root litter provide a significant input of organic carbon to sediments (Kristensen et al. 2008). The degradation of organic matter in mangrove sediments is mediated by aerobic and anaerobic microbial processes using a variety of electron acceptors (Kristensen et al. 2008). The degradation process is mediated by a complex microbiota where each individual microorganism plays an important role. Most of the recent research has focused on the anaerobic microbial degradation process due to the governing conditions in mangrove sediments, and few focused on the aerobic process. Recently, decomposition of organic material, mainly wood, by fungi has become a focal point of research (Nambiar and Raveendran 2009). Fungi use lignocellulose components as their nutrient source by secreting enzymes to degrade components into smaller units, which can be incorporated into their metabolism. In addition, fungal marine borers play an important role in the breakdown of materials in the lower intertidal zone (Hyde 1991).

The kingdom Fungi includes a wide variety of species characterized for being natural decomposers, having a crucial role in recycling organic matter. Manglicolous fungi, which are part of the higher marine fungi, can be found on submerged parts of mangroves. This group includes several species and is the fourth largest ecological group after the wood-, salt-marsh-, and algae-inhabiting species in marine environments (Kohlmeyer & Kohlmeyer, 1979). The majority occur on cellulosic substrates, mainly in submerged and dead mangrove roots and in the damage seeds growing in sea water

(Acevedo Ríos 1987) all around the tropics. These usually invade the wood after the protective bark has been damaged or torn off (Kohlmeyer and Kohlmeyer 1979).

Most studies have focused on the diversity of fungi associated with two or fewer species of mangrove trees (Sarma and Hyde 2001, Nambiar and Raveendran 2008, Madavasamy and Paneerselvam 2012). For example, Kohlmeyer & Kohlmeyer (1979) described just a few host-specific fungi limited to *Rhizophora mangle* such as *Didymosphaeria rhizophorae, Keissleriella blepharospora*, and *Robillarda rhizophorae*. A more recent study by Nambiar and Raveendran (2008) reported the frequency of Ascomycetes occurring on specific mangrove trees such as *Rhizophora mangle*. In Puerto Rico, a study on the southwest coast in La Parguera identified mangrove-wood associated Ascomycota from pure cultures. Nine genera were identified, including *Pleospora, Kymadiscus, Leptosphaeria, Lophiostoma, Didymosphaeria. Macrophoma, Phoma, Phialophorophoma,* and *Tubercularia* (Acevedo Ríos 1987). Most studies involving manglicolous fungi are of a descriptive nature responding to taxonomic and inventory interests (Sahoo and Dhal 2009) and only a few are focused on the role of these fungi in the environment. The present study focused on the role of maglicolous fungi and their ability to produce enzymes on mangrove ecosystems.

1.2.2. Lignolytic Fungi

Every untreated piece of wood that is submerged for a certain period of time in marine or estuarine waters is attacked by higher marine fungi (Kohlmeyer and Kohlmeyer, 1979). This attack is faster and more severe in tropical waters than in temperate ones. Lignin is the most abundant natural aromatic polymer on earth and its degradation is caused in nature primarily by white rot fungi through enzymatic

combustion (Kirk and Farrell 1987). The lignolytic system is an extracellular enzymatic complex that includes peroxidases, laccases and oxidases (Ruiz-Dueñas and Martínez 2009). The enzymes responsible for lignin degradation are mainly lignin peroxidase (LiP), manganese peroxidase (MnP) and a copper containing phenoloxidase, known as laccase (Mendonça Maciel et al. 2010). The process of lignin degradation is a synergism between bacteria and fungi capable of producing the necessary enzymes for this process to occur. These enzymes also play a major role in the food, textile, paper, pharmaceutical and cosmetic industry, as well as for biodegradation of xenobiotics such as PAHs (Mendonça Maciel et al. 2010).

1.2.3. Lignolytic fungi degrading PAHs

Human activities have had a negative impact on mangrove forests. For example, oil spills that occurred near the southwest coast of Puerto Rico in 1962 by Argea Prima and in 1973 by the Zoe Colocotronis caused major damaged to the biota (NOAA 2001). Petroleum and diesel spills from industrial processes can yield PAHs (Bamforth and Singleton 2005) derived from the incomplete combustion of hydrocarbons, such as coal and gasoline. They are also manufactured as part of pesticides, pharmaceuticals and dyes, which are persistent in the environment (Atagana 2009). Polycyclic aromatic hydrocarbons are a class of organic compounds that consist of two or more fused benzene rings and/or pentacyclic molecules arranged in various structural configurations (Bamforth and Singleton 2005). PAHs are an environmental concern because they are toxic to aquatic life and suspected human carcinogens and a mutagenic risk(US EPA, OSWER, Office of Resource Conservation and Recovery 2008). Mangrove sediments are closely tied to human activities and are subjected to PAH contamination (Haritash and

Kaushik 2009). The persistence of PAHs in the environment is dependent on a variety of factors, such as the chemical structure of the PAH, concentration, dispersion of the compound and the bioavailability of the contaminant (Bamforth and Singleton, 2005). Several lignolytic fungi are known to degrade PAHs, including white-rot fungi, *Cunninghamella echinulata* var. *elegans* and other litter-decomposing fungi (Haritash and Kaushik 2009, Pozdnyakova 2012). Due to the irregular structure of lignin, lignolytic fungi produce extracellular enzymes with very low substrate specificity, making them suitable for degradation of different compounds (Haritash and Kaushik 2009). White-rot fungi can oxidize PAHs by generating free radicals, which oxidizes the PAH ring (Bamforth and Singleton 2005). These extracellular enzymes are theoretically able to diffuse into the soil/sediment matrix and potentially oxidize PAHs with low bioavailability (Bamforth and Singleton 2005).

Floating wood from *Rhizophora mangle* is a great substrate for lignolytic fungi in Puerto Rico (Acevedo Ríos 1987), which warrants further investigation of manglicolous fungi in the southwest coast of the island. Manglicolous fungi could be used as alternative microorganisms to produce enzymes necessary to degrade PAHs and thus, help clean up the coastline affected by oil spills. The main objective of this project was to isolate and identify manglicolous fungi that use lignin as their main source of energy and produce lignolytic enzymes that may also act in the presence of contaminants such as PAHs.

1.3. Goals

1.3.1. Hypotheses

1. If fungal samples are isolated from *Rhizophora mangle* wood then the isolated fungi should be able to grow on media supplement with carbon sources with similar structure to lignin such as Congo Red.

2. If fungal isolates are able to use Congo Red dye as their only carbon and energy source, then they could also use naphthalene as their only carbon and energy source.

3. If fungal isolates grow in basal media with Congo Red or naphthalene, then lignolytic enzyme activity should be observed.

1.3.2. Objectives

1. The principal goal of this study was to isolate manglicolous fungi with the capability to use xenobiotics, such as Congo Red dye or naphthalene as their only carbon and energy source.

2. Identify the manglicolous fungi isolated with the sequences obtained from amplifying the ITS region using primers ITS4 and ITS5.

3. Conduct enzymatic assays to determine the production of the lignolytic enzymes such as lignin peroxidases, manganese peroxidase, and laccase.

2 Materials and Methods

2.1. Sampling Area

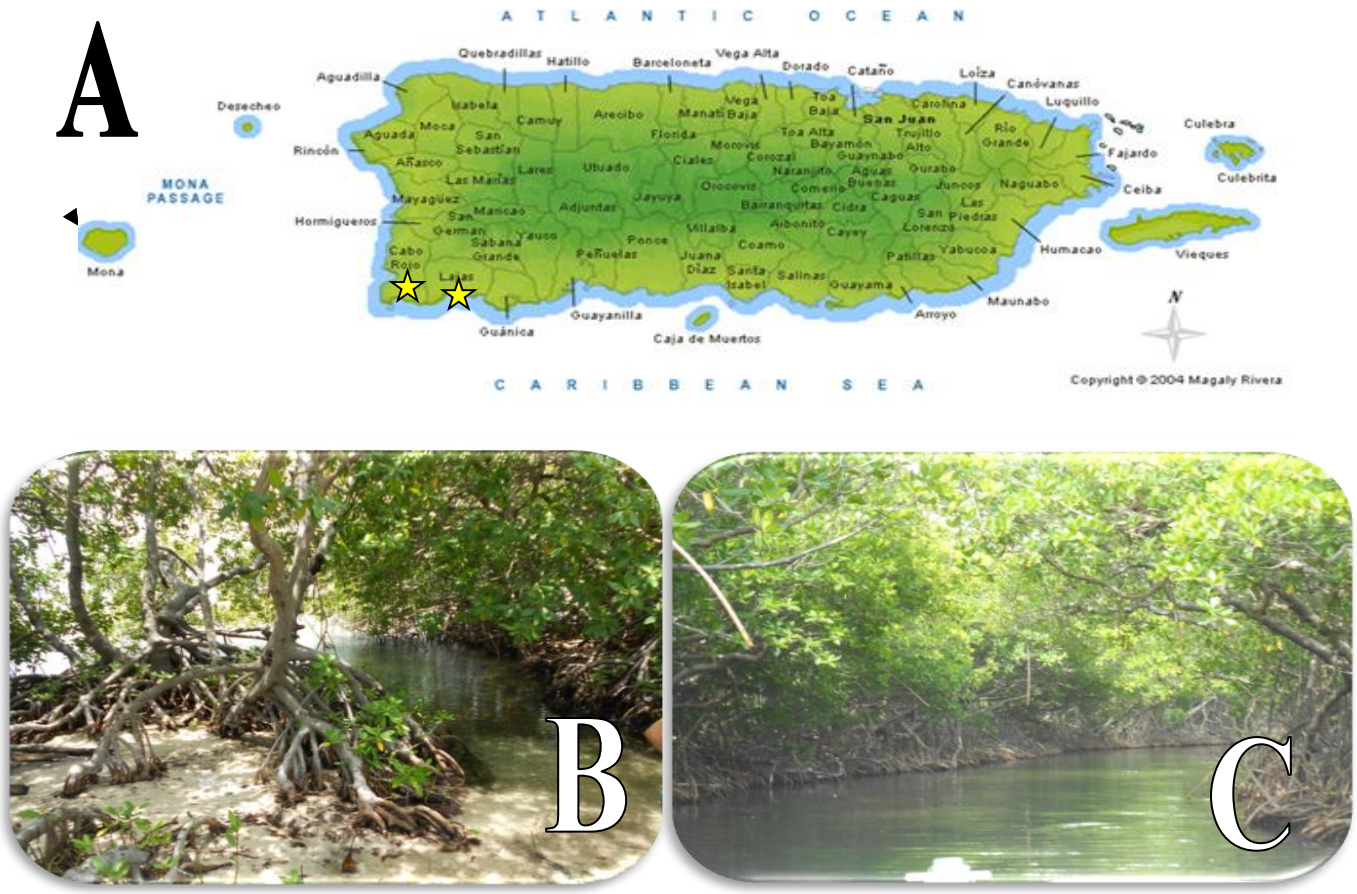

Figure 1. Sampling areas. Map of Puerto Rico (A). The yellow stars mark the two sampling sites in the study. Bahía Salinas, Cabo Rojo sampling site (B) and La Parguera, Lajas sampling site (C).

For this study two sampling areas were selected: Bahía Salinas, Cabo Rojo (17°56'25.18"N 67°11'34.61"W) and La Parguera, Lajas (18º12.745N 067º 08.322W). These are two differently impacted areas with PAHs contaminants. Both areas were impacted by oil spills, in 1962 by Argea Prima and in 1973 Zoe Colocotronis, respectively. Even though many years have passed since these incidents it is known that PAHs have a high persistent in the environment (Włodarczyk-Makuła 2012). In addition, the two areas have different water vehicle activity. La Parguera, Lajas is a tourist attraction with daily high motorboat activity, while Bahia Salinas, Cabo Rojo has little motorboat visits.

2.2. Wood sampling

Wood samples from *Rhizophora mangle* were collected manually from each site. Decaying mangrove-wood was examined on location for fungal activity. Pieces of wood colonized by fungi were place in labeled plastic bags (date, time and collecting site) (Acevedo Ríos 1987). Water temperature and pH were also recorded. Samples were incubated three to four weeks at room temperature in plastic sealed moist bags in the laboratory (Kohlmeyer and Kohlmeyer, 1979) and examined for fruiting bodies or mycelia every 5 days. Mycelia were subsequently transferred into Petri dishes with culture media as described.

2.3. Fungal isolation

Glucose-yeast extract agar (SWYGA) was used for isolating marine fungi (Kohlmeyer & Kohlmeyer, 1979). The composition of this media consists of 1g of glucose, 0.1g of yeast extract, 18g of agar and 1L of aged seawater. Natural seawater was aged and stored for at least 1

week before use (Kohlmeyer & Kohlmeyer, 1979) and filtered with Whatman Filter Papers (1: 11µm). Cultures were incubated at 28ºC for a period of 7 days. Potato dextrose agar (PDA) media (Difco) supplemented with 3.5% of NaCl was used to preserve pure isolates.

2.4. DNA extraction and ITS region amplification

Genomic DNA from all isolates in pure culture was extracted with the Cetyl-Trimethyl Ammonium Bromide (CTAB) modified protocol (Mueller et al. 1998, Vo et al. 2009). PCR amplification of the ITS rDNA fragment as structural marker was done using primers ITS 4 (5'-TCCTCCGCTTATTGATATGCT-3') and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al. 1990). The complete protocol has a duration time of approximately 2hr 3min. The cycling protocol consists of 1) 95°C for 120 sec, 2) 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, and 3) 72°C for 60 sec.

PCR products were sent to the University of Washington High Throughput Genomic Sequencing Center for sequencing. BLAST searches in the database from the National Center for Biotechnology Information (NCBI) were performed to identify the specimens.

2.5. Congo Red degradation screening assay

As a preliminary screening to select isolates that were later used for bioassays, isolates were inoculated in basal media with Congo Red dye as only carbon and energy source. Congo Red is a diazo dye used commonly in the paper and textile industry, which is recalcitrant and known carcinogen because it is an aromatic compound with an amine group (Bhattacharya et al. 2011). It has been shown that the lignin-degrading system of some fungi are able to degrade diverse organic pollutants such as Congo Red.

The AZO dye Congo Red has similar chemical structure to lignin, thus serving as proxy for lignin degradation. Decolorization of the media was observed and measured over a period of 10 days. Under sterile conditions, one-centimeter plugs of the fungal isolates were dropped in a liquid basal medium with Congo Red at 28^{o}C with agitation of 150 rpm. The basal medium (BM) contained $MgSO_4.7H_2O$: 0.5 g, HK_2PO_4: 0.6 g, H_2KPO_4: 0.5g, $NaNO_3$ [10mg/ml]: 200ml, and dH_2O: 800ml. The samples were inoculated in 50 ml Erlenmeyer flasks with 30 ml of media. The concentration of Congo Red used in every trial was 0.01mg/ml. Every two days, one ml of supernatant was extracted to measure absorbance at 490 nm and the percentage of degradation was calculated using the following equation:

$$\%Decolorization = \frac{A_0 - A_t}{A_0}$$

Asperguillus flavus (ATCC 10124) (Eurotiales: Ascomycota) was used as a positive control since it is known to degrade Congo Red (Singh and Singh 2010). A *Trametes* sp. was also used as a second positive control for being a well-known white rot fungi. *Escherichia coli* was used as a negative control. Also flaks with no microorganisms inoculated were used as a second negative control. Positive cultures for Congo Red degradation were then selected for lignolytic enzyme assays.

2.6. Naphthalene as only carbon and energy source

Three isolates from the Congo Red dye screening assay were selected to determine polycyclic aromatic hydrocarbons (PAHs) degradation capacity. The assay was done using a minimal medium ($MgSO_4.7H_2O$: 0.5 g, HK_2PO_4: 0.6 g, H_2KPO_4: 0.5g, $NaNO_3$ [10mg/ml]: 200ml, and dH_2O: 800ml) containing 0.04 mg/ml of naphthalene as the only source of carbon

and energy. Naphthalene was first dissolved in acetone at a concentration of [2.5mg/ml] and then added into the media for a final concentration of [1mg/ml]. For this assay 70 ml serum bottles were used. The bottles had 15ml of the media and then sealed with rubber stoppers to prevent volatilization of naphthalene. Isolates were incubated at 28^{o} C with agitation of 150 rpm for a period of 20 days. Absorbance was measured at 320 nm every five days. To extract the supernatant 1ml syringes were used. Spectrophotometry is a quantitatively way of measuring the amount of naphthalene in the media. If the isolates were able to grow on this medium, then they might be able to use PAHs as their only carbon and energy source.

2.7. Lignolytic enzymatic assay

To establish if lignolytic enzymes play a role in the degradation of Congo Red dye or naphthalene, enzyme activity assays were performed. Lignolytic enzymes are non-specific, which means they will act upon most chemical compounds with similar structure to lignin. The enzymes measured were: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. The total protein concentration in the supernatant was also measured using the Lowry technique.

Fifthteen millitters of supernatant of the six selected isolates and the control were saved in a 15ml centrifuge tube at -20^{o}C until further experiments. One-milliliter samples were analyzed for the three enzymes and total protein by spectrophotometry as follows:

Lignin Peroxidase (LiP) the assay was done using methylene blue as an indicator of oxidation as described by Bello Magalhães et al. (1996). The reaction mixture contained 3.0ml in total: 2.2 ml of the sample supernatant, 0.1 ml of 1.2mM methylene blue and 0.6 ml of 0.5M sodium tartrate buffer (pH4). The reaction was started by the addition of 0.1 ml of 2.7mM H_2O_2. The

change in absorbance was measured at 664nm. In the presence of lignin peroxidase H_2O_2 initiates the oxidation of methylene blue to azure C.

Manganese Peroxidase (MnP) assay reaction mixture contained 2.5ml of a solution of 100mL of succinate buffer (succinic acid 23.6g/L, and NaOH 8g/L), 22.3mg of $SO_4Mn.4H_2O$, and 10 mg of phenol red will be used. A 100µl of the sample were added, and then the tube was incubated in a water bath at 30ºC for 10 minutes. Afterwards 20 µl of H_2O_2 were added to the control and to each sample tube and incubated again in the water bath at 30ºC for 5 minutes. Thereafter, 40µl of HONa 5N were added to end the reaction and the absorbance was read at 610nm (Kuwahara et al. 1984, Glenn and Gold 1985). In the presence of MnP when H_2O_2 is added the $SO_4Mn.4H_2O$ is oxidized from Mn^{+2} to Mn^{+3}.

Laccase activity was determined through the ABTS method (Bourbonnais et al. 1995). The substrate is composed of 0.5mM ABTS [2, 2´-azinobis (3-etilbenztiazoline-6-sulfonate)] and 0.1 M sodium acetate buffer (pH 5) [27.43 mg ABTS/100 ml buffer AcNa, pH 5 (30 A+ 70 B → 200 ml)]. The reaction consisted of 2.5ml of substrate/tube incubated in a water bath at 30°C for 5-10 minutes, 10-50µl of the sample was added and incubated for at least one minute until reaction turns green. In the presence of laccase ABTS is oxidize, hence the change in color to green. Absorbance was read at 420nm and compare to the control (ABTS alone) (Bourbonnais et al. 1995).

Lowry technique was used to measure the total protein concentration in the supernatant. The reaction consists on 1ml of the supernatant, 100µl of water and 1ml of solution I[1] and then incubated at 37°C for 20 min. After the incubation period 100µl of solution II[2] were added, incubated at 37°C for 20 minutes. Absorbance was read at 660 nm. Table 1 shows a breakdown of all the solutions and reagents used for the Lowry technique.

For three enzymatic assays and the total protein concentration assay a standard curve was design using known concentrations of laccase, Mn peroxidases, Li peroxidase and albumin in the Lowry assay. The standard curve obtain of each enzyme was used to determine the concentration of total protein and the concentration of substrate from each enzymatic reaction.

Table 1. Solutions and reagents used for the Lowry technique.

Solution A	60g Na_2CO_3 8g NaOH 1000ml H_2O
Solution B	4g Sodium tartrate 100ml H_2O
Solution C	2g $CuSO_4$ 100ml H_2O
Solution D	Folin-Ciocalteu's phenol reagente
Solution E	40mg BSA 200ml H_2O

[1]Solution I: 9.6ml A + 0.2ml B + 0.2ml C; [2]Solution II: 0.2ml D + 0.8ml H_2O

2.8. Statistical analysis

To establish if there was a significant difference between the fungal isolates using Congo Red or naphthalene, the biomass produced and the concentration of the carbon source levels an analysis of variance (ANOVA) was performed. A Box Cox analysis was done for each isolate to validate the proposed model. The ANOVA is done to observe the variability of the biomass, concentration and the percent of decolorization depending on the fungal isolate used. Also, a desirability analysis was done to determine the best fungal isolate depending on the variable being evaluated. All of the statistical data was evaluated using an Error Type I (α) 0.05. All statistical analyses were done in the program Design Expert® (version 9.0.6).

3 Results

3.1 Molecular identification of samples isolated from *Rhizophora mangle* wood

Sampling sessions were both conducted during rainy season on September 2013. During September 2013 both sampling sites received between 4-7" of rain Temperature and pH were recorded for both sites. Water from Baía Salinas, Cabo Rojo had a pH of 8.5 and temperature ranging between 28.3°- 29.01°C. Water at La Parguera, Lajas also had a pH of 8.5 and temperature ranging between the 27.9°- 29.5°C. From each sampling site 10 pieces of mangrove wood were collected. Table 1 shows the species found in both sampling sites. In both sampling sites Bahía Salinas (BS), Cabo Rojo, and La Parguera (LP), Lajas, 10 fungi were isolated from each site. Isolates were identified using the sequences obtained by amplifying the ITS region (primers ITS 4 and ITS 5). Sequences were compared using NCBI BLAST nucleotide database. Nineteen out of twenty isolates were Ascomyccetes; only one isolate is a Basidiomycete. Samples from BS included *Asperguillus fumigatus*, *A. micronesiensis, A. niger*, *A. terreus*, *Fusarium solani*, *Penicillium citrinum, P. griseofulvum*, and *Purpureocillium lilacinum*. Fungi isolated from La Parguera include *A. caelatus, A. sclerotiorum, A. niger, Engyodotium album, Hypocrea lixii*, *Phoma* sp., *Purpureocillium lilacinum,* and *Tritirachium oryzae*. *A. niger* and *P. lilacinum* were the only isolates to my found in both sampling sites. For both sampling sites *P. lilacinum* had the highest frequency (0.30), while the others had a frequency of 0.1.

Table 2. Fungi isolated from *Rhizophora mangle* wood in this study.

	Distribution of isolates based on the sampling area	
Name of fungi	**Bahia Salinas, Cabo Rojo**	**La Parguera, Lajas**
***Aspergillus caelatus* (Eurotiales, Aspergillaceae)**	0	1
***Aspergillus fumigatus* (Eurotiales, Aspergillaceae)**	1	0
***Aspergillus micronesiensis* (Eurotiales, Aspergillaceae)**	1	0
***Aspergillus niger* (Eurotiales, Aspergillaceae)**	1	1
***Aspergillus sclerotiorum* (Eurotiales, Aspergillaceae)**	0	1
***Aspergillus terreus* (Eurotiales, Aspergillaceae)**	1	0
***Engyodotium album* (Hypocreales, Cordycipitaceae)**	0	1
***Fusarium solani* (Hypocreales, Nectriaceae)**	1	0
***Hypocrea lixii* (Hypocreales, Hypocreaceae)**	0	1
***Penicillium citrinum* (Eurotiales, Aspergillaceae)**	1	0
***Penicillium griseofulvum* (Eurotiales, Aspergillaceae)**	1	0
***Phoma* sp. (Pleosporales)**	0	1
***Purpureocillium lilacinum* (Hypocreales, Ophiocordycipitaceae)**	3	3
***Tritirachium oryzae* (Tritirachiales, Tritiraceae)**	0	1
Total	**10**	**10**

3.2 Congo Red dye screening assay

3.2.1 Bahía Salinas, Cabo Rojo isolates

A preliminary screening assay of the 10 isolates was done using CR dye. Initial media had a red color; 37.5% of the isolates had reach over a 50% of decolorization of the medium by day 10 (Figure 3) but after 10 days of incubation samples range between 87-100% of decolorization leaving the media of those that reached 100% completely transparent (Figure 2). It was also observed that the mycelium mass absorbed the red dye, instead of having their normal cream color (Figure 2). The isolates might be absorbing the dye from the medium; to assure degradation of the dye from the medium further experiments, such as chromatography of the medium and the fungal isolate should be done. Moreover, the fungal isolates are removing the dye from the medium in a fast manner.

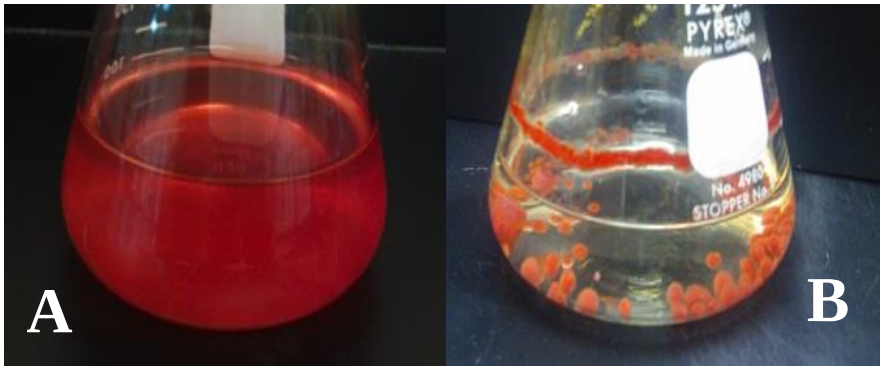

Figure 2. Basal media with Congo Red in 125ml Erlenmeyer flasks. (A) shows day 0 of BM with Congo Red and (B) shows media after 10 days of incubation. Note the red mycelia round bodies at the bottom of the flask.

All isolates from BS have showed the ability of removing Congo Red from the media (Figure 3). The slowest isolate at decolorizing the medium was sample RmBS 6-1, identified as *Asperguillus terreus*. Sample RmBS 2-1-1, identified as *Penicillium citrinum*, showed the highest capability of using Congo Red as its carbon and energy source. By day 2, which was the 2nd day of measurements, *P. citrinum* (RmBS 2-1-1), had reached 57.88mg of biomass, 100% of decolorization and a concentration of Congo Red in the medium of 0.0127 mg/ml. This isolate was faster at removing CR from the medium than the positive control, *Aspergillus flavus* (ATCC 10124), and reaching 99.2% of degradation by day 2.

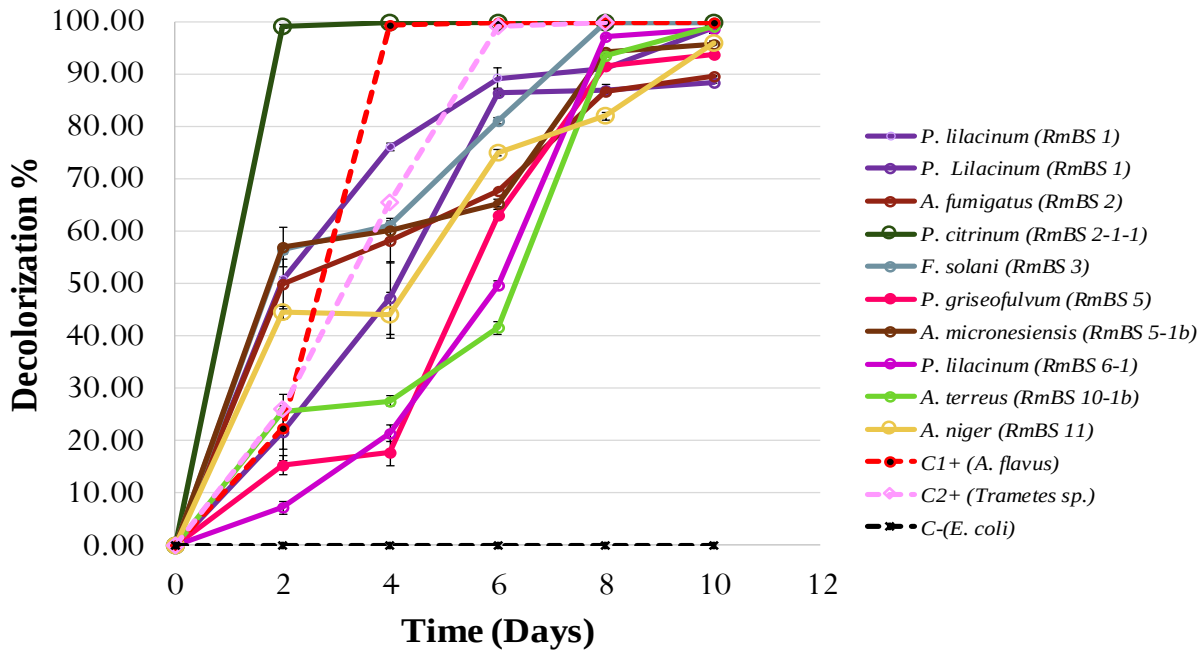

Figure 3. Decolorization percentages of 10 fungal isolates from Bahía Salinas, Cabo Rojo. Isolates were grown in basal media with CR, incubated for a period of 10 days at 28°C with agitation of 150 rpm.

After using Congo Red dye for screening the isolates, only 3 isolates were selected for further studies. They were selected on their rate of decolorizing the media; the fastest, intermediate and slowest. Also isolates were picked based on whether they have not been previously tested for the capability of using Congo Red or naphthalene as their only carbon source. These were *Penicillium citrinum* (RmBS 2-1-1), *Fusarium solani* (RmBS 3), and *Purpureocillium lilacinum* (RmBS 1-2b). Time was a variable considered when selecting the isolates for the experiments. Figure 4 shows the decolorization percentages of the three isolates selected and the controls during a period of 20 days incubated at 28°C, 150 rpm. The percentage of decolorization obtained by day 2 was used as a criterion since the percentage of decolorization obtained by day 10 for the 10 isolates ranged between 87-100%. By day 2, *P. citrinum* had reached 99.2%, *F. solani* 56%, and *P. lilacinum* 22% of media decolorization.

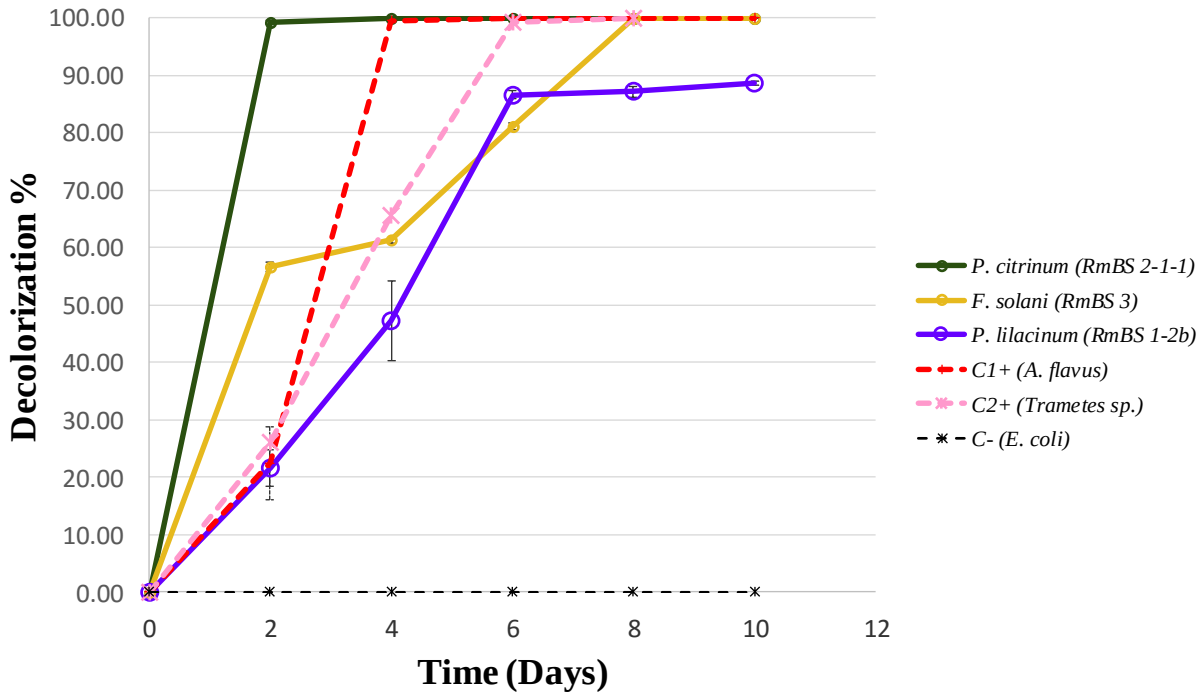

Figure 4. Decolorization percentages of Congo Red by 3 fungal isolates from Bahía Salinas, Cabo Rojo. The isolates were grown in BM with CR, incubated at 28°C for a period of 10 days with and agitation of 150 rpm. Isolates were selected based on their decolorization rate.

Figure 5 shows graphs of the concentration of Congo Red in the media and the biomass of the isolates after 20-day incubation. Isolates showed higher biomass when grown in BM with CR compared to BM with glucose. Results also showed an inversely proportional relation between the concentration of CR left in the media and the biomass. *P. citrinum* (RmBS 2-1-1) growing in BM with CR had the highest biomass by day 20 with 68.10 mg. The fact that *P. citrinum* (RmBS 2-1-1) has the highest biomass relates to the observation of having lowest concentration of Congo Red left in the media meaning that *P. citrinum* RmBS (2-1-1) is using CR as a carbon and energy source to grow. When compared to the growth of the isolate in BM with glucose, the biomass that produces is lower than the one produced when growing in BM with CR. In fact, the 3 isolates show a higher biomass while growing in BM with CR than when growing in BM with glucose. *P. lilacinum* (RmBS 1-2b) and *F. solani* (RmBS 3) had 56.53 mg and 21.63 mg of biomass, respectively. Both *P. lilacinum* (RmBS1-2b) and *P. citrinum* (RmBS2-1-1) had higher biomass by day 20 when compared to the positive control *A. flavus*.

Also, analysis of variance were done to determine if there was a significant difference in biomass produced by the isolates and the concentration of CR left in the media after the 20 days of incubation. The analysis show that all of this factors were significantly different in the experiment.

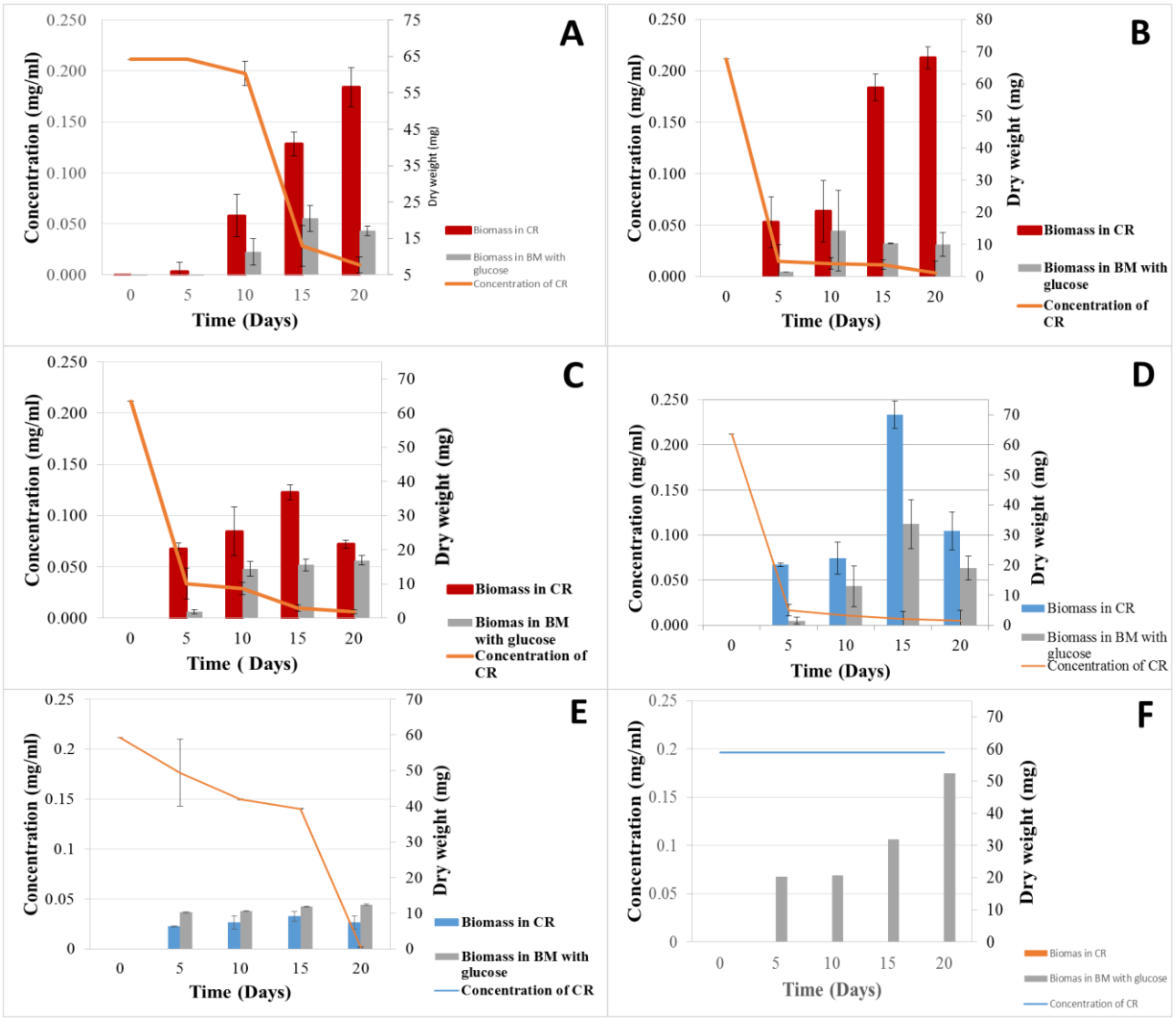

Figure 5. Concentration of Congo Red left in the media in relation with the biomass produce by the isolates. Graphs A-D show the relation between the concentration of Congo Red left in the media and the biomass of the isolates. Blue columns show biomass of isolates BM with CR; gray columns show biomass in BM with glucose. *P. lilacinum* RmBS 1-2b (A), *P. citrinum* RmBS 2-1-1 (B), *F. solani* RmBS 3 (C), *A. flavus* (positive control) (D), *Trametes* sp. (positive control) (E), and *E. coli* (negative control) (F).

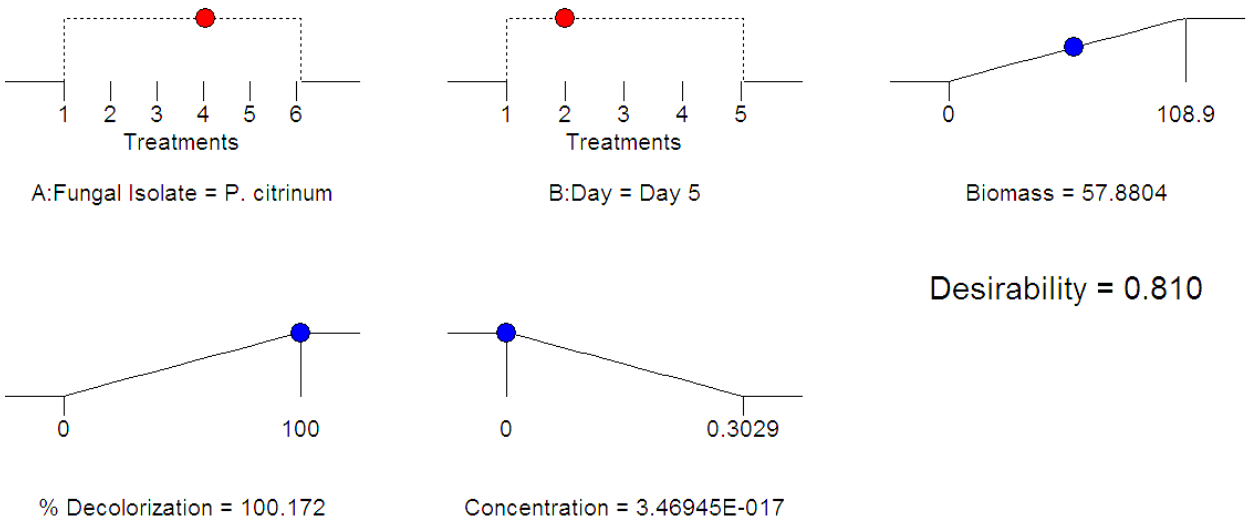

Figure 6. Desirability model arranged in Design Expert®. This model shows that *P. citrinum* (RmBS 2-1-1) was the isolate with the best results in terms of producing a high biomass, lowering the concentration of Congo Red in the medium and with a high decolorization percentage.

3.2.2 La Parguera, Lajas isolates

From the sampling site La Parguera, Lajas 10 isolates were also recovered and screened for Congo Red decoloriztion. Samples were grown in BM with CR, incubated for a period of 10 days, at 28°C with agitation of 150 rpm. In this screening, the isolates had a lower decolorization percentage ranging between 21-60% of decolorization compared to Bahia Salinas, Cabo Rojo samples, which by day 10 had reached 100% of decolorization (Figure 7). Isolate RmPL 5 (25P), identified as *Asperguillus caelatus*, and reached 59.04% of decolorization being the highest of all isolates by day 10. The lowest decolorization % was obtained by sample IIRmPL 2b with 21%.

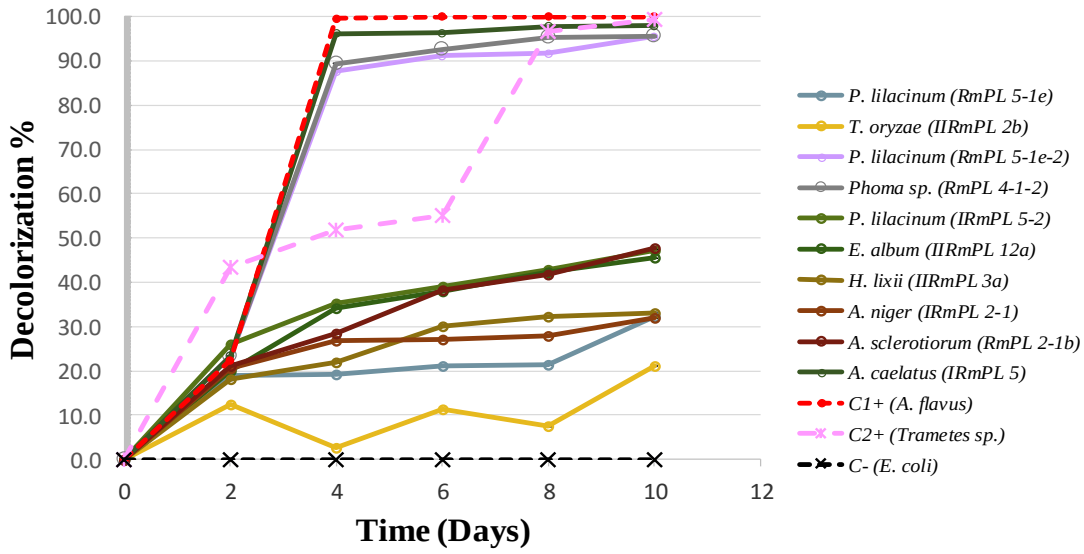

Figure 7. Congo Red decolorization percentages obtained by the 10 isolates from La Parguera, Lajas. Isolates were inoculated in basal media with Congo Red for a period of 10 days, at 28°C with agitation of 150 rpm. The red dotted line represents the control (+) 1 *Aspergillus flavus*, the salmon line represents control (+) 2 *Trametes* sp., and the black dotted line represents the control (-) *E. coli*.

In contrast to BS samples, the 3 isolates with the highest decolorization percentage were selected (Figure 8). These were *Purpureocillium lilacinum* RmPL 5-1e-2 (purple line), *Phoma* sp. RmPL 4-1-2 (orange line), and *Asperguillus caelatus* IRmPL 5 (green line). For this second experiment, where only the 3 selected isolates were used to grow in BM with CR, the decolorization percentage obtained were higher compared to the ones obtained during the screening assay. *Asperguillus flavus* (positive control 1), *Trametes* sp. (positive control 2) and *Escherichia coli* (negative control 1) were the controls used for these experiments. The light blue line represents a second negative control which only has BM with CR, where no fungal isolate was inoculated. Overall, *Phoma* sp RmPL 4-1-2 was the isolate with a higher biomass of 95.00 mg by day 5. In the presence of the isolate the medium had a decolorization percentage of 86.29% and a concentration of CR in the medium of 0.0120 mg/ml.

A desirability model was also prepared in the Design Expert® program which shows the isolate with the best results in terms of low concentration of CR in the medium, high decolorization percentage and high biomass production (Figure 9). When comparing the three isolates from LP, *A. caelatus* IRmPL 5 was the best fit in the model with a desirability of 0.850. By day 20, *A. caelatus* IRmPL 5, had a biomass of 44.98 mg and the concentration of CR in the medium was of 0.0056 mg/ml with a decolorization percentage of 97.52%.

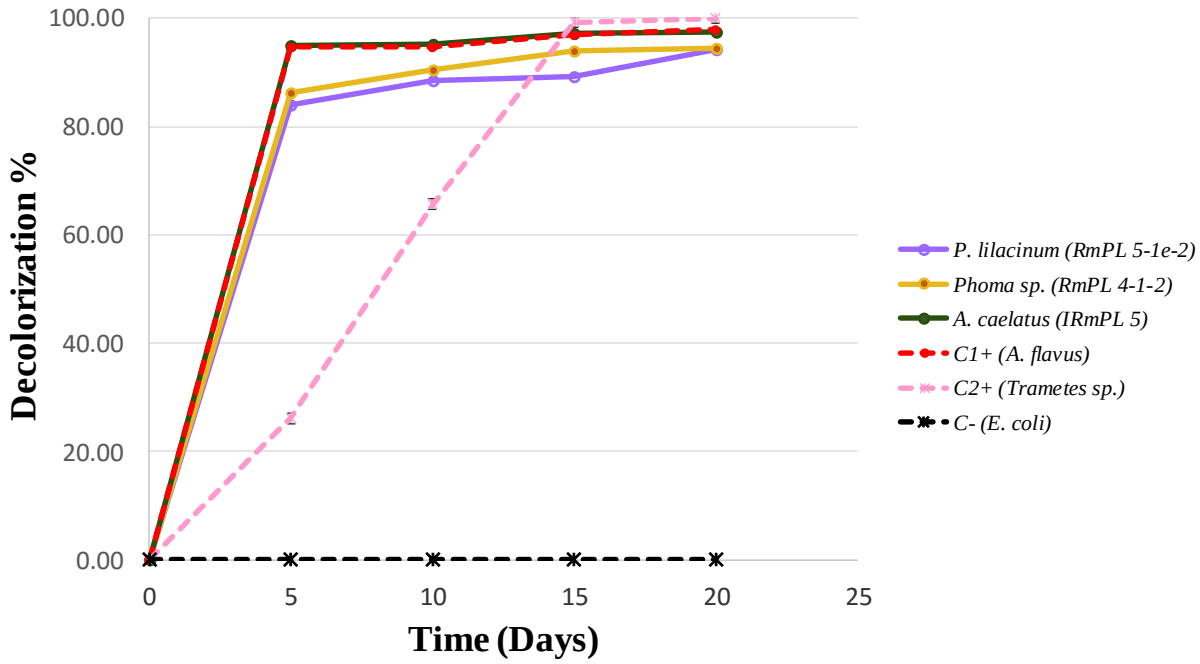

Figure 8. Decolorization percentages of the 3 isolates selected from the La Parguera, Lajas. The graph shows the samples and the controls growing in BM with CR during a period of incubation of 20 days, incubated at 28°C with agitation of 150 rpm.

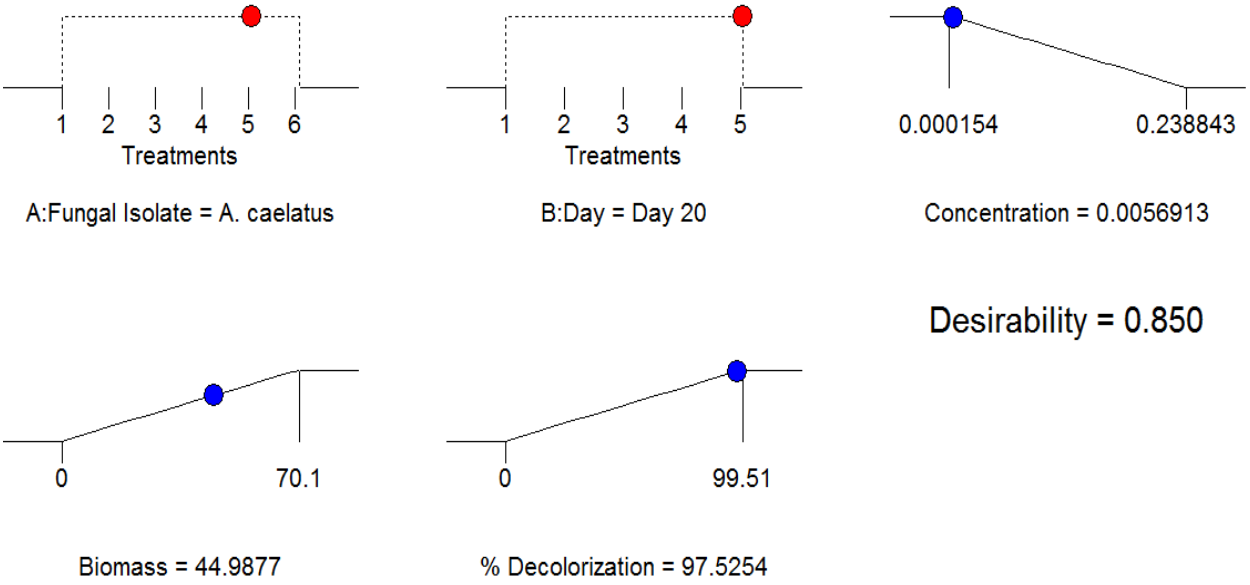

Figure 9. Desirability model arranged in Design Expert®. This model shows that *A. caelatus* IRmPL 5 was the isolate that showed the best results in terms of producing a high biomass, lowering the concentration of Congo Red in the medium and with a high decolorization percentage.

The relation between the concentration of CR left in the media and the isolates biomass produced growing in BM with CR or glucose is presented in a series of graphs (Figure 10). Contrary to the BS samples, these isolates have high biomass between days 5-10 and then they decrease. *Asperguillus caelatus* IRmPL 5, and *Phoma* sp. RmPL 4-1-2, show higher biomass when grown in BM with CR than when grown with glucose, except for day 20 for *A. caelatus* IRmPL 5, which has a higher biomass in glucose. *P. lilacinum* RmPL 5-1e-2 and *Phoma* sp. RmPL 4-1-2, growing in Congo Red media have high biomass by day 5 and then start to decrease. The analyses of variance show that all of these factors (biomass, concentration and decolorization percentage) are significantly different in the experiment after 20 days of incubation (Appendix 1.4-1.6).

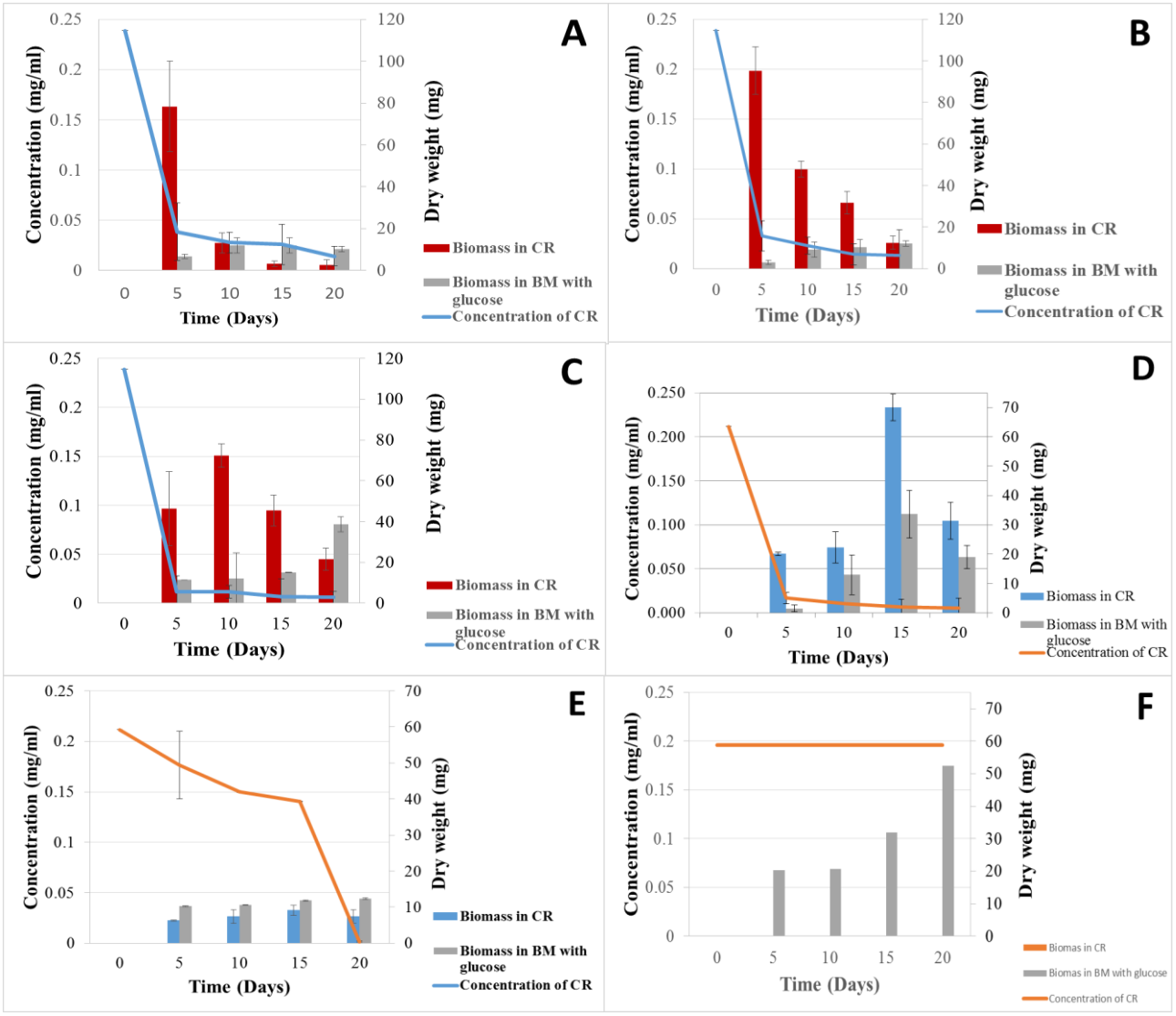

Figure 10. Concentration of Congo Red left in the media in relation with the biomass produce by the isolates. Graphs A-D show the concentration of Congo Red left in the media in relation to the biomass produced by each isolate from La Parguera while growing in basal media with Congo Red or glucose. *P. lilacinum* RmPL 5-1e-2 (A), *Phoma* sp. RmPL 4-1-2 (B), *A. caelatus* IRmPL 5(C), *A. flavus* (positive control) (D), *Trametes sp.* (positive control) (E), and *E. coli* (negative control) (F).

3.3 Naphthalene as only carbon and energy source

Congo Red assays helped in the process of selecting the fungal isolates capable of using a xenobiotic as the only carbon and energy source. From both sampling sites, 3 isolates were selected for experiments using naphthalene as the only carbon and energy source. The selected three isolates from BS were *P. lilacinum* (RmBS 1-2b), *P. citrinum* (RmBS 2-1-1), and *F. solani* (RmBS 3) and from LP the isolates selected were *P. lilacinum* (RmPL 5-1e-2), *Phoma* sp (RmPL 4-1-2), and *A. caelatus* (IRmPL 5). As well as in other experiments, *Asperguillus flavus* and *Trametes* sp. were used as positive controls and the negative controls were *E. coli* and basal media with naphthalene without any fungi. Figure 14 shows how the concentration of naphthalene decreases with time when inoculated with each of the isolates from Bahía Salinas. The graph shows that *F. solani* (RmBS 3) was the isolate that fastest decreased the concentration of naphthalene in the media, followed by *P. citrinum* (RmBS 2-1-1), and lastly by *P. lilacinum* (RmBS 1-2b).

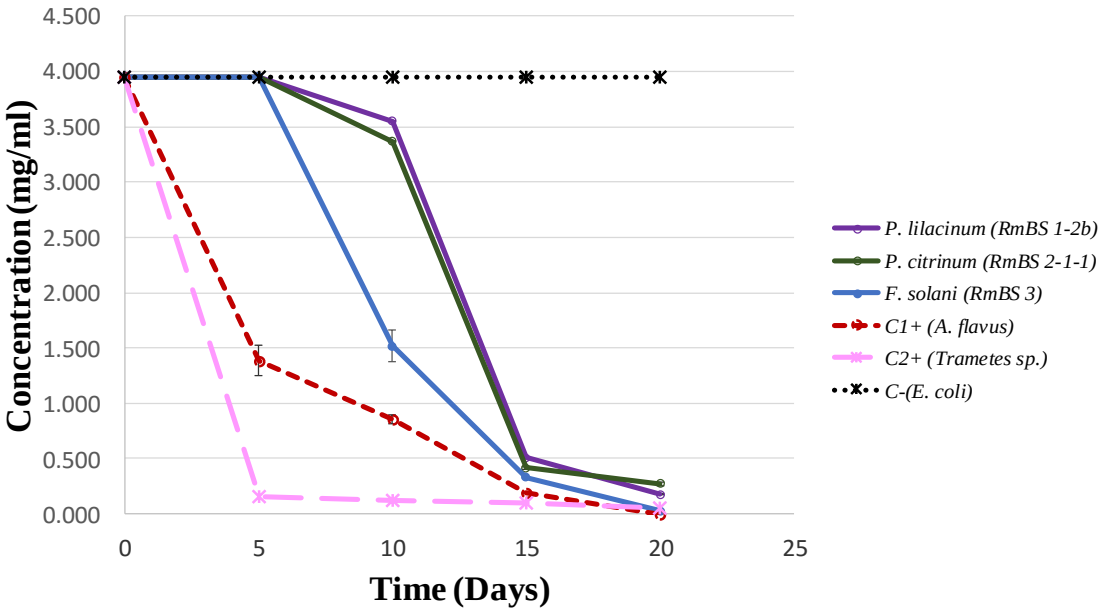

Figure 11. Concentration of naphthalene left in basal media in the presence of each Bahía Salinas, Cabo Rojo isolate. Changes in concentration of naphthalene (left) in the media over a period of 20 days of incubation at 28°C with agitation of 150 rpm.

Here, also to illustrate how the concentration of naphthalene left in the media relates to the biomass produced by each isolate from Bahía Salinas, growing in BM with naphthalene (orange bars) or glucose (gray bars) a series of graphs were design (Figure 12). *P. lilacinum* (RmBS 1-2b) (Figure 5A) growing in BM with naphthalene has a lower biomass by day 5, on day 10 it is similar to the normal growth, on day 15 is lower and by day 20 it is higher than the normal growth with 31.70mg. This result might be due to the toxicity of naphthalene and the isolate trying to stay alive, but to be sure the experiment should be repeated. Overall, *P. lilacinum* (RmBS1-2b) was the isolate with the highest biomass by day 20. *P. citrinum* (RmBS2-1-1) reached 30.53 mg, *F. solani* (RmBS 3)14.97 mg, and *A. flavus* had the lowest biomass with 14.27 mg.

ANOVAs were done to determine if there was a significant difference with the biomass produced (Appendix 2.7) by each of BS isolates growing in BM with naphthalene and the concentration of naphthalene left in the media (Appendix 2.8). Both analyses show that there is a significant difference on the values of the biomass produced by each of the isolates and the concentration of naphthalene in relation to the days.

The desirability model arranged in Design Expert® program shows which isolate from BS was the best fit in terms of both producing a high biomass and lowering the naphthalene concentration in the medium (Figure 13). The results of this analysis show that *F. solani* (RmBS 3) by day 20 has a biomass of 27.32 mg and lowers the concentration of naphthalene to 0.0522 mg/ml.

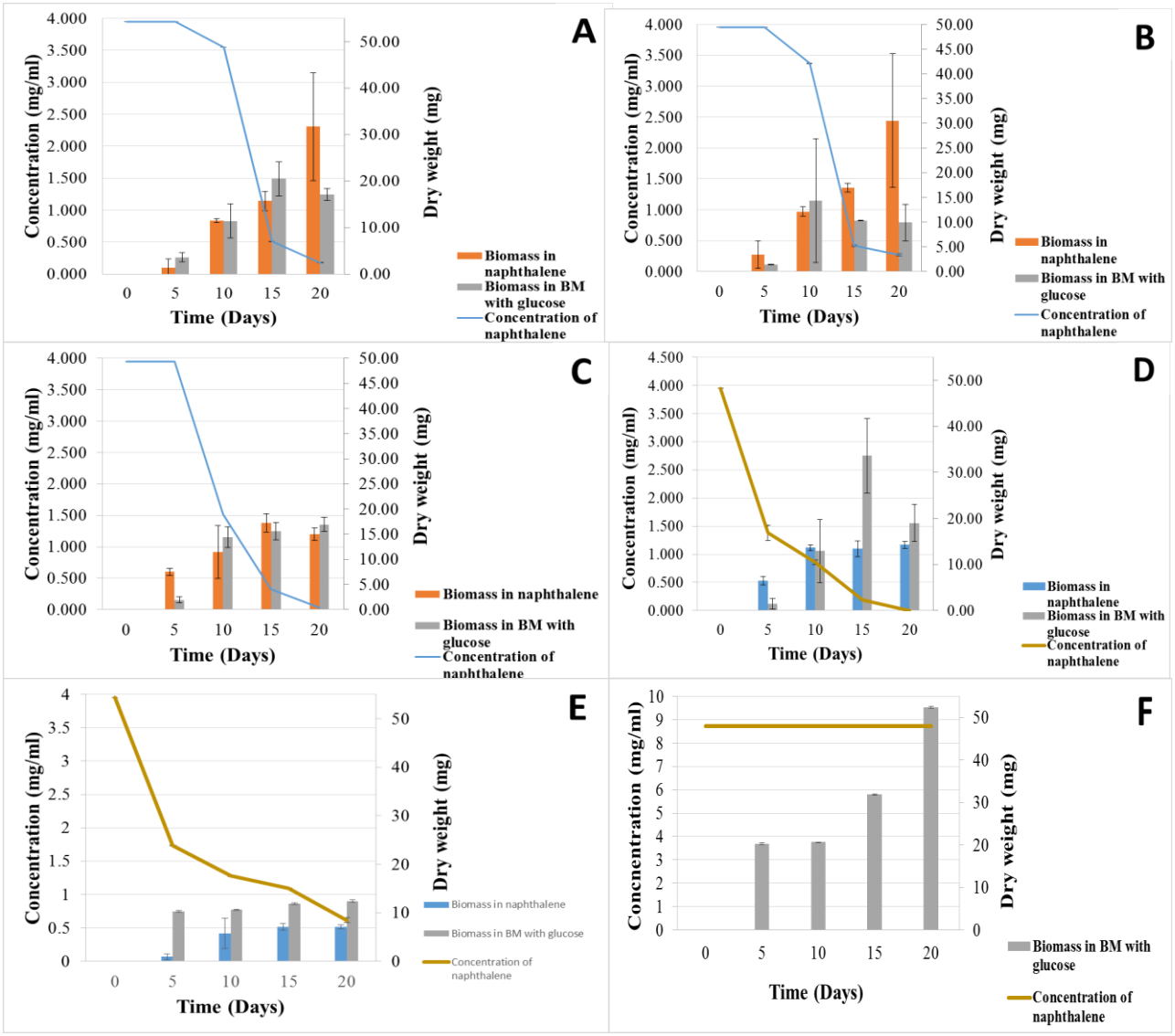

Figure 12. Concentration of naphthalene left in the media in relation with the biomass produce by the isolates. Graphs A-D show the concentration of naphthalene left in the media in relation with the biomass produced of each isolate from Bahía Salinas while growing in basal media with naphthalene or glucose. *P. lilacinum* RmBS 1-2b (A), *P. citrinum* RmBS 2-1-1 (B), *F. solani* RmBS 3 (C), and *A. flavus* (positive control) (D), *Trametes* sp. (positive control) (E), *E. coli* (negative control) (F).

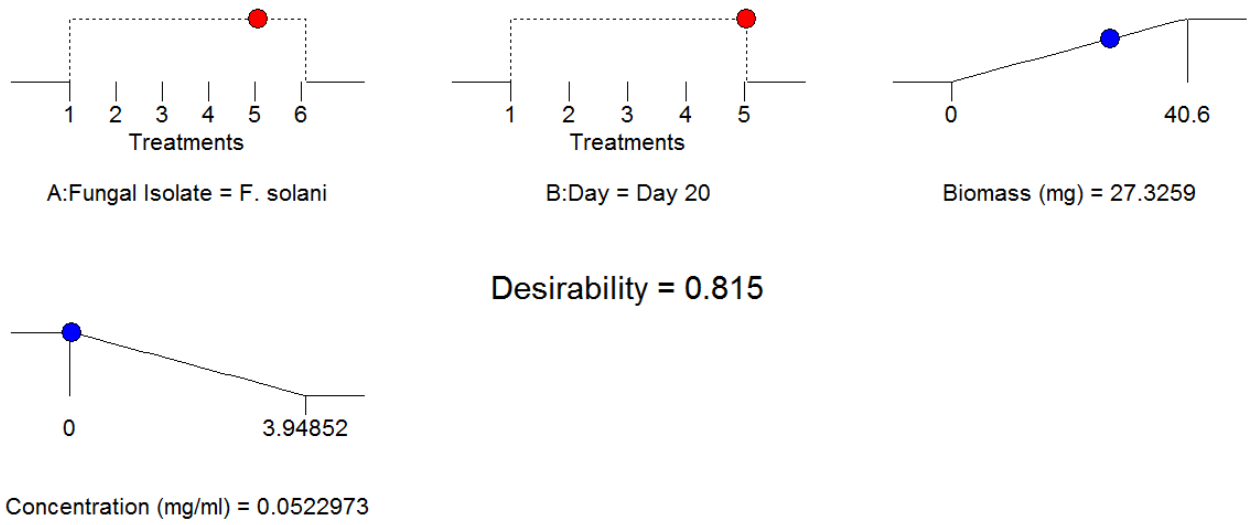

Figure 13. Desirability model arranged in Design Expert®. This model shows that *F. solani* RmBS 3, was the isolate that showed the best results in terms of producing a high biomass, lowering the concentration of naphthalene in the medium from BS samples.

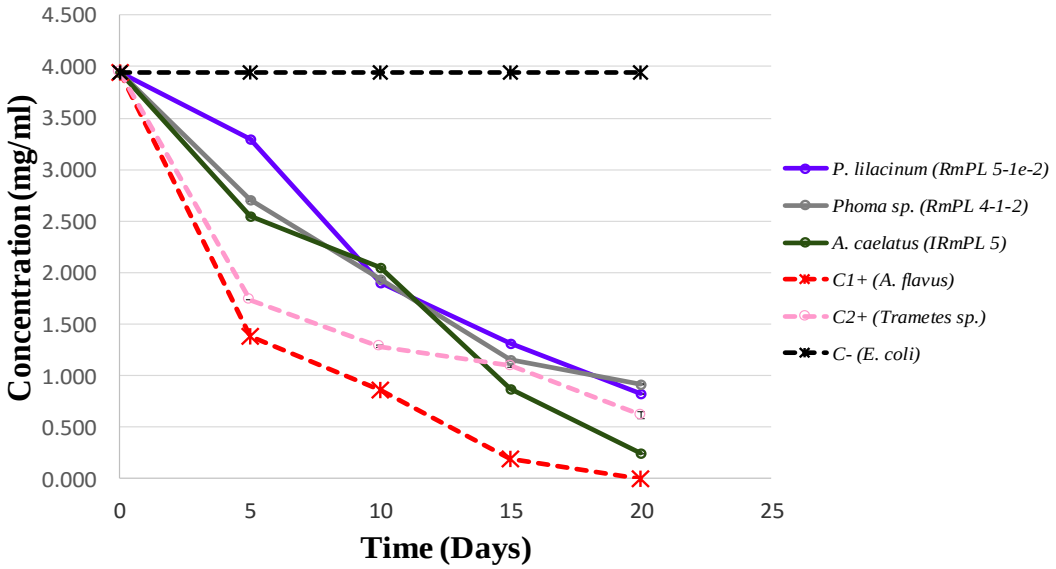

Figure 14. Concentration of naphthalene left in basal media in the presence of each La Parguera, Lajas isolate. Changes in concentration of naphthalene (left) in the media over a period of 20 days of incubation at 28°C with agitation of 150 rpm.

In order to see if there is a relation between the biomass produced by the isolates and the concentration of naphthalene in the media a series of comparative graphs were design (Figure 14). Overall, the positive control *A. flavus* had the highest biomass with 12.03 mg after 20 days of incubation, but from La Parguera isolates *P. lilacinum* (RmPL 5-1e-2) had the highest biomass with 7.13 mg. *Phoma* sp. (RmPL 4-1-2) followed with 3.26 mg and the lowest was *A. caelatus* (IRmPL 5) with 2.53 mg after 20 days of incubation. The lowest concentration of naphthalene left in the media was observed in presence of *A. caelatus* (I RmPL 5) with 0.2410 mg/ml.

Based on the desirability model (Figure 15), *P. lilacinum* (RmPL 5-1e-2) was considered to be the best option in terms of producing a high biomass and lowering the concentration of naphthalene in the medium. It shows that *P. lilacinum* (RmPL 5-1e-2), has a desirability of 0.838. By day 15 it produces a biomass of 184.31 mg and lowers the concentration of naphthalene in the medium to 1.31 mg/ml.

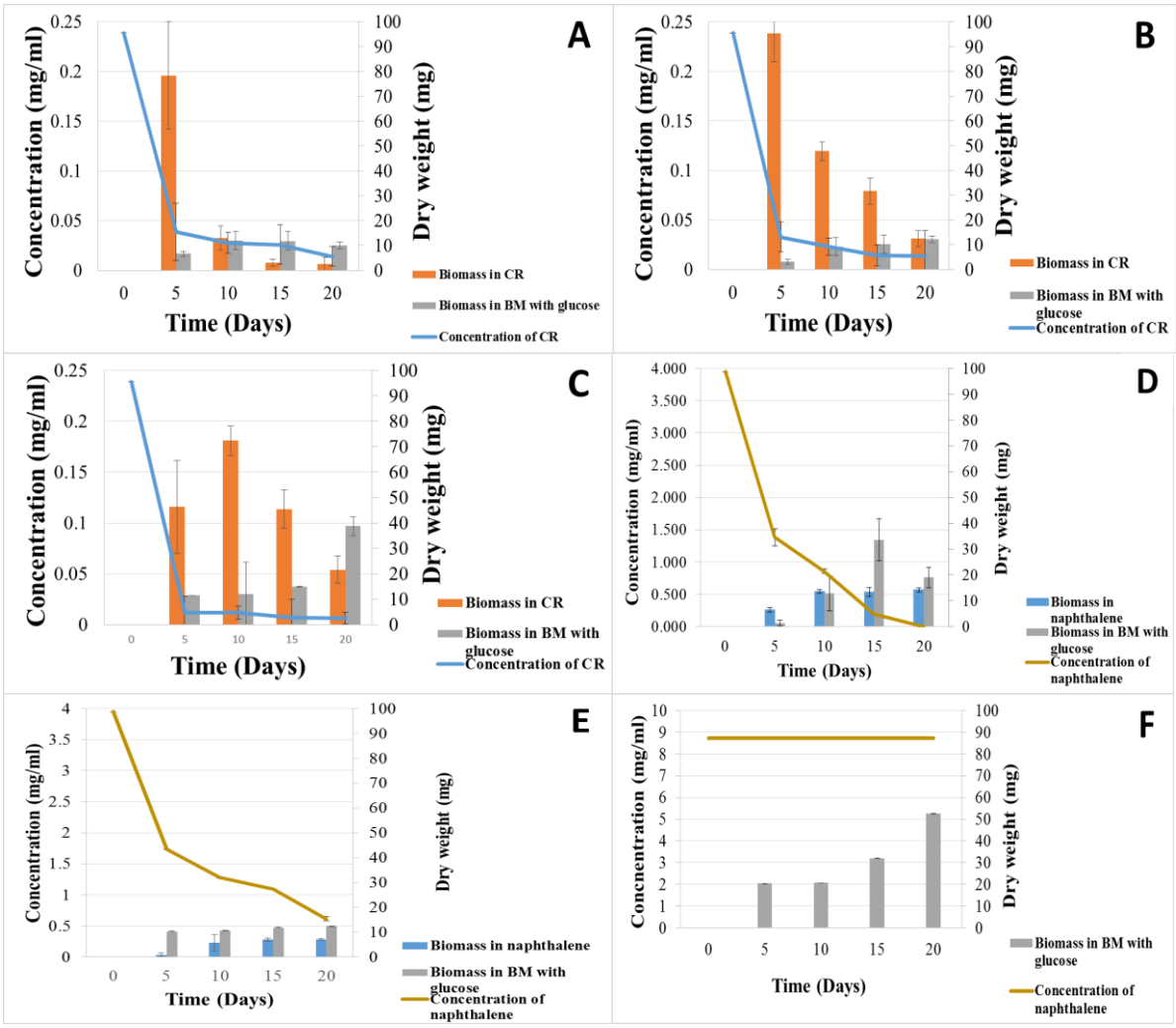

Figure 15. Concentration of naphthalene left in the media in relation with the biomass produce by the isolates. Graphs A-D show the concentration of naphthalene left in the media in relation with the biomass produced of each isolate from La Parguera while growing in basal media with naphthalene or glucose. *P. lilacinum* RmPL 5-1e-2 (A), *Phoma* sp. RmPL 4-1-2 (B), *A. caelatus* IRmPL 5 (C), *A. flavus* (positive control) (D), *Trametes* sp. (positive control) (E), and *E. coli* (negative control) (F).

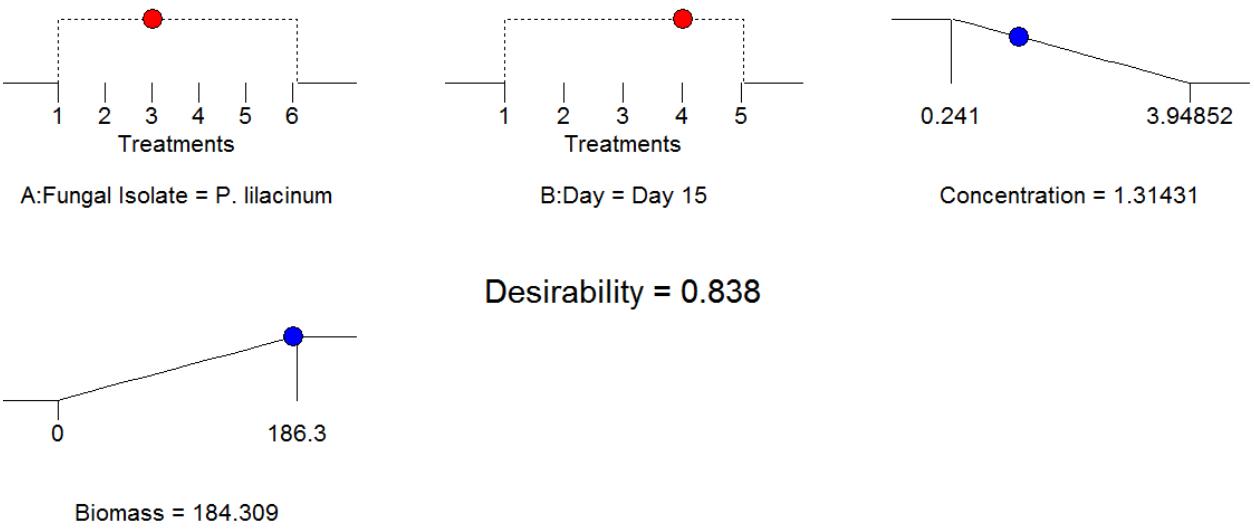

Figure 16. Desirability model arranged in Design Expert® of La Parguera, Lajas isolates. This model shows that *P. lilacinum* RmPL 5-1e-2, was the isolate that showed the best results in terms of producing a high biomass, lowering the concentration of naphthalene in the medium from LP samples.

3.4 Lignolytic enzyme activity

To determine if selected isolates were producing lignolytic enzymes to degrade the xenobiotics from the medium, assays for laccase (Lac) and manganese peroxidase (MnP) activities were done. Also, to detect the presence or absence of the lignin peroxidase (LiP) a colorimetric assay was done using methylene blue. The total protein concentration in the supernatant for each isolate was also measured by Lowry method. The assays were done for isolates grown in both xenobiotics, Congo Red and naphthalene. The objective was to determine if the xenobiotics were inducing the lignolytic enzymes responsible for the lignin degradation pathway. Results include the concentration in the supernatant of the enzymes produced by the selected isolates. The results are presented by fungal isolate growing in different carbon source in order to compare whether or not CR and napthalene are able of inducing the production of these enzymes. The results presented are based on the desirability models of each of the previous experiments. The results presented in this section for the isolates from Bahía Salinas are for *P. citrinum* (RmBS 2-1-1) and *F. solani* (RmBS 3). According to the desirability model *P. citrinum* (RmBS 2-1-1) growing in BM with CR and *F. solani* (RmBS 3) growing in BM with naphthalene were the isolates that produced a high biomass and decreased the most the concentration of the carbon source in the media. From La Parguera, Lajas isoaltes results are shown for *A. caelatus* (IRmPL 5) growing in BM with CR and for *P. lilacinum* (RmPL 5-1e-2). Results for isolates *P. lilacinum* (RmBS 1-2b) and *Phoma* sp. (RmPL 4-1-2) are in the appendix (Appendix 3.1, 3.2, 3.3). The concentration for both laccase and Mn peroxidase reported is the concentration of the end product of the enzymatic reactions. A high concentration equals a high enzymatic activity.

The results of each isolate are presented along with the positive control *Trametes* sp. and *E. coli*. Results for enzymatic concentration in BM with glucose for *E. coli* are the only ones shown because there was no growth in BM with CR or naphthalene. The positive control, *Trametes* sp, has a high total protein concentration, but low or no activity of laccase and Mn peroxidase (Figure 16, B1) except for day 20 where a low activity (3 mg/ml) was detected. *Trametes* sp. when grown in BM with CR (Figure 16, B2) or naphthalene (Figure 16, B3) has different activity levels of laccase and Mn peroxidase, an expected behavior in a well-known lignolytic fungus.

3.4.1 Samples from Bahía Salinas, Cabo Rojo

Penicillium citrinum (RmBS 2-1-1) growing in BM with CR showed a high activity level of laccase of 24.18 mg/ml by day 5 then it starts to decrease, while levels of Mn peroxidase remaind low until day 20 when they increased to 6.66 mg/ml. This correlates with the percentage of decolorization results by day 5 in BM with CR when the fungus reached 99.2 % of decolorization. This observation might indicate that laccases are the first enzymes to act upon the process of absorbing/removing the dye from the media. Later in the process other enzymes keep acting upon the compound residues left in the media.

In comparison, *P. citrinum* (RmBS 2-1-1) growing in BM with naphthalene showed consistently low levels of both laccase and Mn peroxidase from day 5 to day 15 and then in day 20 a spike in the activity levels of both enzymes was observed, which was also reflected also in the total protein concentration. In this case, naphthalene promotes the production of both enzymes, but they might need more time to start acting upon the compounds.

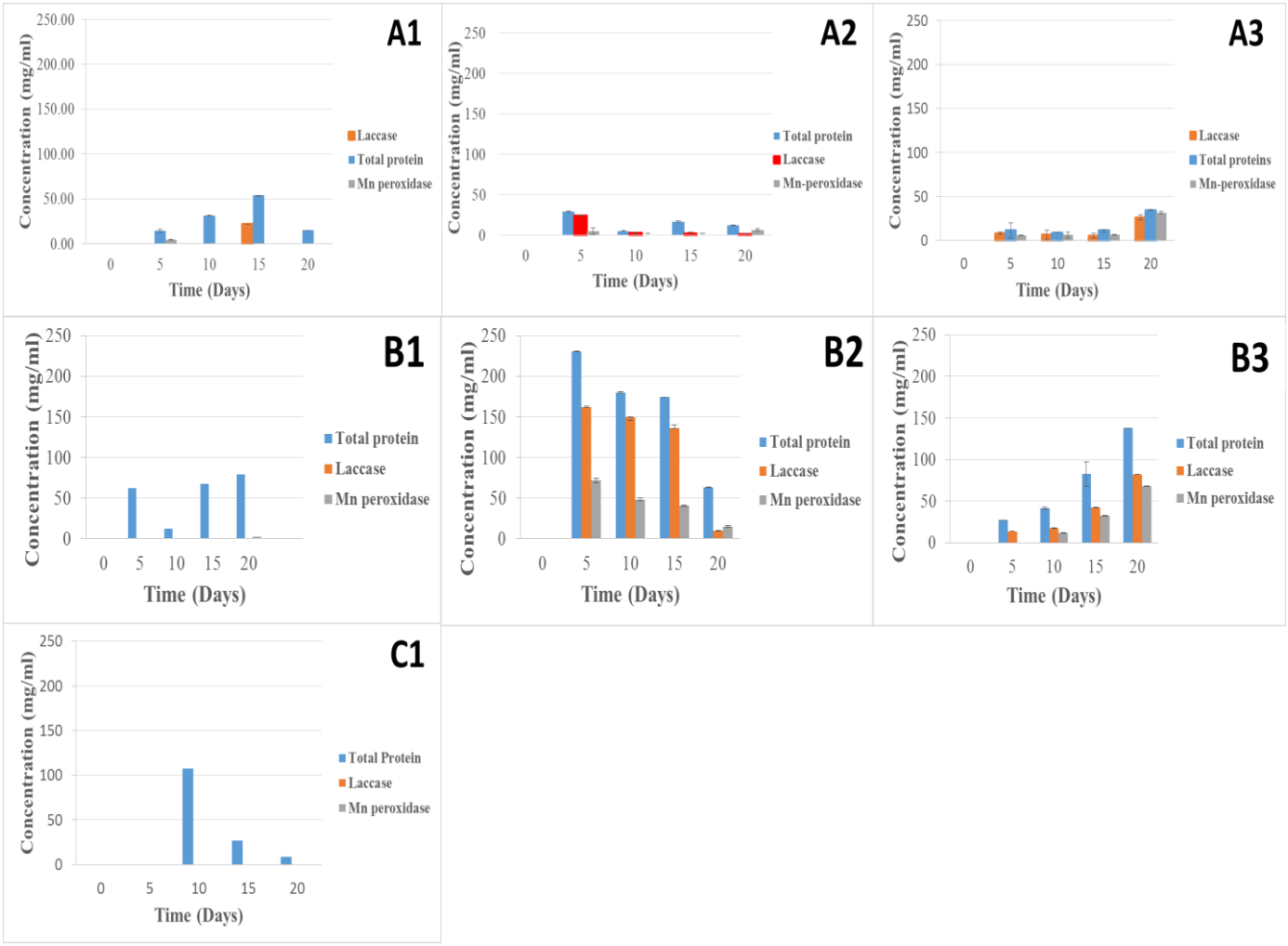

Figure 17. Enzymatic activity and total protein concentration of *P. citrinum* (RmBS 2-1-1). The graph shows total protein concentration, laccase, and Mn peroxidase activity in the supernatant. The isolates in the graphs are *P. citrinum* RmBS 2-1-2(A), *Trametes* sp., positive control (B), and *E. coli*, negative control (C). The 3 microorganims are growing in basal media with different carbon and energy source: glucose (1), Congo Red (2), and naphthalene (3).

F. solani (RmBS 3) growing in BM with naphthalene showed the best ability to produce a high biomass and having a low naphthalene concentration in the media. High concentrations of laccase assay end product are observed, which means there is high enzyme activity. Values range between 13.3 - 47.17 mg/ml (Figure 17, A3). The highest level was seen by day 15 with 47.17 mg/ml. Mn peroxidase activity iswas also observed. The concentrations of end product of the enzymatic reaction were lower than the laccase assay. Values range between 6.94-10.0mg/ml. The highest activity is observed by day 10 with 10.0 mg/ml.

On the other hand, when growing in BM with CR the concentrations of the end products for both laccase and Mn peroxidase assay are lower than when growing with naphthalene (Figure 17, A2). For the laccase assay values range between 0.84 – 7.76 mg/ml, while Mn peroxidase assay values range between 1.6 – 15.6 mg/ml.

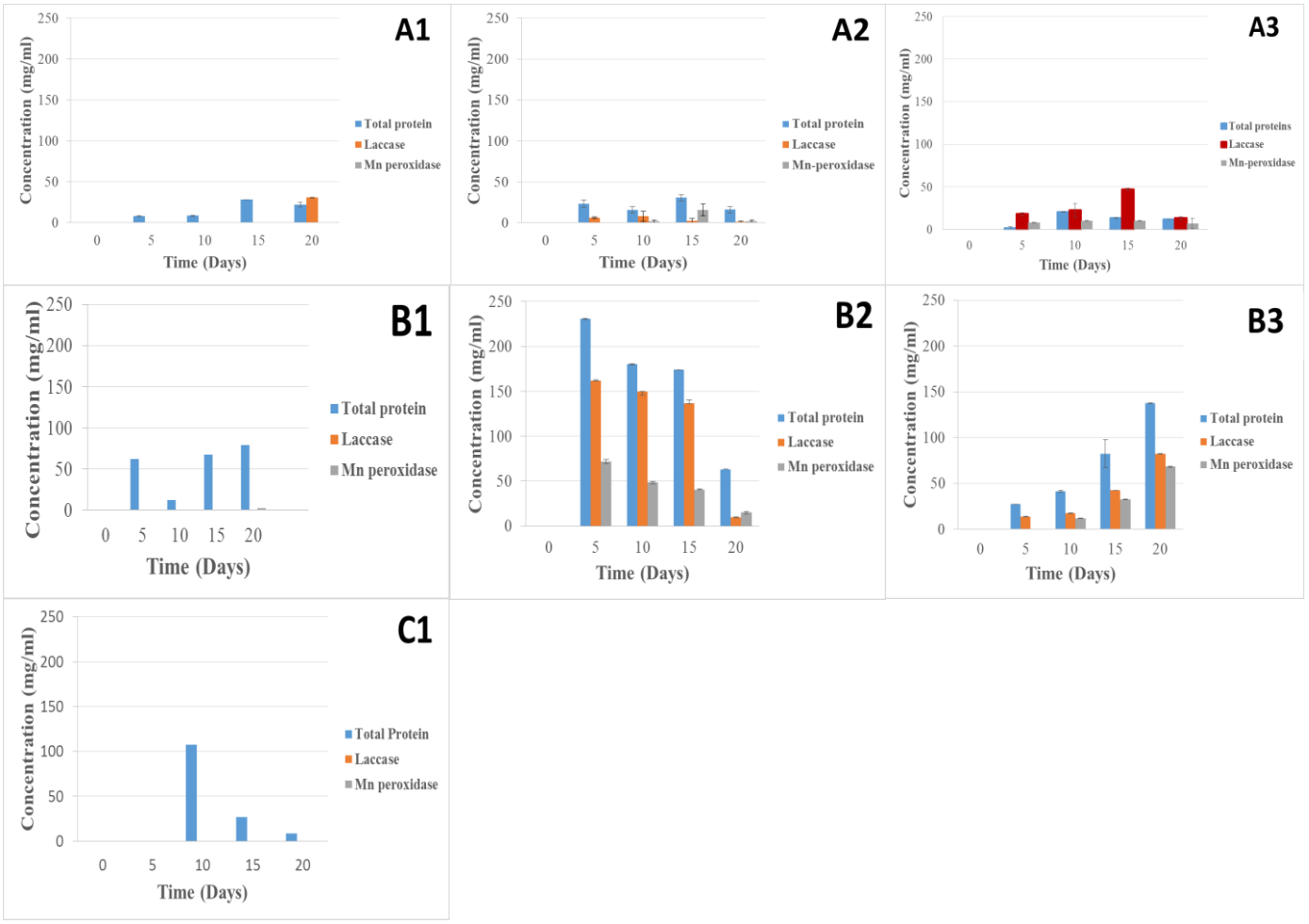

Figure 18. Enzymatic activity and total protein concentration of *F. solani* (RmBS 3). The graph shows total protein concentration, laccase, and Mn peroxidase activity in the supernatant. The isolates in this graphs are *F. solani* RmBS 3 (A), *Trametes* sp., positive control (B), and *E. coli*, negative control (C). The 3 microorganims are growing in basal media with different carbon and energy source: glucose (1), Congo Red (2), and naphthalene (3).

3.4.2 Samples from La Parguera, Lajas

According to the desirability model, *A. caelatus* (IRmPL 5) was the isolate from la LP, when grown in BM with CR, which yielded a high biomass and low concentration of CR in the media. Laccase and Mn peroxidase assays, for both CR and naphthalene, showed concentrations of the reactions end product meaning there is enzymatic activity. *A. caelatus* (IRmPL 5) growing in BM with glucose shows no concentration of the laccase assay and is not until day 20 in the Mn peroxidase assay that a concentration 0f 18.5 mg/ml is seen (Figure 18, A1). For the laccase assay, high concentration were observed between 8- 28.1 mg/ml of end product, which also means there was a high activity of laccase in the supernatant (Figure 18, A2). Mn peroxidase assay end product concentration values range between 6.2-41.2 mg/ml.

Also when looking at the concentration yield when grown in BM with naphthalene there were also high values for the laccase assay end product. Values ranging between 10.8-44.7 mg/ml, the highest was observed on day 5 (Figure 18, A3).

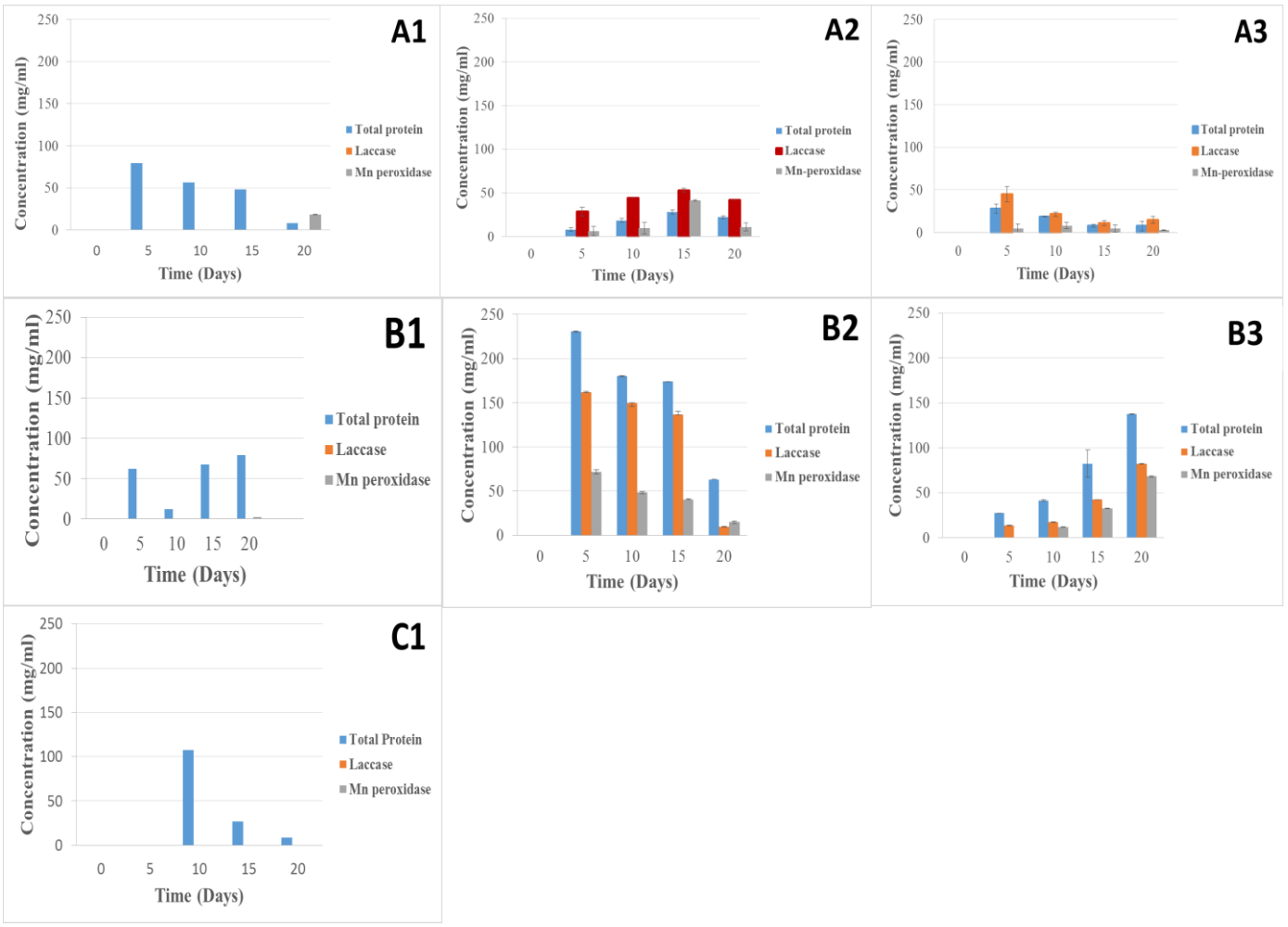

Figure 19. Enzymatic activity and total protein concentration of *A. caelatus* (IRmPL 5). The graph shows total protein concentration, laccase, and Mn peroxidase activity in the supernatant. Is *A. caelatus* (IRmPL 5) (A), is *Trametes* sp., positive control (B), and *E. coli*, negative control (C). The 3 microorganims are growing in basal media with different carbon and energy source: glucose (1), Congo Red (2), and naphthalene (3).

P. lilacinum IRmPL 5 growing in BM with glucose (Figure 1, A1) shows no activity levels of laccase or Mn peroxidase, but it shows different concentrations of total proteins during the 20 days. This isolate is producing other proteins, but not the lignolytic enzymes. On the other hand, when *P. lilacinum* IRmPL 5-1e-2 is growing in BM with Congo Red or naphthalene the activities of laccase and Mn peroxidase are observed. When *P. lilacinum* IRmPL 5-1e-2 is growing in BM with CR the activity levels of laccase are lower than the levels of Mn peroxidase. The highest activity level of laccase was 16 mg/ml by day 15 while the highest level of Mn peroxidase was 26 mg/ml by day 10. However, when *P. lilacinum* IRmPL5-1e-2 is growing in BM with naphthalene the activity levels of laccase were higher that the Mn peroxidase. The concentration levels of laccase ranged between 3.28 mg/ml to 20.30 mg/ml by day 20. The lowest concentration level was observed by day 10. The activity levels of Mn peroxidase were lower than the laccase levels ranging from 5.66 mg/ml to 11.57 mg/ml, this last one on day 10.

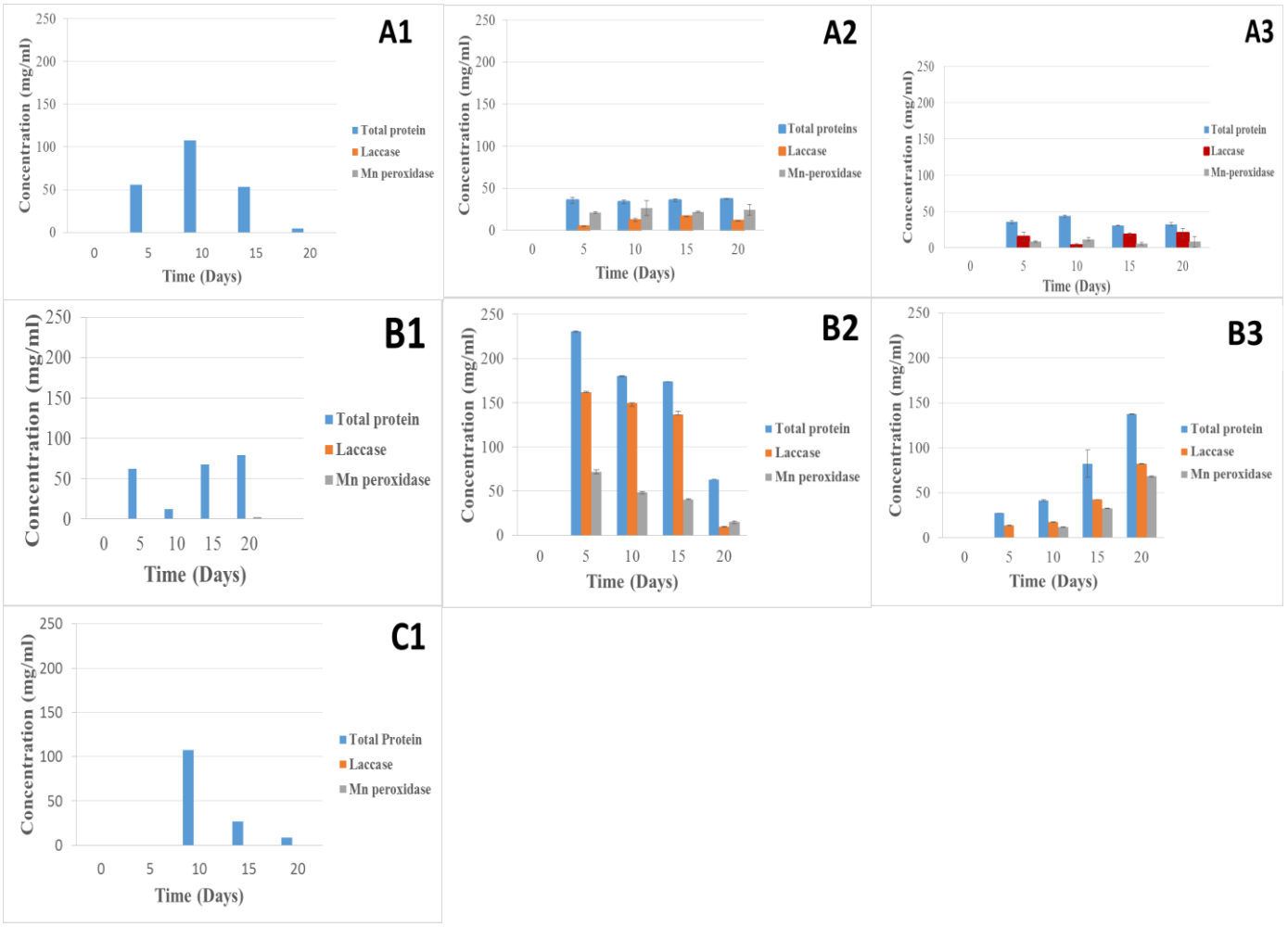

Figure 20. Enzymatic activity and total protein concentration of *P. lilacinum* (RmPL 5-1e-2). The graph shows total protein concentration, laccase, and Mn peroxidase activity in the supernatant. (A) Is *P. lilacinum* (RmPL 5-1e-2), (B) is *Trametes* sp., positive control, and (C) *E. coli*, negative control. The 3 microorganims are growing in basal media with different carbon and energy source: (1) glucose, (2) Congo Red, and (3) naphthalene.

For the lignin peroxidase (LiP) assay, methylene blue was used as substrate and the percentage of decolorization of the reaction is measured at 650 nm. Low percentages represent low levels of lignin peroxidase activity in the supernatant. Experimental samples and controls presented low decolorization percentages of supernatant from BM with glucose (Figure 20, A), which means there is little or no activity of LiP in the medium. Negative control *E. coli* did not show any reaction to lignin peroxidase assay in any of the carbon sources under study.

P. citrinum (RmBS 2-1-1) growing in BM with naphthalene showed low decolorization percentages between 3-7% (Figure 20 A), meaning there was low activity of this enzyme. Meanwhile, *Penicillium citrinum* (RmBS 2-1-1) growing in BM with CR showed around 35% of decolorization on day 15 (Figure 20 A), this being the highest value observed.

F. solani (RmBS 3) showed higher decolorization percentages in BM with naphthalene (5-15%) than in BM with CR (1-5%) (Figure 20B). Higher activity of LiP was observed when grown in BM with CR than wit naphthalene. Meanwhile, *Aspergillus caelatus* (IRmPL 5) growing in BM with CR had decolorization percentages between 2.5-7%, whereas in BM with naphthalene values go from 2-6% and by day 20 it spiked to 15%.

Purpureocillium lilacinum (IRmPL 5-1e-2) (Figure 20D) showed the highest percentage of decolorization between 20-50% while growing in BM with glucose. When growing in BM with CR decolorization percentages ranged between 3-18% and when growing in BM with naphthalene percentages ranged between 8-13%. In contrast to the positive control, *Trametes* sp, the isolates decolorization percentages were low.

None of the samples showed higher than 40% of decolorization indicating that LiP is somewhat induced by the carbon sources but in low levels. Overall, we have observed enzymatic activity by the the fungal isolates when growing in Congo Red or naphthalene.

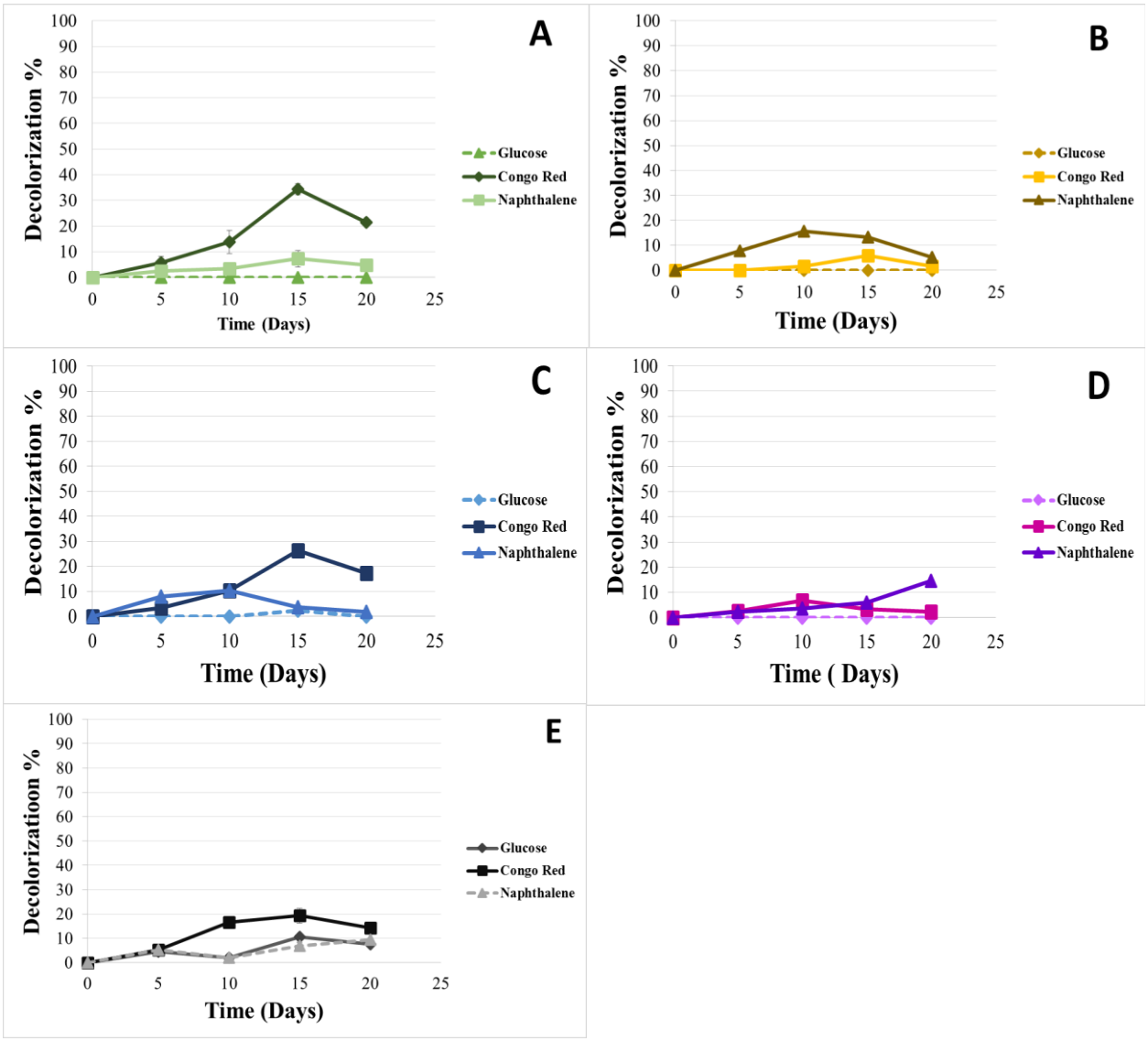

Figure 21. LiP assay with methylene blue decolorization percentages. The figure shows a series of bar graphs illustrating decolorization percentages in the LiP assay using methylene blue as substrate. The assay was done for samples *P. citrinum* (RmBS 2-1-1) (A), *F. solani* (RmBS3) (B), *A. caelatus* (IRmPL 5) (C), and *P. lilacinum* (RmPL 5-1e-2). The assay was also done for positive control *Trametes* sp. (E) and negative control *E. coli* (not shown).

4 Discussion

Most fungal research in mangrove ecosystems in Puerto Rico has mainly focused on characterization and diversity of species. The main objective of this study was to establish the ability of some fungal isolates from *Rhizophora mangrove* wood to use other carbon and energy sources, especially xenobiotic such as Congo Red or naphthalene through the lignin degradation pathway. Since lignolytic enzymes have low substrate specificity, compounds with similar structure to lignin might induce the production of such enzymes. Nineteen out of the twenty studied isolates were Ascomycetes. Normally, when talking about lignin degradation the first thought is about Basidiomycetes, which are well known for having this ability. It follows that not only Basidiomycetes have this ability, but fungi from other phyla can also degrade lignin because they can also produce lignolytic enzymes.

From Bahía Salinas, Cabo Rojo isolates of *Purpureocillium lilacinum*, *Penicillium citrinum*, and *Fusarium solani* were selected. From La Parguera, Lajas isolates of *Purpureocillium lilacinum, Phoma* sp, and *Aspergillus caelatus* were selected. The six isolate species have previously been reported from mangrove ecosystems (Suryanarayanan et al. 1998, Ananda and Sridhar 2002, Wu et al. 2010, 2015, Garzoli et al. 2015). Most of the species have lignolytic abilities previously reported (Kumari et al. 2002, Wu et al. 2010, Junior et al. 2012), except for *Aspergillus caelatus* and *Purpureocillium lilacinum*. These six isolates were selected due to their ability of decolorizing BM with Congo Red compared with the other isolates. Samples from Bahía Salinas, Cabo Rojo by day 10 had reached decolorization percentages between 87- 100%. This shows that all of the isolates from this site have a high capability of using Congo Red as their only carbon and energy source. Samples from La Parguera, Lajas ranged between 21-70%

of medium decolorization for the same period of incubation. The isolates from this site were slower in decolorizing the BM with Congo Red.

When the isolates were growing in BM with CR instead of presenting their normal mycelia color, isolates grew with a red color like the medium. Studies with *Penicillium* use Congo Red as a marker to detect the production of cellulases and xylanases (Sazci et al. 1986, Picart et al. 2007) because of its high affinity for polysaccharides. This can explain why the change in color of the isolates. Since Congo Red has a high affinity to polysaccharides it might be attaching to these compounds (Sazci et al. 1986, Picart et al. 2007).

Previous studies have reported other *Penicillium* species (e.g. *P. chrysogenum* and *P. oxalicum*) as being able to degrade Congo Red from culture media (Bhattacharya et al. 2011, Saroj et al. 2014). Even though only a few *Penicillium* species have been described as being able to degrade azo dyes, others have also been reported to possess a lignin-degrading system (Torres et al., Leitão 2009, Saroj et al. 2014). These enzymes play an important role in hydrolyzing plant cell wall. Most lignolytic fungi show high levels of these enzymes before lignin transformation (Leitão 2009). *Penicillium citrinum* is also found associated to red algae *Actinotrichia fragilis*, known for producing Citrinadin A, an alkaloid anti-cancer compound (Tsuda et al. 2004, Raghukumar 2008), therefore is considered to be common in marine environments such as mangroves.

In the same way, when evaluating the three selected isolates from La Parguera, Lajas, *Aspergillus caelatus* (IRmPL 5) showed the highest biomass when growing in BM with Congo Red of 44. 88 mg, with a decolorization of 97.52% and a concentration of 0.0057 mg/ml of Congo Red left in the medium by day 20. To our knowledge, there are no previous report about

Aspergillus caelatus being able to use Congo Red as its only carbon energy source and promoting lignolytic activity while growing in this source.

Phoma sp. has been reported previously to be found in marine environments (Suryanarayanan et al. 1998, Alias and Jones 2000, Ananda and Sridhar 2002), also it has previously been used in bioreactors to treat textile dye, and proven to be very effective (Junghanns et al. 2012). Torres et al (2011) reported partial degradation of Congo Red by *Phoma* sp. It is also known for producing thiodiketopiperazines (TDKPs), which can function as antiproliferatives, cytotoxic, antibacterial, and histone methyltransferase inhibitors (Kong et al. 2014).

To further study the capability of these isolates to use a different carbon and energy sources other than lignin, the six isolates were tested in an experiment using naphthalene as their only carbon and energy source. In this case for samples of Bahía Salinas growing in BM with naphthalene, *Fusarium solani* produced a higher biomass of 27.33 mg and lower the concentration of naphthalene in the medium to 0.0522 mg/ml by day 20. This result agrees with other reports where *F. solani* is shown to degrade PAHs more complex than naphthalene such as benzoαpyrene (Rafin et al. 2000, Li et al. 2008, Peng et al. 2008, Thion et al. 2012, Olajire and Essien 2014). *Purpureocillium lilacinum* showed to produce the highest biomass (184.31 mg) and lowering the concentration of naphthalene to 1.31 mg/ml after 15 days. *Purpureocillium lilacinum* is an entomopathogen used in biocontrol of agricultural pests, but is also an opportunistic agent of medical importance to humans since it can infect immune compromised patients (Luangsa-Ard et al. 2011, Castillo Lopez et al. 2014). There is little or no information about this species being able of degrading xenobiotics. To our knowledge, this might be the first report of *Purpureocillium lilacinum* being able to use naphthalene as its only carbon and energy

source. The biomass produced by *P. lilacinum* is much higher than the biomass produced by *F. solani* by day 15, but the concentration of naphthalene in the medium is not as low as with *F. solani*. In this case, naphthalene is a better carbon source for *P. lilacinum* resulting in biomass production, but *F. solani* is a better degrader of the xenobiotic.

Penicillium citrinum (RmBS 2-1-1) and *Aspergillus caelatus* (IRmPL5) growing in BM with Congo Red, as well as *Fusarium solani* (RmBS 3) and *P. lilacinum* (IRmPL 5-1e-2) growing in BM with naphthalene were the leading isolates in these experiments. In order to get the best results, meaning a high biomass and low concentration of xenobiotics, these four isolates are the best choices.

It seems that laccases are the primary enzymes to act in the process of removal/degradation of the xenobiotics. In both cases, *P. citrinum* (RmBS 2-1-1) growing in CR and *P. lilacinum* (IRmPL 5-1e-2) growing in naphthalene, higher concentration of laccase was observed during the first days and approaching day 20 the concentrations started to decrease while the concentration of the other enzymes started to increase. Laccases act first on the oxidation/cleavage of phenolic lignin units because they have a low redox potential compared to the lignolytic peroxidases (Martínez et al. 2005).

Interestingly, *P. lilacinum* (IRmPL 5-1e-2) from La Parguera, Lajas had a better performance growing in BM with naphthalene than *P. lilacinum* (RmBS 1-2b) which was isolated from Bahía Salinas, Cabo Rojo. Most of the microbial consortia found in mangrove environments can be comparable between sites but their abilities can change depending on nutrients, compounds and matter available for them to metabolize (Ewel et al. 1998, McKew et al. 2007, Dos Santos et al. 2011, Mendes and Tsai 2014). Even though samples were collected from two *Rhizophora mangle* areas, the environment surrounding them were different. From Bahía Salinas, Cabo Rojo

trees were found along the shore with some of their roots submerged in sea water while the other half was attached to the sand. In La Parguera, Lajas samples were collected from *Rhizophora mangle* trees offshore, more into the sea. Evidently, the matter and compounds the microbes have available in these two zones is different. Both *P. lilacinum* samples were able to grow in BM with naphthalene, but La Parguera sample IRmPL 5-1e-2 has better ability of using naphthalene as it carbon and energy source. This behavior might be due to the constant exposure microbes have in La Parguera to boat activity, meanwhile in the Bahía Salinas there is little or no boat activity. Due to oil spills that happened around this region approximately 50 years ago the capability to degraded PAHs is higher and similar, although many years have passed. Even though PAHs are highly persistent in the environment if there are microbial communities in this area with the ability to degrade them, the number of contaminants might have been reduced along the years. Today microbes are using other compounds and organic matter readily available to them, suggesting that the newer communities are not adapted to using PAHs as their carbon and energy, but they do have the ability to induce the enzymes in their presence. On the other hand, samples from La Parguera are more expose to PAHs because of the boat activity in these area.

Knowing that these isolates possess the capability of using xenobiotics such as Congo Red or naphthalene as only carbon and energy source, they can be used as bioremediadors in polluted mangrove ecosystems. *Aspergillus caelatus* would be a good choice of an isolate to be use as a bioaugmentator.

5 Recommendations

To validate the ability of the fungal isolates screened in this study to use xenobiotics as carbon and energy source, the following suggestions are made.

- The fungal isolates should be tested with PAHs of higher molecular weight to prove their capability to degrade more complex molecules.
- Measure the amount of Congo Red and naphthalene that is mineralized/absorbed by the isolate and the residues left in the media. The remaining PAHs or derivative compounds of the initial contaminant will have to be extracted and to determine the mixture of residue compounds high-pressure liquid chromatography analysis should be performed.

6 Conclusions

In this study, we have presented the capability of some fungal isolates from *Rhizophora mangle* wood to use Congo Red or naphthalene as their only carbon and energy source. Also, the ability to promote lignolytic enzyme activity by these other two compounds was measure.

1. Mangrove possess a bigger diversity than expected of fungi capable of degrading xenobiotics.

2. To our knowledge *A. caelatus* and *P. lilacinum* have not been previously report to having xenobiotic degradation capabilities.

3. The selected four fungal isolates based on their performance would be a good choice for bioremediation studies.

4. They could be biostimulated or bioaugmented in the environment.

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APPENDICES

Appendix 1. Table of raw sequences from the 20 isolates.

Appendix 1.1 Sequences of isolates from Bahía Salinas, Cabo Rojo isolates.

Fungal isolate code	Similar to	Sequence
RmBS1	*Purpureocillium lilacinum*	ctactgatcgaggtcactctaagaagttgggcgttttacggcgtgaccgcctccgcgctccggtgcgaggtg tgtgctactacgcaggggaggctgcggcggggtcgccacwgcatttcgggggcggctggtgtgccgtccccc aacaccgaggcccccgggggggggctcgagggttgaaatgacgctcgaacaggcatgcccgccagaatgctgg cgggcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaattcacattacttatcgcatttcgc tgcgttcttcatcgatgccagaaccaagagatccgttgttgaaagttttgattcatttgtttttgcttgtgc aactcagagaagaaattccgcccgctgggcgtaatgcaagagagtttggggtccctgcggcgggcgcctggg tccggcgccggcgcgggggcaggcggccggggcgttcccgccgaggcaactgaggtaaggttcacagtgggt ttgggagttgtataactcggtaatgatccctccgctggttcaccaacggagaccttgttacgttttt
RmBS 1-2b	*Purpureocillium lilacinum*	tactgatcgaggtcactctaagaagttgggcgttttacggcgtgaccgcctccgcgctccggtgcgaggtgt gtgctactacgcaggggaggctgcggcggggtcgccactgcatttcgggggcggctggtgtgccgtccccca acaccgaggcccccgggggggggctcgagggttgaaatgacgctcgaacaggcatgcccgccagaatgctggc gggcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaattcacattacttatcgcatttcgct gcgttcttcatcgatgccagaaccaagagatccgttgttgaaagttttgattcatttgtttttgcttgtgca actcagagaagaaattccgcccgctgggcgtaatgcaagagagtttggggtccctgcggcgggcgcctgggt ccggcgccggcgcgggggcaggcggccggggcgttcccgccgaggcaactgaggtaaggttcacagtgggtt tgggagttgtataactcggtaatgatccctccgctggttcaccaacggagaccttgtacgctttttt
RmBS 2	*Aspergillus micronesiensis*	aggtcacctgaaaaaaggatgatctgcgtgggctggcgccggccgggcctacagagcgggtgacaaagcccc atacgctcgaggaccggacgcggtgccgccgctgcctttcgggcccgtccccgggggggacgaggacccaata caagccgggcttgagggcagtaatgacgctcggacaggcaygccccccggaataccaggggggcgcaatgtgc gttcaaagactcgatgattcactgaattctgcaattcacattagttatcgcatttcgctgcgttcttcatcg atgccggaaccaagagatccattgttgaaagttttaactgattacaaagaatcggattcatacaggctttca gaaacagtgttcgtgttggggtctccggcgggcgcgggcccgggggcagaagccccccggcggccagctagg ctggcgggcccgccgaagcaacagtggtacagtagtcacgggtgggaggttgggccacgaggaccctcactc ggtaatgatccttccgcaggttcacctacggaaaccttgtmkttttttt
RmBS 2-1-1	*Penicillium citrinum*	tccgaggtcacctgagataattaaaggttgggggtcggctggcgccggccgggcctactagagcgggtgacg aagccccatacgctcgaggaccggacgcggtgccgccgctgcctttcgggcccgtccccccggcgggggggga

Appendix 1.1 (cont.)

		cggggcccaacacacaagccgggcttgagggcagcaatgacgctcggacaggcatgccctccggaataccag agggcgcaatgtgcgttcaaagactcgatgattcactgaattctgcaattcacattagttatcgcatttcgc tgcgttcttcatcgatgccggaaccaagagatccgttgttgaaagttttaactaatttcgttataggtctca gactgcaacttcagacagcgttcaggggggccgtcggcgggcgcggggcccgccgaggcaacataggttcgg gcaacacgggtgggaggttgggccccgaggggcccgcactcggtaatgatccttccgcaggttcacctacgg aaaccttgttacgattttt
RmBS 3	*Fusarium solani*	acctgattcgaggtcacttcagaagagttgggtgttttacggcgtggccgcgccgctctccagtcgcgaggt gttagctactacgcgatggaagctgcggcgggaccgccactgtatttgggggacggcgtgtgcccacagaga ggctccgccgatccccaacgccaggcccgggggcctgagggttgtaatgacgctcgaacaggcatgcccgcc agaatactggcgggcgcaatgtgcgttcaaagattcgatgattcactgaattctgcaattcacattacttat cgcatttcgctgcgttcttcatcgatgccagagccaagagatccgttgttgaaagttttaatttatttgctt gttttactcagaagaaacattatagaaacagagttaagggtcctctggcgggggcggcccgtttcacggggc cgtctattcccgccgaagcaacgtttaggtatgttcacagggttgatgagttgaataactcggtaatgatcc ctccgctggttcaccaacggagaccttgttckwtttt
Rm BS 5	*Purpureocillium lilacinum*	ggcggggtcgccactgcatttcgggggcggctggtgtgccgtcccccaacaccgaggcccccgggggggctc gagggttgaaatgacgctcgaacaggcatgcccgccagaatgctggcgggcgcaatgtgcgttcaaagattc gatgattcactgaattctgcaattcacattacttatcgcatttcgctgcgttcttcatcgatgccagaacca agagatccgttgttgaaagttttgattcatttgtttttgcttgtgcaactcagagaagaaattccgcccgct gggcgtaatgcaagagagtttggggtccctgcggcgggcgcctgggtccggcgccggcgcgggggcaggcgg ccggggcgttcccgccgaggcaactgaggtaaggttcacagtgggtttgggagttgtataactcggtaatga tccctccgctggttcaccaacggagaccttgttacgttttttwwmwytyymaa
RmBS 5-1b	*Penicillium griseofulvum*	actgatcgaggtcacctgagataattaaaggttgggggtcggctggcgccggccgggcctactagagcgggt gacgaagccccatacgctcgaggaccggacgcggtgccgccgctgcctttcgggcccgtccccccggcgggg gggacggggcccaacacacaagccgggcttgagggcagcaatgacgctcggacaggcatgccctccggaata ccagagggcgcaatgtgcgttcaaagactcgatgattcactgaattctgcaattcacattagttatcgcatt tcgctgcgttcttcatcgatgccggaaccaagagatccgttgttgaaagttttaactaatttcgttataggt ctcagactgcaacttcagacagcgttcaggggggccgtcggcgggcgcggggcccgccgaggcaacataggt tcgggcaacacgggtgggaggttgggccccgaggggcccgcactcggtaatgatccttccgcaggttcacct acggaaaccttgttagttttttttt

Appendix 1.1 (cont.)

RmBS 6-1	*Aspergillus terreus*	cctgatccgaggtcacctggaaaaacaagttgcaaataaatgcgtcggcgggcgccggccgggc ctacggagcggaagacgaagccccatacgctcgaggaccggacgcggtgccgccgctgcctttc gggcccgtcccccgggagccgggggacgagggcccaacacacaagccgggcttgagggcagcaa tgacgctcggacaggcatgccccccggaataccaggggggcgcaatgtgcgttcaaagactcgat gattcactgaattctgcaattcacattagttatcgcatttcgctgcgttcttcatcgatgccgg aaccaagagatccattgttgaaagttttaactgattgcaaagaatcacactcagactgcaagct ttcagaacagggttcatgttggggtctccggcgggcacgggcccgggggcgagtcgcccccggg cggccagcaacgctggcgggcccgccgaagcaacaaggtacaatagtcacgggtgggaggttgg gccataaagacccgcactcggtaatgatccttccgcaggttcacctacggaaaccttgttacga tttttwmcwccc
RmBS 10-1b	*Aspergillus fumigatus*	atgccccccggaataccgggggcgcawtgtgsgttcaaagactcratgattcactgaattctg caattcacattacttatcgcatttcgctgcgttcttcatcgatgccsgaaccragagatccgkt gktgaaagttttmactgattactatwatcmactcasactgcwtactttyagaacmscsttcatg ttgggstcttcggcgggcgcgggcccgggggcgcaaggcctctccgggggccgtcraaacgggg ggcccscccsaagcwacwaggtacgatagagmcgygtgggasgctgaacttagagggctctcact cgacwmtgattcttccrggggttcccctacaaamacrttgttacracttttwcttacc
RmBS 11	*Aspergillus niger*	tacctgatccgaggtcacctggaaaaatggttggaaaacgtcggcaggcgccggccaatcctac agagcatgtgacaaagccccatacgctcgaggatcggacgcggtgccgccgctgcctttcgggc ccgtccccccggagaggggacggcgacccaacacacaagccgggcttgagggcagcaatgacg ctcggacaggcatgcccccggaataccaggggggcgcaatgtgcgttcaaagactcgatgattc actgaattctgcaattcacattagttatcgcatttcgctgcgttcttcatcgatgccggaacca agagatccattgttgaaagttttaactgattgcattcaatcaactcagactgcacgctttcaga cagtgttcgtgttggggtctccggcgggcacgggcccgggggggcaaaggcgcccccccggcggc cgacaagcggcgggcccgccgaagcaacagggtataatagacacggatgggaggttgggcccaa aggacccgcactcggtaatgatccttccgcaggttcacctacggaaaccttgttacgttttttw yyttwccaa

Appendix 1.2. Sequences of isolates from La Parguera, Lajas

Fungal isolate code	Similar to	Sequence
RmPL 5-1e	*Tritirachium oryzae*	aaataagaatttggatttttttcaatccaattctgtatttttaaattcactaatgatccttccg cagttcacctacggaaaccttgttacgatttttwmytyccaagg
IIRmPL 2b	*Hypocrea lixii*	tgatccgaggtcacatttcagaagttgggtgtttaacggctgtggacgcgccgcgctcccgatgc gagtgtgcaaactactgcgcaggagaggctgcggcgagaccgccactgtatttcggagacggcca cccgctaagggagggccgatccccaacgccgacccccccggaggggttcgagggttgaaatgacgc tcggacaggcatgcccgccagaatactggcgggcgcaatgtgcgttcaaagattcgatgattcac tgaattctgcaattcacattacttatcgcatttcgctgcgttcttcatcgatgccagaaccaaga gatccgttgttgaaagttttgattcattttcgaaacgcctacgagaggcgccgagaaggctcaga ttataaaaaacccgcgaggggtatacaaaaagagttttggttggtcctccggcgggcgccttgg tccggggctgcgacgcacccggggcagagatcccgccgaggcaacagtttggtaacgttcacatt gggtttgggagttgtaaactcggtaatgatccctccgctggttcaccaacggagaccttgttacg tttttwwmytycca
RmPL5-1e-2	*Purpureocillium lilacinum*	gaaagttgggcgttttacggcgkgaccgcctccgcgctccggtgcgaggtgtgtgctactacsca rgggaggctgcggsggggtcgccactgcatttcgggggcggctggtgtgccgtcccccaasascs aggccccggggggggctcgagggttgaaatgacgctcgracasgcatgsccgccmgaatgctggc gggcgcaatgtgsgttcaaagattcsatgattcactgrattctgcaattcmcattacttatcgca tttcgctgcgttcttcatcgatgccagaaccaagagatccgttgttgaaagttktgawtcatttg tttttgcttgtgcaactcagagaagaaattmcgcccgctgggcgtratgcaagagagtttggggt ccctgcsgcgggcgcctgggtccggcgccggcgcgggggcaggcggccggggcgktcccgccrag gcaactgaggtaaggttcacagtgggkttgggagttgtataastcggtaatgatccctccgctgg ttcaccracggasaccttgttacsacwtttacttccca
RmPL 4-1-2	*Phoma sp.*	cctgatccgaggtcaaagtggaagaaggagcttcctggagagccgacctgccaatygcctagggc gcgacwgtgctgcgctcacggccggcgggtgcgggctgccactgattttgaggtgagtcgccgcg cgggggcgggacaagcacccaacaccaagctgagcttgagggtttaaatgacgctcgaacaggca tgccccacggaataccgaggggcgcaatgtgcgttcaaagattcgatgattcactgaattctgca attcacactacttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccattgtt

Appendix 1.2 (cont.)

		gaaagttttgattattggtatgtttttcagacaaatactgcaaactgcaaagcgtttaggggtc ctcgctggcgggcgaacccgccgaggaaacaaaaggtgctcaaagtcaagggtggtagaacgctt gcctggagtcggtgtctcctcgcaggcatcgtacttaggtaatgatccttccgaaggttcaccta cggaaaccttgttacgttttttwwmmyyycca
IRmPL 5-2	*Aspergillus niger*	tacctgatccgaggtcacctggaaaaatggttggaaaacgtcggcaggcgccggccaatcctaca gagcatgtgacaaagccccatacgctcgaggatcggacgcggtgccgccgctgcctttcgggccc gtccccccggagaggggggacggcgacccaacacacaagccgggcttgagggcagcaatgacgctc ggacaggcatgcccccccggaataccaggggggcgcaatgtgcgttcaaagactcgatgattcactg aattctgcaattcacattagttatcgcatttcgctgcgttcttcatcgatgccggaaccaagaga tccattgttgaaagttttaactgattgcattcaatcaactcagactgcacgctttcagacagtgt tcgtgttggggtctccggcgggcacgggcccgggggggcaaaggcgcccccccggcggccgacaag cggcgggcccgccgaagcaacagggtataatagacacggatgggaggttgggcccaaaggacccg cactcggtaatgatccttccgcaggttcacctacggaaaccttgttacgttttttwyyttwccaa
IIRmPL 12a	*Engyodontium album*	acctgattcgaggtcacattcggaaggtggggtgttttacggcgtggccgcgcccgggtcccggt gcgagtggttgttactacgcagaggtcgccgcggacgggccgccactccatttcggggccggcgt tgttagcgccgggtccccaacgccgggctccccccgaaaggggagctcgagggttgaaatgacgc tcgaacaggcatgcccgccagaattctggcgggcgcaatgtgcgttcaaagattcgatgattcac tgaattctgcaattcacattacttatcgcatttcgctgcgttcttcatcgatgccagaaccaaga gatccgttgttgaaagttttgattcatttgtatactgccttgcggcacactcagaagaaactgta aaaacagaagagtttgtggcctccggcgggcgccttggttccgggccgcgggaccgcaggggtt ccgggcgccggggcggctccgccgaagcaacatagaggtaatgttcacatgggtttgggagttgt aaactcggtaatgatccctccgctggttcaccaacggagaccttgttacgacttttacwtcc
IIRmPL 3a	*Purpureocillium lilacinum*	cctactgatcgaggtcactctaagaaagttgggcgttttacggcgtgaccgcctccgcgctccgg tgcgaggtgtgtgctactacgcaggggaggctgcggcggggtcgccactgcatttcgggggcggc tggtgtgccgtcccccaacaccgaggcccccgggggggctcgagggttgaaatgacgctcgaaca ggcatgcccgccagaatgctggcgggcgcaatgtgcgttcaaagattcgatgattcactgaattc tgcaattcacattacttatcgcatttcgctgcgttcttcatcgatgccagaaccaagagatccgt tgttgaaagttttgattcatttgtttttgcttgtgcaactcagagaagaaattccgcccgctggg cgtaatgcaagagagtttggggtccctgcggcgggcgcctgggtccggcgccggcgcgggggcag gcggccggggcgttcccgccgaggcaactgaggtaaggttcacagtgggtttgggagttgtataa ctcggtaatgatccctccgctggttcaccaacggagaccttgttacgtttttwwmytycaaa

Appendix 1.2 (cont.)

IRmPL 2-1	*Purpurecillium lilacinum*	ttcstgatcgaggtcaccggaagagacgagccccaaagggccgcctgggggaagaccagcaccgaccgagtccct cccgagcgggtgacagagccccatacgctcgaggacccgacgcggcgccgccactgcttttggggcgtgtccccg gggggacagcacccatcacccagccgtgctggagggcagaaatgacgctcggacaggcatgccccccggaatgcc agggggcgcaatgtgcgttcaaagattcgatgattcacggaattctgcaattcacattacttatcgcatttcgct gcgttcttcatcgatgccggaaccaagagatccgttgttgaaagttttaatgattcaatctctcactcagactca ctattcagacagggttccagggcgcttcggcgggcgcgggcccgggggcaagaaccccccggcgaccggggcgag gccccagtgggcccgccgaagcaacgcggtaacagtagacacgggtgggaggttgggctcgttcgaacccgcact cagtaatgatccttccgcaggttcacctacggaacggcttgaagttcagtgtacttgtccccccgatctctcggg gagcccgactatatcttaagcggaggctcggcctccacccactcacacttagtctgtgaacgttccccatggccc gcgagggctttaggggcttcgctgcggattgcccatggtctctgcgggaggcagaagtatccagatcgtctgtta ccgtaccctgtgcggtta acacaggcccccgagggatttctcccggcgggttggcgcgactggctttagggtgtccccgtcaattcgagagtg tcgccccgggggctttccgggagggaaagccctgggacttgcaacggcgccgacttgtttagagagtcgctcggg cctaccgcttgcgcggctgcagaggcttcggtaagtgtacactgcagaaggaatcaccattccagcagacttgca cggaaacctgtcrtmtt
IRmPL 2-1b	*Aspergillus sclerotiorum*	acctgatcgaggtcacctggagaaatgatggttgcttttcagcgtcggccagcgccggccgggcctacgagagcg gtgtgacaaagccccatacgctcgaggaccggacgcggtgccgccgctgcctttcgggcccgtccccccgggggg ggggacgaggacccaacacacaagccgggcttgagggcagcaatgacgctcggacaggcatacccccggaatac caggggtgcaatgtgcgttcaaagactcgatgattcactgaattctgcaattcacattaattatcgcatttcgc tgcgttcttcatcgatgccggaaccaararatccattgttgaaagttttaactgattgcgatacaatcgaactca gacgacaaaacttcagacagtgttcacgttggggtctccggcgggcgctcgcccgggggagggggttcccccccg gcggccgcgcgaacggcgggcccgccgaagcaacttggtacggtatacaagggtgggaggttgggccccgaagga accctcactcagtaatgatccttccgcaggttcacctacggaaaccttgttacgacttttamwtcac
IRmPL 5	*Aspergillus caelatus*	cctgatccgaggtcacctggaaaaatggttgttttgcgttcggcaagcgccggccgggcctacagagcgggtgac aaagccccatacgctcgaggatcggacgcggtgccgccgctgcctttggggcccgtccccccccgaagaggggac gacgacccaacacacaagccgtgcttgatgggcagcaatgacgctcggacaggcatgccccccggaataccaggg ggcgcaatgtgcgttcaaagactcgatgattcacggaattctgcaattcacactagttatcgcatttcgctgcgt tcttcatcgatgccggaaccaagagatccattgttgaaagttttaacwgattgcgatacaatcaactcagacttc actagatcagacagagttcgtggtgtctccggcgggcgcgggcccgggggctgatgccccccggcggccgtaaac ggcgggcccgccgaagcaactaaggttacagtaaacacgggtgggaggttgggctcgctaggaaccctacactcg gtaatgatccttccgcaggttcacctacggaaaccttgttacgatttttacttccaa

Appendix 2. ANOVA

Appendix 2.1. ANOVA of biomass produced by the Bahía Salinas, Cabo Rojo isolates growing in BM with Congo Red. N=5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 1: Biomass
Transform: Natural Log **Constant:** 0.1089
ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]

	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	502.19	29	17.32	62.51	< 0.0001	significant
A-Fungal Isolate	*82.80*	*5*	*16.56*	*59.78*	*< 0.0001*	
B-Day	*255.60*	*4*	*63.90*	*230.65*	*< 0.0001*	
AB	*163.79*	*20*	*8.19*	*29.56*	*< 0.0001*	
Pure Error	16.62	60	0.28			
Cor Total	518.82	89				

Appendix 2.2. ANOVA of decolorization percentages of Congo Red by the Bahía Salinas, Cabo Rojo isolates. N=5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 2: %Decolorization
ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]

	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	1736.57	29	59.88	347.97	< 0.0001	significant
A-Fungal Isolate	*713.31*	*5*	*142.66*	*828.99*	*< 0.0001*	
B-Day	*790.41*	*4*	*197.60*	*1148.25*	*< 0.0001*	
AB	*232.86*	*20*	*11.64*	*67.65*	*< 0.0001*	
Pure Error	10.33	60	0.17			
Cor Total	1746.90	89				

Appendix 2.3. ANOVA of Congo Red concnetrations in basal media inoculated with Bahía Salinas, Cabo Rojo isolates. N= 5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 3: Concentration

ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]						
	Sum of		**Mean**	**F**	**p-value**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	151.06	29	5.21	47.11	< 0.0001	significant
A-Fungal Isolate	*60.87*	*5*	*12.17*	*110.10*	*< 0.0001*	
B-Day	*58.06*	*4*	*14.52*	*131.28*	*< 0.0001*	
AB	*32.13*	*20*	*1.61*	*14.53*	*< 0.0001*	
Pure Error	6.63	60	0.11			
Cor Total	157.69	89				

Appendix 2.4. ANOVA of Congo Red concentration in basal media inoculated with La Parguera, Lajas isolates. N= 5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 1: Concentration
ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]

	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	0.98	29	0.034	189.09	< 0.0001	significant
A-Fungal Isolate	*0.41*	*5*	*0.081*	*457.85*	*< 0.0001*	
B-Day	*0.42*	*4*	*0.11*	*591.90*	*< 0.0001*	
AB	*0.15*	*20*	*7.357E-003*	*41.34*	*< 0.0001*	
Pure Error	0.011	60	1.780E-004			
Cor Total	0.99	89				

Appendix 2.5. ANOVA of biomass produced by La Parguera, Lajas isolates growing in BM with Congo RedN= 5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 2: Biomass
Transform: Inverse Sqrt **Constant:** 00701
ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	239.41	29	8.26	4310.94	< 0.0001	significant
A-Fungal Isolate	*95.67*	*5*	*19.13*	*9990.93*	*< 0.0001*	
B-Day	*119.11*	*4*	*29.78*	*15549.44*	*< 0.0001*	
AB	*24.64*	*20*	*1.23*	*643.25*	*< 0.0001*	
Pure Error	0.11	60	1.915E-003			
Cor Total	239.53	89				

Appendix 2.6. ANOVA of decolorization percentages of basal media with Congo Red inoculated with La Parguera, Lajas isolates. N= 5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 3: Decolorization %
Transform: Square Root **Constant:** 0.09951
ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1675.51	29	57.78	335.77	< 0.0001	significant
A-Fungal Isolate	*690.01*	*5*	*138.00*	*802.01*	*< 0.0001*	
B-Day	*762.84*	*4*	*190.71*	*1108.33*	*< 0.0001*	
AB	*222.67*	*20*	*11.13*	*64.70*	*< 0.0001*	
Pure Error	10.32	60	0.17			
Cor Total	1685.83	89				

Appendix 2.7. ANOVA of biomass produced by Bahía Salinas, Cabo Rojo isolates growing in basal media with naphthalene. N= 5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 1: Biomass
Transform: Base 10 Log Cosntant: 0.0406
ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	104.32	9	11.59	49.06	< 0.0001	significant
A-Fungal Isolate	*44.62*	*5*	*8.92*	*37.77*	*< 0.0001*	
B-Day	*59.70*	*4*	*14.93*	*63.17*	*< 0.0001*	
Residual	18.90	80	0.24			
Lack of Fit	*15.21*	*20*	*0.76*	*12.34*	*< 0.0001*	*significant*
Pure Error	*3.70*	*60*	*0.062*			
Cor Total	123.22	89				

Appendix 2.8. ANOVA of naphtahlene concentration in basal media inoculated with Bahía Salinas, Cabo Rojo isolates. N= 5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 2: Concentration
Transform: Square Root Constant: 0.00394852
ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	41.16	29	1.42	39.58	< 0.0001	significant
A-Fungal Isolate	*10.08*	*5*	*2.02*	*56.19*	*< 0.0001*	
B-Day	*22.62*	*4*	*5.65*	*157.66*	*< 0.0001*	
AB	*8.47*	*20*	*0.42*	*11.81*	*< 0.0001*	
Pure Error	2.15	60	0.036			
Cor Total	43.32	89				

Appendix 2.9. ANOVA of naphtahlene concentration in basal media inoculated with La Parguera, Lajas isolates. N= 5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 1: Concentration
Transform: Power Lambda: 1.68 Constant: 0
ANOVA for selected factorial model

Analysis of variance table [Classical sum of squares - Type II]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1446.27	29	49.87	1.408E+005	< 0.0001	significant
A-Fungal Isolate	*468.25*	*5*	*93.65*	*2.643E+005*	*< 0.0001*	
B-Day	*761.47*	*4*	*190.37*	*5.373E+005*	*< 0.0001*	
AB	*216.55*	*20*	*10.83*	*30561.55*	*< 0.0001*	
Pure Error	0.021	60	3.543E-004			
Cor Total	1446.29	89				

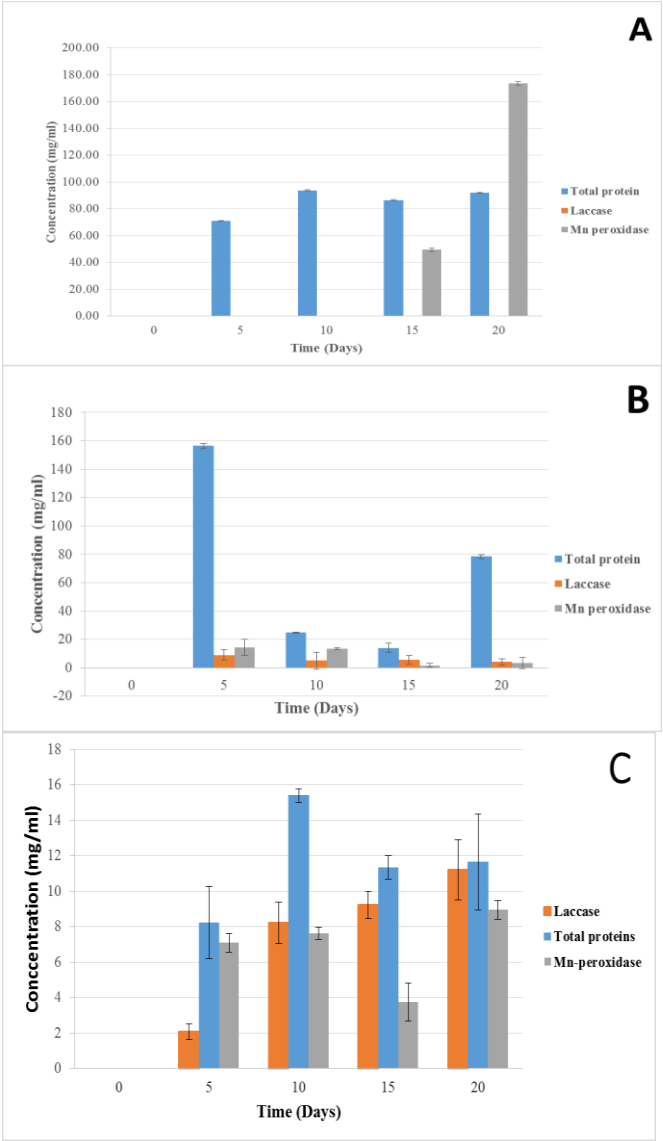
Appendix 2.10. ANOVA of biomass produced by La Parguera, Lajas isolates growing in basal media with naphthalene. N= 5, number of isolates measured for their biomass and n=3 is the number of replicates for each isolate.

Response 2: Biomass
Transform: Square Root Constant: 0.1863
ANOVA for selected factorial model

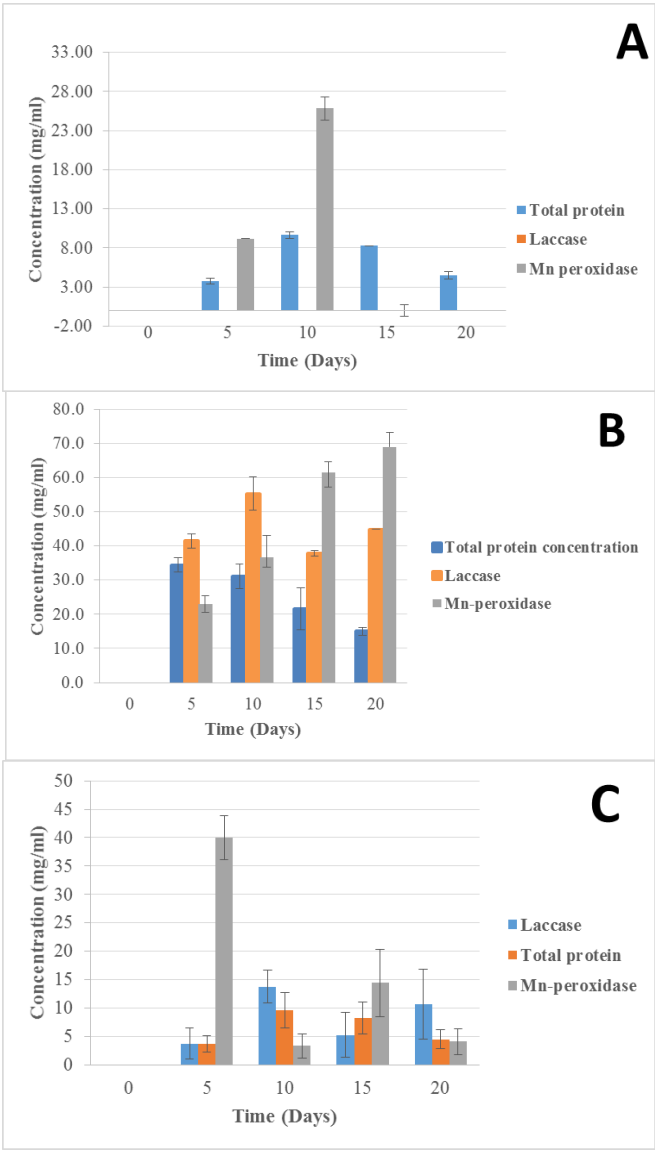
Analysis of variance table [Classical sum of squares - Type II]

	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	727.84	29	25.10	549.84	< 0.0001	significant
A-Fungal Isolate	*154.43*	*5*	*30.89*	*676.62*	*< 0.0001*	
B-Day	*240.33*	*4*	*60.08*	*1316.28*	*< 0.0001*	
AB	*333.08*	*20*	*16.65*	*364.85*	*< 0.0001*	
Pure Error	2.74	60	0.046			
Cor Total	730.58	89				

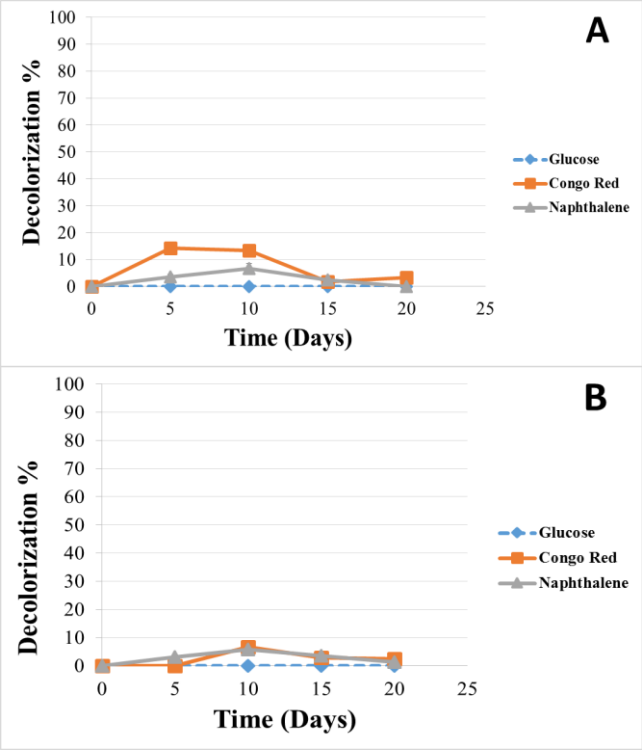
Appendix 3. Enzyme activity of samples *Purpureocillium lilacinum* (RmBS 1-2b) and *Phoma* sp. (RmPL4-1-2).



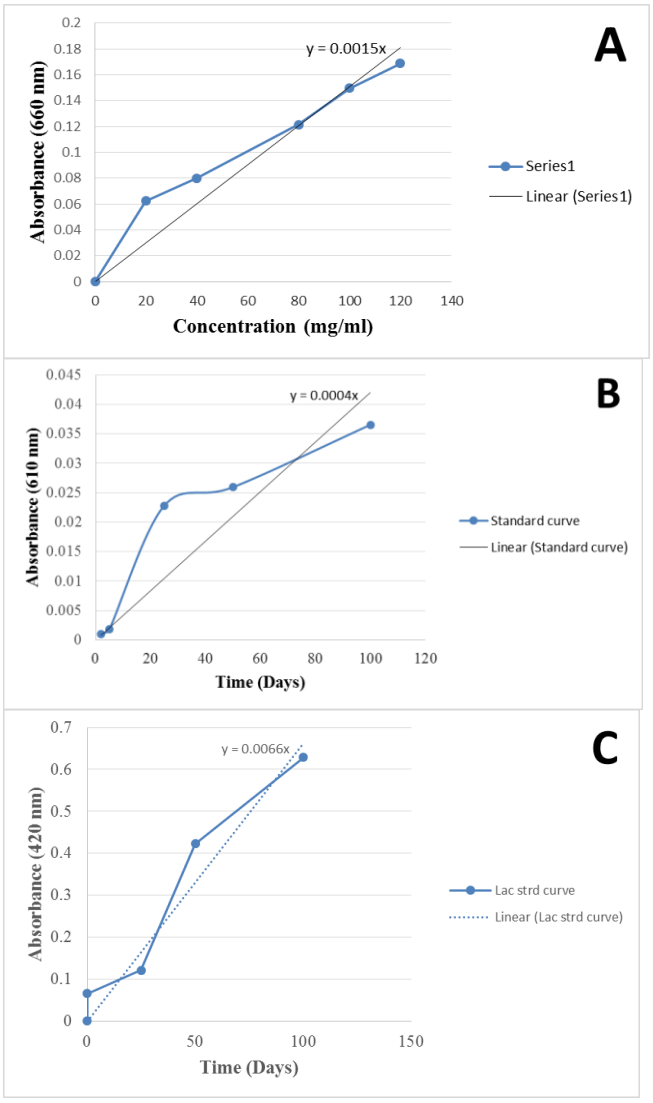
Appendix 3.1. Enzyme activity and total protein concentrations of *P. lilacinum* (RmBS 1-2b). The isolate was grown in basal media with glucose (A), Congo Red (B), and naphthalene (C).



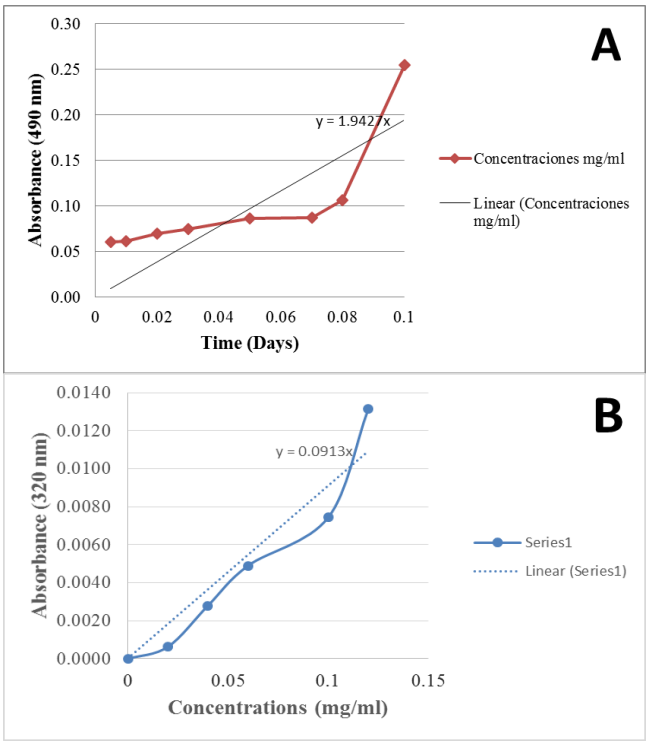
Appendix 3.2. Enzyme activity and total protein concentrations of *Phoma* sp. (RmPL 4-1-2). The isolate was grown in basal media with glucose (A), Congo Red (B), and naphthalene (C).



Appendix 3.3. LiP enzyme assay using methylene blue. The graphs show decolorization percentages of the reaction using methylene blue. *Purpurecillium lilacinum* (RmBS 1-2b) (A), and *Phoma* sp. (RmPL 4-1-2) (B).



Appendix 3.4. Standard curve to determine concentrations. The figure shows graphs of the standard curves design to determine concentration of total protein (A), Mn peroxidase (B), and laccase (C) in the supernatant.



Appendix 3.5. Standard curve of Congo Red and naphthalene. This figure shows the standard curves designed for known Congo Red (A) and naphthalene (B) concentrations.