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SUSAN GRACE KARP

DEVELOPMENT OF A BIOTREATMENT FOR DELIGNIFICATION OF SUGARCANE BAGASSE AND PRODUCTION OF LACCASES

CURITIBA
2012

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DEVELOPMENT OF A BIOTREATMENT FOR DELIGNIFICATION OF SUGARCANE BAGASSE AND PRODUCTION OF LACCASES

Tese apresentada como requisito parcial à obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia, do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Área de Concentração Agroindústria, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Carlos Ricardo Soccol
Co-orientadora: Prof.ª Dr.ª Vincenza Faraco

CURITIBA

2012

UNIVERSIDADE FEDERAL DO PARANÁ
Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia
Setor de Tecnologia

UFPR

RELATÓRIO DE DEFESA DE TESE DE DOUTORADO

Universidade Federal do Parana
Setor de Tecnologia
Curso de Doutorado em Proc s
Biotecnológicos

Aos quinze dias do mês de março de 2012, no Salão Nobre do Setor de Tecnologia, Segundo Andar do Prédio da Administração do Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pela Profª Drª Luciana Porto de Souza Vandenberghe, Coordenadora do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, a banca examinadora para a Septuagésima Quarta Defesa de Tese de Doutorado, Área de Concentração: Agroindústria e Biocombustíveis. Estiveram presentes no Ato, além da Coordenadora do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do colegiado do Curso de Doutorado em Engenharia de Bioprocessos e Biotecnologia, ficou constituída pelos Professores Doutores Fernando Araripe Gonçalves Torres (UNB), José Rodriguez Leon (UP), Adenise Lorenci Woiciechowski (UFPR), Luciana Porto de Souza Vandenberghe (UFPR) e Carlos Ricardo Soccol (UFPR - orientador da tese).

Às 9h00, a banca iniciou os trabalhos, convidando a candidata **Susan Grace Karp** a fazer a apresentação da Tese intitulada: **"Development of a Biotreatment for Delignification of Sugarcane Bagasse and Production of Laccases"**. Encerrada a apresentação, iniciou-se a fase de argüição pelos membros participantes.

Tendo em vista a tese e a argüição, a banca composta pelos professores Dr Fernando Araripe Gonçalves Torres, Dr José Rodriguez Leon, Drª Adenise Lorenci Woiciechowski, Drª Luciana Porto de Souza Vandenberghe e Dr Carlos Ricardo Soccol declarou a candidata aprovada (de acordo com a determinação dos Artigos 59 a 68 da Resolução 65/09 de 30.10.09).

Curitiba, 15 de Março de 2012

Prof Dr Fernando A Gonçalves Torres

Prof Dr José Rodriguez Leon

Profª Drª Luciana P S Vandenberghe

Profª Drª Adenise Lorenci Woiciechowski,

Prof Dr Carlos Ricardo Soccol

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ABSTRACT

The valorization of agro-residues by biological routes is a key technology that contributes to the development of sustainable processes and the generation of value-added products. Sugarcane bagasse is a lignocellulosic agro-residue generated in high amount by the sugar and alcohol industry in Brazil. The utilization of sugarcane bagasse as a feedstock has been limited because of the presence of lignin and hemicelluloses that restrain the efficient hydrolysis of cellulose. Delignification is a necessary pretreatment step in the process of converting plant biomass into fermentable sugars. The biological degradation of lignin can be performed by white-rot fungi that produce oxidative enzymes, especially laccases, manganese peroxidases and lignin peroxidases. Laccases receive much attention since they oxidize both phenolic and non-phenolic compounds and use oxygen as the final electron acceptor. They present many industrial applications related to the oxidation of phenolic substances. The objectives of this research were: to select a strain of basidiomycete able to degrade lignin and produce laccase in sugarcane bagasse and to perform its molecular identification by DNA sequencing; to optimize the production of laccase and to determine its mathematical model, through the process of solid state fermentation of sugarcane bagasse; to evaluate the effect of inducers and different concentrations of nitrogen on the pattern of laccase isoforms, produced in solid state fermentation of sugarcane bagasse by the selected strain; to evaluate the kinetics of biological delignification of sugarcane bagasse by the selected strain on solid state fermentation, under optimized conditions for laccase production; to recover and concentrate laccases and perform the enzymatic delignification of sugarcane bagasse. Among 45 strains of basidiomycetes, one of *Pleurotus* sp. presented a laccase activity significantly higher than all the other strains (183 U/L after 5 days of growth in semi-solid condition). The ITS region of the fungal rDNA was amplified by PCR and the strain was identified as *Pleurotus ostreatus* through ITS1-5.8rDNA-ITS2 sequence analysis. The production of laccase by the selected *P. ostreatus* was evaluated in solid state fermentation of sugarcane bagasse and the peak of enzymatic activity was reached at the 5^{th} day of fermentation (2.05 U/g). Among eight variables, $CuSO_4$ and $(NH_4)_2SO_4$ concentrations demonstrated to significantly influence laccase production. The replacement of ammonium sulfate by yeast extract and the addition of ferulic acid as inducer provided increases of, respectively, 5.7 and 2.0 fold in laccase production. Optimization of laccase production as a function of yeast extract, copper sulfate and ferulic acid concentrations was performed by response surface methodology and optimal concentrations were 6.4 g/L, 172.6 μM and 1.86 mM, respectively, the maximum laccase activity predicted by the model (R^2 0.8753) being 161.3 U/g. Experimentally, the maximum laccase activity of 151.6 U/g was produced at the 5^{th} day of solid state fermentation. Zymograms indicated the presence of six laccase isoforms (POXA1b, POXA3, POXC and three other isoforms). Results of protein identification by mass spectrometry confirmed the presence of POXC and POXA3 as the main isoenzymes, and also identified a glyoxal oxidase and three galactose oxidases. The fact that the isoenzyme POXA1b was not identified in the analyzed samples can be possibly explained by its sensitivity to protease degradation. Lignin content was reduced from 31.89% to 26.36% after 5 days and to 20.79% after 15 days by the biological treatment of solid state fermentation. Enzymatic hydrolysis reduced the lignin content from 31.89% to 14.98% in 12h.

Keywords: Biological delignification, sugarcane bagasse, laccase, solid state fermentation

RESUMO

A valorização de agro-resíduos por rotas biológicas é uma tecnologia chave que contribui para o desenvolvimento de processos sustentáveis e para a geração de produtos de alto valor agregado. O bagaço de cana é um agro-resíduo lignocelulósico produzido em grandes quantidades pela indústria sucroalcooleira no Brasil. A utilização do bagaço de cana como matéria-prima tem sido limitada pela presença de lignina e hemiceluloses, que restringem a hidrólise eficiente da celulose. A deslignificação é um pré-tratamento necessário no processo de conversão da biomassa vegetal em açúcares fermentescíveis. A degradação biológica da lignina pode ser realizada pelos fungos da podridão branca, que produzem enzimas oxidativas, especialmente lacases, manganês peroxidases e lignina peroxidases. As lacases recebem muita atenção pois oxidam tanto compostos fenólicos como não fenólicos e usam oxigênio como aceptor final de elétrons. Elas apresentam muitas aplicações industriais relacionadas à oxidação de substâncias fenólicas. Os objetivos desta pesquisa foram: selecionar uma cepa de basidiomiceto capaz de degradar lignina e produzir lacase no bagaço de cana, e realizar sua identificação molecular por sequenciamento de DNA; otimizar a produção de lacase e determinar seu modelo matemático, pelo processo de fermentação sólida do bagaço de cana; avaliar o efeito de indutores e diferentes concentrações de nitrogênio no padrão de isoformas de lacases produzidas em fermentação sólida do bagaço de cana pela cepa selecionada; avaliar a cinética de deslignificação biológica do bagaço de cana pela cepa selecionada em fermentação sólida, sob condições otimizadas para a produção de lacase; recuperar e concentrar a lacase e realizar a deslignificação enzimática do bagaço de cana. Dentre 45 cepas de basidiomicetos, uma cepa de *Pleurotus* sp. apresentou atividade de lacase significativamente superior às demais cepas (183 U/L após 5 dias de crescimento em condição semi-sólida). A região ITS do rDNA fúngico foi amplificada por PCR e a cepa foi identificada como *Pleurotus ostreatus* pela análise da sequência ITS1-5.8rDNA-ITS2. A produção de lacase pela cepa selecionada de *P. ostreatus* foi avaliada em fermentação sólida do bagaço de cana e o pico de atividade enzimática foi atingido ao 5º dia de fermentação (2.05 U/g). Dentre oito variáveis, as concentrações de $CuSO_4$ e $(NH_4)_2SO_4$ demonstraram influenciar significativamente a produção de lacase. A substituição do sulfato de amônio por extrato de levedura e a adição de ácido ferúlico como indutor conferiram aumentos de 5,7 e 2,0 vezes, respectivamente, na produção de lacase. A otimização da produção de lacase como função das concentrações de extrato de levedura, sulfato de cobre e ácido ferúlico foi realizada pela metodologia da superfície de resposta e as concentrações ótimas foram 6,4 g/L, 172,6 μM e 1,86 mM, respectivamente, e a atividade máxima de lacase prevista pelo modelo (R^2 0,8753) foi de 161,3 U/g. Experimentalmente, a máxima atividade de lacase de 151,6 U/g foi produzida ao 5º dia de fermentação no estado sólido. Zimogramas indicaram a presença de seis isoformas de lacases (POXA1b, POXA3, POXC e três outras isoformas). Os resultados de identificação protéica por espectrometria de massa confirmaram a presença de POXC e POXA3 como as principais isoenzimas, e também identificaram uma glioxal oxidase e três galactose oxidases. O fato de a enzima POXA1b não ter sido identificada nas amostras analisadas pode ser possivelmente explicado por sua sensibilidade à degradação por proteases. O teor de lignina foi reduzido de 31,89% para 26,36% após 5 dias e para 20,79% após 15 dias pelo tratamento biológico de fermentação sólida. A hidrólise enzimática reduziu o teor de lignina de 31,89% para 14,98% em 12h.

Palavras-chave: Deslignificação biológica, bagaço de cana, lacase, fermentação sólida

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INTRODUCTION

The valorization of agro-residues by biological routes is a key technology that contributes to the development of sustainable processes and the generation of value-added products. Sugarcane bagasse is a lignocellulosic agro-residue generated in high amount by the sugar and alcohol industry in Brazil. The utilization of sugarcane bagasse as a feedstock has been limited because of its lignocellulosic structure – presence of lignin and hemicelluloses – that restrain the efficient hydrolysis of cellulose, unless the material is previously treated. The aim of the pretreatment is to separate lignin and break the structure of lignocellulose, and can be performed by thermo-chemical processes or by the biological route, using enzymes or microorganisms.

The main objective of this research was to develop a process for biological delignification of the sugarcane bagasse with simultaneous production of laccases, which are enzymes that can perform enzymatic delignification and present many other industrial applications.

The specific objectives of this research were: to select a strain of basidiomycete able to degrade lignin and produce laccase in sugarcane bagasse and to perform its molecular identification by DNA sequencing; to optimize the production of laccases and to determine its mathematical model, through the process of solid state fermentation of sugarcane bagasse; to evaluate the effect of known inducers of laccase expression (copper sulfate and ferulic acid) and organic nitrogen (yeast extract) on the pattern of laccase isoforms – identified by zymograms and mass spectrometry – produced in solid state fermentation of sugarcane bagasse by the selected strain; to evaluate the kinetics of biological delignification of sugarcane bagasse under optimized conditions; to recover and concentrate the laccases to perform the enzymatic hydrolysis of sugarcane bagasse.

CHAPTER I

Up-to-date pretreatment strategies for delignification of sugarcane bagasse: a review

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Abstract

The valorization of agro-residues by biological routes is a key technology that contributes to the development of sustainable processes and the generation of value-added products. Sugarcane bagasse is an agro-residue generated by the sugar and alcohol industry in Brazil (186 million tons per year), composed essentially of cellulose (32-44%), hemicellulose (27-32%) and lignin (19-24%) (Soccol et al., 2011[b]). The conversion of sugarcane bagasse into fermentable sugars requires essentially two steps: pretreatment and hydrolysis. The aim of the pretreatment is to separate lignin and break the structure of lignocellulose, and it is one of the most critical steps in the process of converting biomass to fermentable sugars. The aim of this review is to describe different pretreatment strategies to promote delignification of the sugarcane bagasse by thermo-chemical and biological processes.

Keywords: Sugarcane bagasse, lignocellulose, pretreatment, delignification

1.1. Introduction

The valorization of agro-residues by biological routes is a key technology that contributes to the development of sustainable processes and the generation of value-added products. Brazilian economy is one of the most important agricultural-based economies in the world, being the first ranked worldwide in production and exportation of coffee, sugarcane, tropical fruits, beans and meat; second ranked in the production of ethanol, soybean and cassava and the third ranked in the production of corn. The residues generated by this intense agricultural activity represent potential feedstock that could be inserted in diverse production chains instead of being discarded (Singhania et al., 2009; Soccol and Vandenberghe, 2003).

Agro-industrial wastes are interesting substrates for fermentative processes since they are easily available, rich in carbon and often represent a problem of disposal (Gassara et al., 2010). There are several publications describing bioprocesses that use wastes such as hulls and bagasse as raw materials to produce ethanol, single-cell protein, mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, among other products (Soccol and Vandenberghe, 2003). An important example is the production of second-generation biofuels, i.e. those produced from non-food resources such as sugarcane bagasse.

Sugarcane bagasse is a lignocellulosic agro-residue generated in high amount by the sugar and alcohol industry in Brazil. One of the main challenges in the utilization of lignocellulosic biomass in fermentative processes is the transformation of the complex polysaccharides into simple sugars that can be assimilated by microorganisms. This can be achieved by chemical or enzymatic hydrolysis, preceded by appropriate

pretreatments that enhance the efficiency of hydrolysis. The aim of the pretreatment is to separate lignin and break the structure of lignocellulose, and it is one of the most expensive and least technologically mature steps in the process of converting biomass to fermentable sugars (Binod et al., 2012). The aim of this review is to describe different pretreatment strategies to promote delignification of the sugarcane bagasse by thermo-chemical and biological processes.

1.2. Characteristics of the sugarcane bagasse

Sugarcane bagasse is an agro-residue generated in high amount (186 million tons / year) by the sugar and alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane (Soccol et al., 2011[b]). Sugar mills generate approximately 270-280 kg of bagasse (50% moisture) per metric ton of sugarcane (Rodrigues et al., 2003).

Considering that about 92% of the sugarcane bagasse is burned in the industry for heat generation, if the remaining 8% were used for second generation ethanol production an additional ethanol yield of 2,200 L could be expected per hectare of sugarcane. The production of ethanol from sugarcane bagasse, through hydrolysis and fermentation, could yield about 280-330 L per ton of dry bagasse considering a cellulose content of 40% in the bagasse (Leite et al., 2009). Table 1.1 presents the chemical composition of sugarcane bagasse reported by different authors.

Table 1.1 – Chemical composition of sugarcane bagasse.

Component	Soccol et al. (2011[b])	Rocha et al. (2011)	Bertoti et al. (2009)
Cellulose	32-44%	45.5%	47.5-51.1%
Hemicellulose	27-32%	27%	26.7-28.5%
Lignin	19-24%	21.1%	20.2-20.8%
Extractives	-	4.6%	0.8-3% other
Ashes	4.5-9%	2.2%	compounds

Lignin is present in the cell wall and confers structural support, impermeability and resistance against microbial attack and oxidative stress, and among the components of lignocellulose it is the most recalcitrant to biodegradation. Lignin is formed from three precursor alcohols: *p*-hydroxycinnamyl (coumaryl) alcohol, which forms *p*-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units; and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, the syringyl units. Free radical copolymerization of these alcohols produces the heterogeneous, optically inactive, cross-linked and highly polydisperse polymer (Soccol et al., 2011[a]; Lee, 1997).

Cellulose is a linear polymer composed of D-glucose subunits linked by β-1,4 glycosidic bonds forming the dimmer cellobiose. These form long chains (or elemental fibrils) linked together by hydrogen bonds and intra- and intermolecular van der Waals forces. This polymer is usually present as a crystalline form and a small amount of non-organized cellulose chains forms amorphous cellulose. In the latter conformation, cellulose is more susceptible to enzymatic degradation (Pérez et al., 2002).

Hemicellulose is a polysaccharide with a lower molecular weight than cellulose. It is formed from D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-

methyl-glucuronic, D-galacturonic and D-glucuronic acids, depending on the hemicellulose source. Sugars are linked together by β-1,4- and sometimes by β-1,3-glycosidic bonds (Soccol et al., 2011[a]).

The conversion of sugarcane bagasse into fermentable sugars requires essentially two steps: pretreatment and hydrolysis. Hydrolysis is conducted in the presence of enzymes (exo-glucanases, endo-glucanases and cellobiases) or mineral acids and releases glucose units from the cellulose molecules (Hernández-Salas et al., 2009). Cellulose is the main targeted polysaccharide because it is composed only by D-glucose units, which is a fermentable sugar for most microorganisms. Sugars released from hemicellulose, most of them pentoses but also hexoses, however, are not fermented by most of the yeasts used for ethanol production.

The enzymatic hydrolysis of cellulose is a multi-step reaction that takes place in a heterogeneous system, in which insoluble cellulose is initially broken down at the solid-liquid interface via the synergistic action of endoglucanases (EC 3.2.1.4) and exo-glucanases / cellobiohydrolases (EC 3.2.1.91). Subsequently a liquid phase hydrolysis of soluble intermediate products takes place, i.e. short cellulo-oligosaccharides and cellobiose, that are catalytically cleaved to produce glucose by the action of β-glucosidase (EC 3.2.1.21) (Andric et al., 2010). The cost of utilities for enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because it is usually conducted at mild conditions and does not cause corrosion problems (Duff and Murray, 1996), however the cost of enzymes can limit their application.

1.3. Pretreatment strategies for delignification of sugarcane bagasse

Pretreatment is a very important step in the conversion of lignocellulose to fermentable sugars because the crystallinity of cellulose, degree of polymerization, moisture content, available surface area and lignin content are factors that hinder the action of the hydrolysis agents. The aim of the pretreatment is to separate lignin, break the structure of lignocellulose and enhance the accessibility to the cellulose chains, and it is usually performed by thermo-chemical processes. It is one of the most expensive and least technologically mature steps in the process of converting biomass to fermentable sugars. In order to be effective, pretreatment should avoid the need for reducing the size of biomass particles, preserve the pentose (hemicellulose) fractions, limit the formation of degradation products, minimize energy demands and the pretreatment agent should have low cost and be capable of recycling inexpensively (Binod et al., 2012).

Many methods have been used for pretreating lignocellulosic materials. These are steam explosion (Hernández-Salas et al., 2009; Hendriks and Zeeman, 2009; Balat et al., 2008; Ramos et al., 1992, 2000; Glasser and Wright, 1997), hot alkali washing (Hernández-Salas et al., 2009; Hendriks and Zeeman, 2009; Balat et al., 2008), dilute acid hydrolysis (Hernández-Salas et al., 2009; Balat et al., 2008; Zhang et al., 2007), ammonia fiber expansion (Hendriks and Zeeman, 2009; Balat et al., 2008), liquid hot water and wet oxidation (Hendriks and Zeeman, 2009; Martín et al., 2007), among others.

1.3.1. Steam explosion

Steam explosion is one of the most common methods for the pretreatment of lignocellulosic biomass (Soccol et al., 2011[a]), and can be performed in the presence or absence of a catalyst (alkali or acid). The grinded biomass is treated with high-pressure saturated steam, at temperatures varying from 160 to 260°C and pressures of 0.69 to 4.83 MPa, and then the pressure is quickly reduced, which makes the material undergo an explosive decompression. The process causes the disrupting of the material's structure, the partial degradation of hemicellulose and lignin transformation due to the high temperature, thus facilitating the subsequent hydrolysis of cellulose (Öhgren et al., 2007).

Rocha et al. (2012) reported the results of a steam explosion treatment of the sugarcane bagasse containing approximately 50% of moisture. Steam was injected in the reactor up to a pressure of almost 1.3 MPa equivalent to 190ºC, and was maintained for 15 min. After this period the reactor was suddenly depressurized. The treatment solubilized an average of 82.7±4.3% of the hemicelluloses. Cellulose was hydrolyzed at the ratio of 11.8±3.7%, this amount probably corresponding to the polymer amorphous region. Lignin solubilized at the proportion of 7.9±9.1%.

A study developed by Kaar et al. (1998) varying temperature and residence time in the steam explosion treatment of sugarcane bagasse showed that the furfural content, that represents the degradation of sugars, reported as a percentage of original xylose, was higher for higher severity and/or low temperature samples. The observed relationship with respect to temperature at constant severity could be due to the thermal decomposition/polymerization of the furfural in the reactor during the runs.

1.3.2. Alkaline pretreatment

Alkaline pretreatment has received a lot of attention lately because it can remove lignin from biomass, thus improving the reactivity of the remaining polysaccharides and removing acetyl groups and various uronic acid substitutions on hemicellulose (Chen et al., 2011). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross-linking xylan hemicelluloses and other components, for example, lignin and other hemicelluloses. Dilute NaOH treatment of lignocellulosic material causes swelling, leading to an increase of internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates and disruption of the lignin structure (Soccol et al., 2011[a]; Fan et al., 1987).

Regarding the delignification process developed by Rocha et al. (2012), the steam explosion pretreated bagasse was reacted with a NaOH solution 1.0% (w/v), using a solid-liquid ratio of 1:10 (w/v). The operation was carried out at 100ºC for 1 h, and there was an excellent removal of lignin from the biomass (92.7±3.9%). The process hydrolyzed 31.1±3.5% of the cellulose and the percentage of hemicellulose hydrolysis was 94.7±0.9%.

The chemical pretreatment for sugarcane bagasse developed by Aguiar et al. (2010) to produce exoglucanases and endogluconases resulted in better breakage of the fibers when using 2% H_2O_2 together with 1.5% NaOH, at 121ºC for 15 min. This treatment increased the cellulose level up to 1.2 times and decreased the hemicellulose content 8.5 times, promoting a better accessibility of the fungi to the fibers.

1.3.3. Acid pretreatment

Dilute acid hydrolysis can be an effective pretreatment process for sugarcane bagasse. Sulfuric acid is the most commonly used acid in pretreatment of sugarcane bagasse (Lavarack and Griffin, 2002) but other reagents, such as hydrochloric, nitric and phosphoric acids can also be used (Gámez et al., 2006; Rodríguez-Chong et al., 2004). The use of acetic acid and hydrogen peroxide has been reported by Tan et al. (2010) as a method for removing lignin prior to bagasse enzymatic hydrolysis.

According to Chen et al. (2011), the pretreatment using dilute sulfuric acid has been considered as one of the most cost-effective methods. The mixture of biomass and dilute acid solution is usually controlled at a moderate temperature by means of conventional heating or microwave-assisted heating, which is another effective route to pretreat biomass. The electromagnetic field used in microwaves may create non-thermal effects that also accelerate the destruction of crystal structures. The process developed by Binod et al. (2012) using microwave-alkali (1% NaOH) followed by acid pretreatment (1% H_2SO_4) and enzymatic hydrolysis gave an overall reducing sugar yield of 0.83 g/g dry sugarcane bagasse.

During hot acid pretreatment, some of the polysaccharides are hydrolyzed, mostly hemicelluloses. The resulting free sugars can degrade to furfural (from pentoses) and to 5-hydroxy-methyl-furfural or HMF (from hexoses) at high temperatures. These compounds are inhibitory for microorganisms, and their production means loss of fermentable sugars. Organic acids such as maleic and fumaric have been suggested as alternatives to avoid HMF formation (Kootstra et al., 2009).

1.3.4. Ammonia fiber expansion

Ammonia fiber expansion (AFEX) is a process in which liquid ammonia is added to the biomass under moderate pressure (100 to 400 psi) and temperature (70 to 200°C) before rapidly releasing the pressure (Bals et al., 2010). This process decrystallizes the cellulose, hydrolyses hemicellulose, removes and depolymerises lignin and increases the size and number of micropores in the cell wall, thereby significantly increasing the rate of enzymatic hydrolysis (Mosier et al., 2005).

As reported by Krishnan et al. (2010), the AFEX pretreatment improved the accessibility of cellulose and hemicelluloses in sugarcane bagasse during enzymatic hydrolysis by breaking down the ester linkages and other lignin carbohydrate complex bonds. The maximum glucan conversion of the AFEX pretreated bagasse and cane leaf residue by cellulases was approximately 85%, and the supplementation with hemicellulases during enzymatic hydrolysis improved the xylan conversion to 95-98%.

1.3.5. Organosolv

The treatment with organosolvents involves the use of an organic liquid and water, with or without the addition of a catalyst (acid or alkali). Organosolv pretreatments efficiently remove lignin from lignocellulosic materials through the partial hydrolysis of lignin bonds, resulting in a pulp enriched in cellulose. The addition of a catalyst can enhance the selectivity of the solvent with respect to lignin. Most of the hemicellulose sugars are also solubilized by this process (Mesa et al., 2011; Sun and

Cheng, 2002). This technique presents advantages when compared with aqueous based processes. In particular, the recovery of lignins and polyoses from the liquor is easily performed by distillation with the simultaneous recycling of solvents (Novo et al., 2011).

Mesa et al. (2011) demonstrated that the combination of a dilute-acid pretreatment followed by the organosolv pretreatment with NaOH under optimized conditions (60 min, 195ºC, 30% (v/v) ethanol) was efficient for the fractionation of sugarcane bagasse for subsequent enzymatic hydrolysis, yielding a residual solid material containing 67.3% (w/w) glucose, which was easily recovered by enzymatic hydrolysis. Novo et al. (2011) developed a process using glycerol-water mixtures and obtained a pulp with a residual lignin amount lower than 8%; extent of delignification close to 80%; and residual cellulose content higher than 80%.

1.3.6. Liquid hot water

Liquid hot water is a hydrothermal pretreatment, where pressure is applied to maintain water in the liquid state at elevated temperature. Temperatures in the range of 170-230°C and pressure higher than 5 MPa are commonly used (Talebnia et al., 2010). This subcritical fluid presents particular properties in relation to water at ambient conditions, i.e. dielectric strength and ionic product, and these properties can be easily controlled as functions of pressure and temperature (Schacht et al., 2008). The treatment presents high yields and low generation of undesired products (Hamelinck et al., 2005).

Allen et al. (1996) described a process to fractionate sugarcane bagasse and leaves using liquid hot water at 190-230ºC by rapid immersed percolation (45 s to 4

min). Over 50% of the biomass could be solubilized, and all of the hemicellulose together with more than 60% of the acid-insoluble lignin was solubilized, while less than 10% of the cellulose entered the liquid phase. The recovery of the hemicellulose as monomeric sugars after a mild post-hydrolysis exceeded 80% and less than 5% of the hemicellulose was converted to furfural.

1.3.7. Wet oxidation

Wet oxidation is a hydrothermal treatment, the process of treating material with water and air or oxygen at temperatures above 120ºC (McGinnis et al., 1983). Two types of reactions occur during wet oxidation: a low-temperature hydrolytic reaction and a high-temperature oxidative reaction (Martín et al., 2007).

Martín et al. (2007) demonstrated that alkaline wet oxidation at 195ºC during 15 min yielded a solid material with nearly 70% of cellulose, with a solubilization of approximately 93% of hemicelluloses and 50% of lignin, and an enzymatic cellulose convertibility of around 75%. Acid wet oxidation at 195ºC for 15 min gave a good fractionation of bagasse, but a significant part of the polysaccharides was lost, and the enzymatic convertibility of the pretreated material was poor.

1.3.8. Biological treatments

Delignification can be performed not only by thermo-chemical processes but also by the biological route, using enzymes or microorganisms. An example is the bleaching process of the wood pulp with ligninolytic enzymes that can provide mild and

clean strategies for pretreatment (Kuhad et al., 1997). The advantages of biological delignification over the previous methods may include mild reaction conditions, higher product yields and quality and fewer side reactions, less energy demand and less reactor resistance to pressure and corrosion (Lee, 1997). Moreover, the use of environmentally friendly technologies is a requisite to be assured in a sustainable industry.

Lignin decomposition in nature is primarily attributed to the metabolism of microorganisms. Among all other organisms, white-rot basidiomycetes degrade lignin more rapidly and extensively than other groups (Falcón et al., 1995). These microorganisms produce several ligninolytic enzymes (laccases, manganese peroxidases and lignin peroxidases) that catalyze one-electron oxidation of lignin units, producing aromatic radicals (Giardina et al., 2000). Lignin degradation is mainly attributed to the secondary metabolism or to restricted availability of nitrogen, carbon or sulphur, and it is normally not degraded as sole carbon and energy sources, requiring additional co-substrates such as cellulose, hemicellulose or glucose (Silva et al., 2010).

Some white-rot fungi preferentially attack lignin more readily than hemicellulose and cellulose. *Ceriporiopsis subvermispora*, *Phellimus pini*, *Phlebia* spp. and *Pleurotus* spp. belong to this group. Many white-rot fungi, however, exhibit a pattern of simultaneous decay characterized by degradation of all cell wall components. Examples of this group include *Trametes versicolor*, *Heterobasidium annosum* and *Irpex lacteus* (Wong, 2009).

Solid state fermentation is an interesting process to perform biological delignification because it mimics the natural environment of lignin-degrading fungi. The advantages of the solid state fermentation process over submerged fermentation include: smaller fermenter volume; lower sterilization energy costs; easier aeration;

reduced or eliminated costs for stirring and effluent treatment; lower costs for product recovery and drying; less favorable environment for many bacteria, lowering the risk of contamination (Lee, 1997; Soccol and Vandenberghe, 2003). The guiding principles to design solid-state fermenters for biological delignification should be, first, to provide optimum conditions for the activity of the fungus through effective mixing, heat removal and oxygen supply and, second, to keep the equipment as simple and inexpensive as possible (Reid, 1989).

One of the main disadvantages of the biological delignification by fermentation is the long incubation time. The process developed by Pellinen et al. (1989) to delignify kraft pulp and chemithermo-mechanical pulp (CTMP) using *Phanerochaete chrysosporium* presented delignification times of around two weeks, the kappa number being reduced from 33 to less than 10 for the kraft pulp and the lignin content decreasing from 26.5% to 21.3% for the CTMP. Reid (1989) estimated a cost of C$ 720 per batch for a hypothetical process to delignify 10 tons of aspen wood. The duration of the batch was considered as 8 weeks and the highest cost was attributed to the wood (C$ 56 per ton).

In comparison with solid state fermentation, enzymatic delignification processes demand less incubation time. Kuila et al. (2011) reported a maximum delignification of *Bambusa bambos* of 84% using laccase from *Pleurotus* sp. at 400 international units per mililiter (U/mL), after 8h of incubation. Highest reducing sugar yield from the enzyme-pretreated biomass was 818.01 mg/g dry substrate after 8 h of incubation time using the following enzyme loading: endoglucanase 1.63 U/mL; β-glucosidase 1.28 U/mL; exoglucanase 0.08 U/mL and xylanase 47.93 U/mL.

Recently, Moniruzzaman and Ono (2012) described an enhanced method to promote enzymatic delignification of wood chips, using ionic liquid swollen biomass in aqueous systems with the aim of overcoming difficulties with accessibility of the enzyme and solubility of substrates and products. Enzymatic delignification in ionic liquid aqueous media containing 5% ionic liquid resulted in around 50% delignification of wood biomass after 24 h in the presence of laccase, whereas only 10% delignification was obtained when original wood chips (without ionic liquid pretreatment) were used.

1.4. Ligninolytic enzymes

There are four major groups of ligninolytic enzymes produced by the white-rot fungi: lignin peroxidase (LiP; 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol:hydrogen-peroxide oxidoreductase; EC 1.11.1.14), manganese dependent peroxidase (MnP; Mn(II):hydrogen-peroxide oxidoreductase or manganese peroxidase; EC 1.11.1.13), versatile peroxidase (VP; EC 1.11.1.16) and laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2). However, the process of lignin biodegradation can be further enhanced by the action of other enzymes such as glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (veratryl alcohol oxidase; EC 1.1.3.7), pyranose 2-oxidase (glucose 1-oxidase; EC 1.1.3.4), cellobiose/quinone oxidoreductase (EC 1.1.5.1) and cellobiose dehydrogenase (EC 1.1.99.18) (Wong, 2009).

Both LiP and MnP belong to the class of peroxidases that oxidize their substrates by two consecutive one-electron oxidation steps with intermediate cation radical formation. Due to its high redox potential, the preferred substrates for LiP are nonphenolic methoxyl-substituted lignin subunits and the oxidation occurs in the

presence of H_2O_2 (Tuor et al., 1995; Wong et al., 2009) whereas MnP acts exclusively as a phenoloxidase on phenolic substrates using Mn^{2+}/Mn^{3+} as an intermediate redox couple (Tuor et al., 1995). Versatile peroxidases are a group of enzymes, primarily recognized as manganese peroxidases, which exhibit activities on aromatic substrates similar to that of LiP. These enzymes are not only specific for Mn (II), but also oxidize phenolic and non-phenolic substrates that are typical for LiP, including veratryl alcohol, methoxybenzenes and lignin model compounds in the absence of manganese (Wong, 2009).

Laccases are blue multicopper oxidases able to oxidize a variety of phenolic compounds including polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, with concomitant reduction of molecular oxygen to water (Autore et al., 2009; Dwivedi et al., 2011). They oxidize phenols and phenolic lignin substructures by one-electron abstraction with formation of radicals that can repolymerize or lead to depolymerization (Higuchi, 1989). These enzymes have been found to oxidize also non-phenolic compounds in the presence of a mediator (e.g., 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate or ABTS) (Wong, 2009). Laccases are more readily available and easier to manipulate than both LiP and MnP. Moreover, these enzymes find many industrial applications in the areas of food products, pulp and paper, textiles, nanobiotechnology, soil bioremediation, synthetic chemistry and cosmetics (Couto and Herrera, 2006).

The overall reaction catalyzed by these phenoloxidases is 4 benzenediol + O_2 ↔ 4 benzosemiquinone + $2H_2O$ (Wong, 2009). The laccase molecule is a dimeric or tetrameric glycoprotein, which usually contains four copper atoms per monomer distributed in three redox sites named T1, T2 and T3. In the resting enzymes, all four

coppers are in the 2^+ oxidation state (Couto and Herrera, 2006; Wong, 2009). Both intracellular and extracellular isoenzymes may be produced from a single organism. The monomeric proteins have a molecular mass ranging from 50 to 110 kDa (Thurston, 1994).

As described by Wong (2009), the first step of catalysis is an electron transfer between the reducing substrate and copper (Cu^{2+} to Cu^{+}) at the T1 site, which is the primary electron acceptor. The electrons extracted from the reducing substrate are transferred to the T2/T3 site, and the enzyme changes from its fully oxidized form to a fully reduced state. A successive four-electron oxidation, from four substrate molecules, is required to fully reduce the enzyme. The rate-limiting step in the catalytic cycle is the intramolecular electron transfer from T1 to the T2/T3 trinuclear copper site. The reduction of oxygen occurs at the T2/T3 site, and the diffusion of oxygen to the trinuclear site is also a rate-limiting step.

The highest amounts of laccases are produced by white-rot fungi, which are the only organisms able to mineralize all components of lignin to carbon dioxide and water. Fungal laccases are secreted into the medium by the mycelium of filamentous fungi (Couto and Toca-Herrera, 2007). Examples of microorganisms that produce laccase with high activity are *Trametes pubescens* (740,000 U/L) (Galhaup et al., 2002), *Coriolus hirsutus* (83,830 U/L) (Koroleva et al., 2002), *Trametes hirsuta* (19,400 U/L) (Rodríguez-Couto et al., 2006), *Trametes versicolor* (16,000 U/L) (Font et al., 2003), *Pycnoporus cinnabarinus* (10,000 U/L) (Meza et al., 2006), *Neurospora crassa* (10,000 U/L) (Luke and Burton, 2001), *Pleurotus ostreatus* (3,500 U/L) (Lenz and Hölker, 2004). *Pleurotus ostreatus* belongs to a class of white-rot fungi that produces laccases, manganese peroxidases but not lignin peroxidases (Giardina et al., 2000).

Laccases are secreted in multiple isoforms depending on the fungal species and the environmental conditions, and this variety is related to the diversity of their roles: lignin degradation/synthesis, fruiting bodies development, pigment production, cell detoxification (Piscitelli et al., 2011). The biochemical diversity of laccase isoenzymes appears to be due to the multiplicity of laccase genes; however, regulation of their expression can be substantially diverse between fungal species (Palmieri et al., 2003). The sequencing and annotation of the *Pleurotus ostreatus* PC 15 genome version 2.0 (Joint Genome Institute, 2011) indicates the presence of at least twelve genes of multicopper oxidases. Some of the corresponding enzymes have been purified and characterized, and these include POXA1b (Giardina et al., 1999), POXA1w and POXA2 (Palmieri et al., 1997), POXA3 (Palmieri et al., 2003) and POXC, previously named POX2 (Giardina et al., 1996) where POX means phenol oxidase. Other isoenzymes whose sequences have been determined are POX1 (Giardina et al., 1995), POX3 and POX4 (Joint Genome Institute, 2011). POXC is the most abundantly produced under all growth conditions examined according to Giardina et al. (1999).

Laccases can be industrially employed in the degradation of phenolic compounds, promoting their oxidation. Enzymatic oxidation is advantageous in relation to chemical because of its specificity, biodegradability and requirement of mild conditions. In the food and beverages industry laccases can be applied to remove undesired phenolics which are responsible for browning, haze formation and turbidity. In the pulp and paper industry, the biopulping and biobleaching processes use enzymes as alternatives to the conventional chemical processes. In the textile industry, the decolourization of dyes is the main application of laccases, and there are also applications in the fields of nanotechnology, bioremediation and synthetic chemistry

(Couto and Herrera, 2006). All these features indicate that the production of ligninolytic enzymes, especially laccases, could be considered as a process to integrate biological delignification within the concept of a lignocellulose biorefinery.

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CHAPTER II

Selection and molecular identification of a laccase-producing basidiomycete cultivated in sugarcane bagasse

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Abstract

The biological degradation of lignin can be performed by white-rot fungi that produce oxidative enzymes, especially laccases, manganese peroxidases and lignin peroxidases. Laccases receive much attention since they oxidize both phenolic and non-phenolic compounds and use oxygen as the final electron acceptor. They present many industrial applications related to the oxidation of phenolic substances. The objective of this work was to select a strain of basidiomycete able to degrade lignin and produce laccase in sugarcane bagasse and to perform its molecular identification by DNA sequencing. Among 45 strains of basidiomycetes, one of *Pleurotus* sp. presented a laccase activity significantly higher than all the other strains (183 U/L after 5 days of growth in semi-solid condition). The ITS region of the fungal rDNA was amplified by PCR and the strain was identified as *Pleurotus ostreatus* through ITS1-5.8rDNA-ITS2 sequence analysis. The production of laccase by the selected *P. ostreatus* was evaluated in solid state fermentation using sugarcane bagasse. The peak of enzymatic activity was reached at the 5th day of fermentation (2049 U/kg).

Keywords: Agro-industrial wastes, sugarcane bagasse, laccase, basidiomycete, molecular identification.

2.1. Introduction

Agro-industrial wastes are interesting substrates for fermentative processes since they are easily available, rich in carbon and often represent a problem of disposal (Gassara et al., 2010). There are several publications describing bioprocesses that use wastes such as hulls and bagasse as raw materials to produce ethanol, single-cell protein (SCP), mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, among other products (Soccol and Vandenberghe, 2003). An important example is the production of second-generation biofuels, i.e. those produced from non-food resources such as sugarcane bagasse.

Sugarcane bagasse is an agro-residue generated in high amount (186 million tons / year) by the sugar and alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane, and is composed of 19-24% lignin, 27-32% hemicellulose, 32-44% cellulose and 4.5-9% ashes (Soccol et al., 2011). The conversion of sugarcane bagasse into fermentable sugars requires essentially two steps: pre-treatment and hydrolysis. The aim of the pre-treatment is to separate lignin and break the structure of lignocellulose, and is usually performed by thermo-chemical processes. Pretreatment is one of the most expensive and least technologically mature steps in the process of converting biomass to fermentable sugars (Binod et al., 2012). Hydrolysis is conducted in the presence of enzymes (exo-glucanases, endo-glucanases and cellobiases) or mineral acids and releases glucose units from the cellulose molecules (Hernández-Salas et al., 2009).

Delignification can be performed not only by thermo-chemical processes but also by the biological route, using enzymes or microorganisms. An example is the

bleaching process of the wood pulp with ligninolytic enzymes that can provide mild and clean strategies for pretreatment (Kuhad et al., 1997). Lignin decomposition in nature is primarily attributed to the metabolism of microorganisms. Among all other organisms, white-rot basidiomycetes degrade lignin more rapidly and extensively than other groups (Falcón et al., 1995). These microorganisms produce several ligninolytic enzymes (laccases, manganese peroxidases and lignin peroxidases) that catalyze one-electron oxidation of lignin units, producing aromatic radicals (Giardina et al., 2000). Laccases are more readily available and easier to manipulate than both lignin peroxidase and manganese-dependent peroxidase (Couto and Herrera, 2006).

Laccases (E.C. 1.10.3.2, p-diphenol: dioxygen oxidoreductases) are blue multicopper oxidases able to oxidize a variety of phenolic compounds including polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, with concomitant reduction of molecular oxygen to water (Autore et al., 2009; Dwivedi et al., 2011). The research of microorganisms as natural producers of laccases is to be developed, and there is no clear comparative study or evidence showing that the recombinant technology is superior to the natural homologous laccase producer (Herpoel et al., 2000). Moreover, ligninolytic enzymes are difficult to overexpress heterologously in an active form (Jönsson et al., 1997). Particularly, fungal laccases are glycosylated enzymes whose sugar moieties are involved in the stabilization process against proteolysis, so the heterologous expression requires eukaryotic microorganisms as hosts, which are able to perform such post-translational modifications (Couto and Toca-Herrera, 2007).

The objective of this work was to select a strain of basidiomycete able to degrade lignin and produce laccase in sugarcane bagasse and to perform its molecular

identification. Amplification by polymerase chain reaction (PCR) was performed using universal primers targeted to conserved regions within fungal ribosomal DNA (rDNA) followed by DNA sequencing.

2.2. Materials and Methods

2.2.1. Screening for basidiomycetes presenting ligninolytic activity

The strains of basidiomycetes available at the culture collection of the Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná (DEBB-UFPR), were reactivated from the maintenance media stored at 4ºC in PDA dishes and incubated at 30ºC. After significant growth (around 7 days), mycelium disks of 7 mm diameter were transferred to solid media containing 2% lignin (alkali, Sigma-Aldrich). Considering that lignin is not degraded as the sole carbon and energy source, additional co-substrates such as cellulose, hemicellulose or glucose are required (Silva et al., 2010). Media were thus supplemented with 0.2% carboxymethyl cellulose (Sigma-Aldrich), and also with inorganic nitrogen source and minerals as reported by Soccol et al. (1994), with all concentrations in g/L: $(NH_4)_2SO_4$ 1.32; KH_2PO_4 0.15; $MgSO_4.7H_2O$ 0.25; $ZnSO_4.7H_2O$ 0.04, final pH 5.5. Dishes were incubated at 30ºC for 7 days. After this period, the diameter of the colonies was measured.

2.2.2. Screening for basidiomycetes producing laccase in sugarcane bagasse

Sugarcane bagasse was previously washed with water (seven times) in order to remove the residual sucrose, dried at 60°C and grinded to < 0.8 mm particle size. Strains were cultivated in pretreated sugarcane bagasse as the sole carbon source, in semi-solid condition (Couto et al., 1998; 2002), with a basal medium containing the following salts: $(NH_4)_2SO_4$ (1.4 g/L), KH_2PO_4 (2 g/L), $MgSO_4.7H_2O$ (0.3 g/L), $CaCl_2$ (0.3 g/L), $FeSO_4.7H_2O$ (0.005 g/L), $MnSO_4.H_2O$ (0.00156 g/L), $CoCl_2$ (0.002 g/L) and $ZnSO_4.7H_2O$ (0.0014 g/L), final pH 5.5 (Menezes et al., 2009). The basal medium was sterilized by filtration and the bagasse was autoclaved. The concentration of bagasse in the fermentation was 20 g/L. Strains were reactivated in Petri dishes containing PDA medium and three disks of 7mm diameter were used as inoculum. The flasks were incubated at 30ºC, 120 rpm and samples of 3, 5 and 7 days were taken for enzymatic quantification.

The enzymatic activity of laccases was assayed by the oxidation of ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). The reaction mixture contained 100 μL of ABTS 20mM (in sodium citrate buffer 0.1M, pH 3.0), sample (usually 20-50 μL) and sodium citrate buffer ($C_6H_8O_7.H_2O$ 0.1M, pH 3.0) up to 1 mL. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}). The enzyme activity was expressed in International Units (U), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.

2.2.3. Molecular identification of the selected fungus

2.2.3.1. DNA extraction

Fresh fungal mycelium was collected from the surface of a plate culture and transferred to sterile 2 mL screw cap microtubes containing around 1.5 g of 0.70 mm garnet beads (Mo-Bio Laboratories, Carlsbad, CA) and 600 µL of CTAB extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM sodium EDTA pH 8.0, 1.5 M NaCl, 2% CTAB and 1 % polyethylenglycol 8000). Microtubes were fixed horizontally with tape on a flat-bed vortex and cells were submitted to physical disruption at maximum speed for 1-2 minutes. Supernatants were collected after centrifugation at 12,000×g for 10 min at room temperature and transferred into a new microtube. Then, an equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added, tubes were homogenized by inversion and centrifuged as described before. Aqueous phase from the supernatant was transferred to a new microtube and one volume of isopropanol was added. Microtubes were centrifuged at 12,000×g for 20 min, the supernatant was removed and the DNA pellet was washed two times with 500 µL ethanol 70% (v/v) and dried. The DNA pellet was resuspended in 20 µL of sterile ultrapure water.

2.2.3.2. DNA amplification

Primers ITS4 (5’ TCCTCCGCTTATTGATATGC 3’) and ITS5 (5’ GGAAGTAAAAGTCGTAACAAGG 3’) were used to amplify the internal transcribed spacer (ITS) regions of the fungal rDNA (White et al., 1990). Dilutions (1:2, 1:5, 1:10 and 1:20) of the extracted chromosomal DNA were prepared using sterile ultrapure

water and, from these, 2 µL were used as DNA template for the PCR. Amplification was performed in 20 µL PCR mixtures containing 200 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1x PCR buffer, 325 nM forward primer ITS5, 325 nM reverse primer ITS4, 1 U of Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY) and sterile ultrapure water to complete 20 µL. PCRs were performed in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA) at the following conditions: 94ºC for 2 min, 30 cycles of 96ºC for 15 s, 60ºC for 30 s, then 72°C for 1 min followed by 72°C for 10 min. The amplified products were analyzed by electrophoresis on a 1% agarose gel in 1x TBE buffer with 1 kb Plus DNA ladder (Invitrogen, Grand Island, NY) used as a standard. Gels were stained with ethidium bromide and visualized under UV light using a L-Pix HE Photodocumentation System (Loccus Biotecnologia, São Paulo, SP, Brazil).

2.2.3.3. ITS rDNA sequencing and sequence analysis

ITS amplicons were pooled and purified by precipitation with 0.6 volumes of 7.5 M ammonium acetate (pH 8.0) and 1.2 volumes of absolute ethanol followed by 70% ethanol rinsing. Direct sequencing of ITS-PCR products was performed by ACTGene Análises Moleculares Ltd. (Centro de Biotecnologia, Porto Alegre, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic (Applied Biosystems, Foster City, CA). DNA sequencing reaction was carried out using Big Dye® Terminator v3.1 Cycle Sequencing RR-100 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. ITS sequences were analyzed and compared with the Assembling Fungal Tree of Life (AFTOL, available at http://aftol.org) database using

the BLAST interface of Web Accessible Sequence Analysis for Biological Inference (WASABI) (Kauff et al., 2007).

2.2.4. Kinetics of laccase and manganese peroxidase production by the selected fungus in solid state fermentation

Glass flasks containing 1 g of sugarcane bagasse (< 0.8 mm particle size) were autoclaved and humidified with a sterile saline solution (15 mL/g bagasse) presenting the following constant composition: $(NH_4)_2SO_4$ (1.4 g/L), KH_2PO_4 (2 g/L), $MgSO_4.7H_2O$ (0.3 g/L), $CaCl_2$ (0.3 g/L), $FeSO_4.7H_2O$ (0.005 g/L), $MnSO_4.H_2O$ (0.00156 g/L), $CoCl_2$ (0.002 g/L) and $ZnSO_4.7H_2O$ (0.0014 g/L), final pH 5.5 (Menezes et al., 2009). The selected strain was reactivated in PDA medium and after 7 days of growth, 4 disks of 7 mm diameter were transferred to Czapek liquid medium containing the antibiotic cephalexin (0.08 g/L). After 5 days of growth at 30ºC and 120 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a spatula and resuspended in the residual medium to a lower final volume (10% of the initial volume). 0.2 mL of the homogenized mycelium was transferred to the fermentation flasks, which were manually homogenized and incubated at 30ºC.

The enzymatic activity of laccases was assayed according to item 2.2.2 and the activity of manganese peroxidase was measured in the presence of phenol red 0.1 mM, $MnSO_4$ 0.1 mM and hydrogen peroxide 50 mM as described by Mercer et al. (1996). The oxidation of phenol red was followed by absorbance variation at 610 nm (ε = 22,000 M^{-1} cm^{-1}). Enzyme activities were expressed in International Units (U), where

one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.

2.3. Results and Discussion

2.3.1. Screening for basidiomycetes presenting ligninolytic activity

A preliminary screening was performed among the strains of basidiomycetes available at the DEBB-UFPR culture collection. Strains of the following genera were evaluated (identification codes in parentheses): *Auricularia* sp. (CC267); *Ganoderma curtisii*; *Ganoderma* spp. (Gan112; GanCC264); *Grifola frondosa*; *Grifola* sp. (Fan155); *Hypsizygus* sp. (HMCC182); *Lentinula edodes* (L465; L609; L790; L797; L1022; L6121; L6602; L6608; L6609; L6613; L6614; L6624; L6635; L6646; L6647); *Lentinula* spp. (L1Fan; L2Fan; L4Fan; L5Fan; L6Fan; L10Fan; LCC18; LCC57; LCC265; LCC299); *Pleurotus* spp. (Pl 20; Pl 22; Pl 22 Em; Pl 23; Pl 35; Pl 0850; Pl 3501; Pl 3824); *Phanerochaete* spp. (Ph HO; Ph Bk); *Trametes* spp. (247; TICC266); *Tremella fuciformis* (Tf Fan4). The screening method for ligninolytic activity demonstrated to be inappropriate since almost 70% of the strains presented significant growth in lignin-based medium, with colony diameters higher than 5 cm after 7 days of incubation. Considering that laccase is the ligninolytic enzyme of major interest, another quantitative test was performed to evaluate the laccase activity produced in sugarcane bagasse by semi-solid fermentation.

2.3.2. Screening for basidiomycetes producing laccase in sugarcane bagasse

Table 2.1 presents the results of the screening for laccase-producing basidiomycetes in sugarcane bagasse. The strain of *Pleurotus* sp. (Pl 22 Em) presented a laccase activity (183 U/L after 5 days) significantly higher than all the other strains, so it was selected for molecular identification and for the subsequent studies in sugarcane bagasse. Some literature values of laccase activity produced by *Pleurotus* species are 1200 U/L in submerged fermentation of organic municipal wastes (Olivieri et al., 2006); 1403 U/L in submerged fermentation of commercial medium (Prasad et al., 2005); 3500 U/L in solid state fermentation (trickle-film processing) of sugarcane bagasse (Lenz and Hölker, 2004). These values suggest that the laccase activity presented by *Pleurotus* sp. could be significantly enhanced by optimization studies.

The genus *Pleurotus* includes edible and medicinal species belonging to the group of white-rot fungi that produce not only laccase, but also manganese-dependent peroxidase (MnP), versatile peroxidase (VP) and aryl-alcohol oxidase (AAO) (Stajic et al., 2006). *Pleurotus* species present high adaptability for growth and fructification within a wide variety of agro-industrial lignocellulosic wastes (Menolli Junior et al., 2010), and preferentially attack lignin more readily than hemicellulose and cellulose (Wong, 2009). *Pleurotus ostreatus*, also called oyster mushroom, is the second most cultivated edible mushroom worldwide after *Agaricus bisporus*. Particularly, the cultivation of *P. ostreatus* is advantageous in relation to other mushrooms because it requires a shorter growth time, the substrate used for its cultivation does not require sterilization, only pasteurization and few environmental controls are demanded (Sánchez, 2010).

Table 2.1 – Laccase activities after 3, 5 and 7 days of growth on sugarcane bagasse in semi-solid condition.

Strain	Code	Laccase activity 3 days, U/L	Laccase activity 5 days, U/L	Laccase activity 7 days, U/L
Auricularia sp.	CC267	0	0.278	0
Ganoderma spp.	*Ganoderma curtisii*	0	0	0
	Gan112	0	0	1.22
	GanCC264	0	0.278	0
Grifola spp.	*Grifola frondosa*	0.0833	0.275	0.833
	Fan155	0	0	0
Hypsizygus sp.	HMCC182	0	0	0
Lentinula spp.	L1Fan	0	0	0
	L2Fan	0.278	0.290	0.417
	L4Fan	0	0	0
	L5Fan	0.25	0	0
	L6Fan	0	0.139	0
	L10Fan	0	0.389	0.639
	LCC18	0	0	0
	LCC57	0	0.389	0
	LCC265	0.0833	0	0
	LCC299	0.278	0.331	0.333
Lentinula edodes	L465	0	0	0
	L609	0	0	0
	L790	0	0	0
	L797	0.139	0	0
	L1022	0	0	0
	L6121	11.6	29.3	80.8
	L6602	0	0	24.3
	L6608	0	0	16.5
	L6609	0	0	7.61
	L6613	0.278	0.321	0.361
	L6614	0.333	0.399	0.5
	L6624	0	0.139	0.389
	L6635	0	0.0833	0.333
	L6646	0	0	27.9
	L6647	0.278	0.330	0.333
Pleurotus spp.	Pl 20	0	0	16.2
	Pl 22	14.9	42.8	26.8
	Pl 22 Em	10.7	183	17.5
	Pl 23	8.61	116	6.67
	Pl 35	0	0.278	0
	Pl 0850	0	0	0.778
	Pl 3501	0	50.1	48.8
	Pl 3824	0.278	0.367	0.389
Phanerochaete spp.	Ph HO	0	0.278	0
	Ph Bk	0	0	0
Trametes spp.	247	0	0.278	0
	TICC266	0	0.278	0
Tremella sp.	Tf Fan4	0	0	0.389

2.3.3. ITS amplification and molecular identification of the selected fungus

Molecular identification was based on the sequence of the nuclear ribosomal DNA repeat unit spanning, the internal transcribed spacer regions (ITS1 and ITS2), and the 5.8S gene. Mechanical disruption with beads in CTAB extraction buffer followed by organic extraction and isopropanol precipitation of the DNA, yielded DNA suitable for amplification of a fragment with approximately 700 bp in size when different DNA template dilutions (1:2, 1:5, 1:10 and 1:20) were tested (Figure 2.1). Direct sequencing of the ITS amplicon and its sequence analysis (630 bp length) revealed high sequence identity (99%, E-value 0.0) with nuclear internal transcribed spacer (*nuc*ITS) from *Pleurotus ostreatus* (GenBank accession code AY854077, AFTOL-ID 564). Therefore, by comparing ITS1-5.8rDNA-ITS2 sequences the fungus was identified as *Pleurotus ostreatus*. The ITS amplicon sequence was submitted to the National Center for Biotechnology Information (NCBI) (GenBank accession code JQ316531).

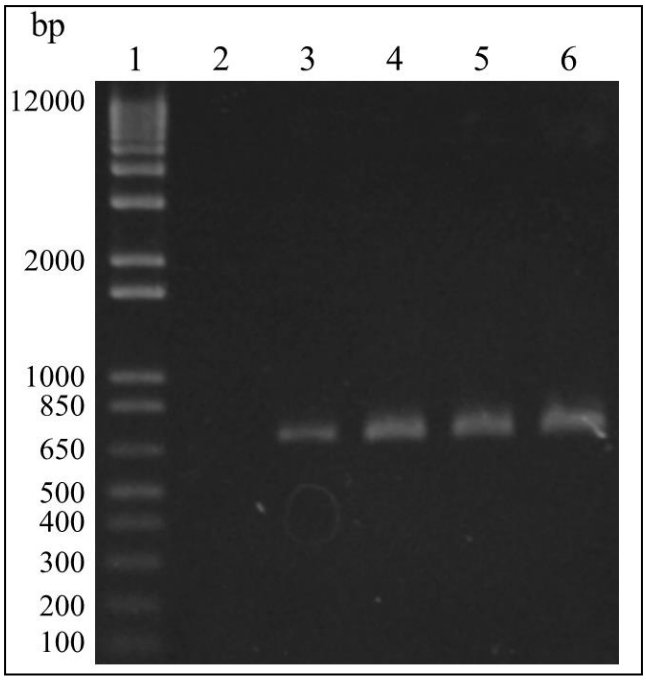

Figure 2.1. ITS-PCR amplification of nuclear rDNA from chromosomal DNA isolated from fungus using different DNA template dilutions. Lane 1: 1 kb Plus DNA ladder (Invitrogen, Grand Island, NY); lane 2: undiluted DNA template; lane 3: diluted DNA template (1:2); lane 4: diluted DNA template (1:5); lane 5: diluted DNA template (1:10); lane 6: diluted DNA template (1:20).

2.3.4. Kinetics of laccase and manganese peroxidase production by the selected fungus in solid state fermentation

The kinetics of laccase production by *P. ostreatus* 22 Em was also evaluated in solid state condition. Considering that *P. ostreatus* belongs to a class of white-rot fungi that produces laccases and also manganese peroxidases, but not lignin peroxidases (Giardina et al., 2000), the production kinetics of these two enzymes was followed in solid state fermentation of sugarcane bagasse, and results are presented in Table 2.2. Laccase was the enzyme produced in highest amount (2049 U/kg), and the peak of

laccase activity was reached at the 5th day of fermentation. Results obtained by Stajic et al. (2004) about a screening of laccase, manganese peroxidase and versatile peroxidase activities using *Pleurotus* spp. showed that conditions of solid state fermentation were more favorable for ligninolytic activity than those in submerged fermentation owing to their similarity to natural conditions on wood substrates. Here, although the semi-solid condition demonstrated to be favorable, the solid state fermentation was chosen as the technology to be optimized.

Table 2.2 – Laccase and Mn peroxidase activities, produced after 4, 5 and 6 of solid state fermentation on sugarcane bagasse by *P. ostreatus* 22 Em.

Enzyme	Activity 4 days, U/kg	Activity 5 days, U/kg	Activity 6 days, U/kg
Laccase	968	2049	856
Mn peroxidase	241.5	152.5	134.5

Note: Enzyme activities in international units (U) per kilogram of dry bagasse.

2.4. Conclusions

Among 45 strains of basidiomycetes available at the culture collection of DEBB-UFPR, one of *Pleurotus* sp. (coded Pl 22 Em) presented a laccase activity on sugarcane bagasse significantly higher than all the other strains, i.e. 183 U/L after 5 days of growth in semi-solid condition. Direct sequencing of the ITS amplicon and its sequence analysis revealed high sequence identity (99%, E-value 0.0) with *nuc*ITS from *Pleurotus ostreatus* (GenBank accession number AY854077, AFTOL-ID 564). The sequence containing ITS1-5.8rDNA-ITS2 is available at the NCBI GenBank (accession code JQ316531). Cultivation of the fungus in solid state fermentation on sugarcane bagasse resulted in a maximum laccase activity of 2049 U/kg after 5 days of fermentation.

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CHAPTER III

Optimization of culture conditions for laccase production in solid state fermentation of sugarcane bagasse by *Pleurotus ostreatus*

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Abstract

The objective of this work was to optimize the production of laccase and to determine its mathematical model, through the process of solid state fermentation of sugarcane bagasse using a selected strain of *Pleurotus ostreatus* (coded Pl 22 Em). Among eight variables (pH, water activity, temperature and concentrations of $CuSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, asparagine and yeast extract), copper sulfate and ammonium sulfate concentrations demonstrated to significantly influence laccase production. The replacement of ammonium sulfate by yeast extract and the addition of ferulic acid as inducer provided increases of, respectively, 5.7 and 2.0 fold in laccase production. Optimization of laccase production as a function of yeast extract, copper sulfate and ferulic acid concentrations was performed by response surface methodology and optimal concentrations were 6.4 g/L, 172.6 μM and 1.86 mM, respectively, the maximum laccase activity predicted by the model (R^2 0.8753) being 161.3 U/g. Experimentally, the maximum laccase activity of 151.6 U/g was produced at the 5th day of solid state fermentation.

Keywords: Laccase, solid state fermentation, sugarcane bagasse, optimization, mathematical modeling

3.1. Introduction

The utilization of agro-industrial wastes as substrates for fermentative processes has been widely explored, since they are easily available, rich in carbon and often represent a problem of disposal (Gassara et al., 2010). There are several publications describing bioprocesses that use wastes such as hulls and bagasse as raw materials to produce ethanol, single-cell protein, mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, among other products (Soccol and Vandenberghe, 2003). Sugarcane bagasse is an agro-residue generated in high amount (186 million tons / year) by the sugar and alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane, and is composed of 19-24% lignin, 27-32% hemicellulose, 32-44% cellulose and 4.5-9% ashes (Soccol et al., 2011). Sugar mills generate approximately 270-280 kg of bagasse (50% moisture) per metric ton of sugarcane (Rodrigues et al., 2003).

Solid state fermentation is an interesting technology to be applied in the valorization of agro-industrial residues and can be economically feasible for the production of many biotechnological products (Soccol and Vandenberghe, 2003). The advantages of the solid state fermentation process over submerged fermentation include: smaller fermenter volume; lower sterilization energy costs; easier aeration; reduced or eliminated costs for stirring and effluent treatment; lower costs for product recovery and drying; less favorable environment for many bacteria, lowering the risk of contamination (Lee, 1997; Soccol and Vandenberghe, 2003).

Laccases are blue multicopper oxidases able to oxidize a variety of phenolic compounds including polyphenols, methoxy-substituted phenols, diamines and a

considerable range of other compounds, with concomitant reduction of molecular oxygen to water (Autore et al., 2009; Dwivedi et al., 2011). They oxidize phenols and phenolic lignin substructures by one-electron abstraction with formation of radicals that can repolymerize or lead to depolymerization (Higuchi, 1989). These enzymes have been found to oxidize also non-phenolic compounds in the presence of a mediator (e.g., 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate or ABTS) (Wong, 2009). It is known that the production of laccases by fungi, especially the white-rot basidiomycetes, can be affected by the type and concentration of the carbon and nitrogen sources, and also by the presence of copper and organic compounds that act as inducers of laccase activity (Palmieri et al., 2000; Hou et al., 2004; Lettera et al., 2011; Piscitelli et al., 2011).

Laccases can be applied in the food and beverages industry to remove undesired phenolics which are responsible for browning, haze formation and turbidity. In the pulp and paper industry, the biopulping and biobleaching processes use enzymes as alternatives to the conventional chemical processes. In the textile industry, the decolourization of dyes is the main application of laccases, and there are also applications in the fields of nanotechnology, bioremediation and synthetic chemistry (Couto and Herrera, 2006). The objective of this work was to optimize the production of laccases and to determine its mathematical model, through the process of solid state fermentation of sugarcane bagasse using a selected strain of *Pleurotus ostreatus* (coded Pl 22 Em).

3.2. Materials and Methods

3.2.1. Solid state fermentations

Erlenmeyer flasks containing 1 g of sugarcane bagasse (particle size between 0.8 and 2 mm 50% and < 0.8 mm 50%) were autoclaved and humidified with a sterilized saline solution presenting the following constant composition: $MgSO_4.7H_2O$ (0.3 g/L), $FeSO_4.7H_2O$ (0.005 g/L), $MnSO_4.H_2O$ (0.00156 g/L), $ZnSO_4.7H_2O$ (0.0014 g/L), $CaCl_2$ (0.3 g/L), $CoCl_2$ (0.002 g/L) (Menezes et al., 2009). For the experiments designed according to Plackett-Burman (Table 3.1), the saline solution presented some differences regarding the following variables: $CuSO_4.5H_2O$ as the inducer (0, 75 or 150 μM – concentrations defined according to previous experiments), $(NH_4)_2SO_4$ as the nitrogen source (1.5, 2.0 or 2.5 g/L), KH_2PO_4 as the source of potassium and phosphorus (1, 1.5 or 2 g/L), asparagine as the supplementary aminoacid (0, 0.3 or 0.6 g/L), yeast extract as the source of vitamins and aminoacids (0, 0.25 or 0.5 g/L) and pH (5.0, 5.5 or 6.0). pH was adjusted with HCl 1 M or NaOH 1 M. These variables were chosen on the basis of the composition of the Basidiomycetes Synthetic Medium. In order to evaluate the effect of water activity (A_w) and temperature, the bagasse was humidified with different volumes of saline solution (10, 15 and 20 mL/g, corresponding to initial A_w of 0.993, 0.996 and 0.999, respectively) and the cultures were incubated at 25, 29 and 33ºC. For the subsequent experiments, the concentration of KH_2PO_4 and the pH were set at 1.5 g/L and 5.5, respectively, no asparagine was added, initial A_w was fixed at 0.993 or 10 mL/g and temperature at 29ºC. The studied variables were: nitrogen source (yeast extract, 1.96 – 12.04 g/L or $(NH_4)_2SO_4$, 2.5 g/L), inorganic

inducer ($CuSO_4$, 24 – 276 μM) and organic inducer (ferulic acid, 0.32 – 3.68 mM, added after 48h of fermentation).

The strain of *P. ostreatus* (Pl 22 Em) was reactivated in PDA dishes and after 7 days of growth, 4 disks of 7 mm diameter were transferred to Czapek liquid medium containing the antibiotic cephalexin (0.08 g/L). After 5 days of growth at 30ºC and 120 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a spatula and resuspended in the residual medium to a lower final volume (10% of the initial volume). 0.2 mL of the homogenized mycelium was transferred to the fermentation flasks, which were manually homogenized and incubated for 5 days (optimization studies) or for 3 to 7 days (kinetic study). Analysis of the results and determination of the mathematical model were performed using the software Statistica 5.0 (Statsoft, USA), and determination of the optimal levels was performed through the Solver Excel tool (Microsoft, USA).

3.2.2. Experimental designs

Fermentations were prepared as described in item 3.2.1. Tables 3.1 and 3.2 present the chosen variables and levels for the Plackett-Burman Design and Central Composite Design experiments, respectively.

Table 3.1 – Plackett-Burman Design to select significant variables to be optimized in the production of laccase by solid state fermentation of sugarcane bagasse.

Levels	Variables							
	pH	A_w[a]	T ºC	$CuSO_4$ μM	$(NH_4)_2SO_4$ g/L	KH_2PO_4 g/L	Asn[b] g/L	YE[c] g/L
-1	5.0	0.993	25	0	1.5	1	0	0
+1	6.0	0.999	33	150	2.5	2	0.6	0.5

[a]Water activity; [b]asparagine; [c]yeast extract.

Table 3.2 – Central Composite Design for the modeling of laccase production in solid state fermentation of sugarcane bagasse.

Levels	Variables		
	Yeast extract g/L	$CuSO_4$ μM	Ferulic acid mM
-1.68	1.96	24	0.32
-1	4	75	1
0	7	150	2
+1	10	225	3
+1.68	12.04	276	3.68

3.2.3. Extraction of the enzymes

Enzymes produced by solid fermentation were extracted by solid-liquid extraction using sodium phosphate buffer as solvent ($NaH_2PO_4.H_2O$, 50 mM, pH 7.0) (Iandolo et al., 2011[b]). The fermented material was manually homogenized, weighed (around 1 g) and the extraction buffer was added in the proportion of 1:10 (w/w). A protease inhibitor (phenylmethylsulfonyl fluoride, PMSF 1mM) was added to the extraction mixture. The mixture was homogenized in vortex for 1 min and centrifuged for 7,500 g, 4ºC, 45 min. The supernatant was separated and submitted to analyses.

3.2.4. Laccase activity assay

The enzymatic activity of laccase was assayed by the oxidation of ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). The reaction mixture contained 100 μL of ABTS 20mM (in sodium citrate buffer 0.1M, pH 3.0), sample (usually 20-50 μL) and sodium citrate buffer ($C_6H_8O_7.H_2O$ 0.1M, pH 3.0) up to 1 mL. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}). The enzyme

activity was expressed in International Units (U), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.

3.3. Results and Discussion

3.3.1. Screening of significant variables affecting laccase production – Plackett-Burman Design

Table 3.3 presents the results of the Plackett-Burman experiments to select significant variables to be optimized for laccase production. According to the Pareto chart of standardized effects (Figure 3.1), the variables that presented significant effects on laccase production at the confidence level of 90% were copper sulfate and ammonium sulfate concentrations, both presenting positive effects.

Table 3.3 – Results of laccase activity obtained for the Plackett-Burman experiments after 5 days of solid state fermentation on sugarcane bagasse.

	Variables and corresponding levels								Activity
	pH	A_w[a]	T[b]	$[Cu^{2+}]$[c]	[N][d]	[PK][e]	[Asn][f]	[YE][g]	U/g
1	1	-1	1	-1	-1	-1	1	1	2.091
2	1	1	-1	1	-1	-1	-1	1	7.420
3	-1	1	1	-1	1	-1	-1	-1	3.278
4	1	-1	1	1	-1	1	-1	-1	7.366
5	1	1	-1	1	1	-1	1	-1	11.45
6	1	1	1	-1	1	1	-1	1	4.294
7	-1	1	1	1	-1	1	1	-1	6.825
8	-1	-1	1	1	1	-1	1	1	10.94
9	-1	-1	-1	1	1	1	-1	1	11.18
10	1	-1	-1	-1	1	1	1	-1	3.729
11	-1	1	-1	-1	-1	1	1	1	1.914
12	-1	-1	-1	-1	-1	-1	-1	-1	2.755
C[h]	0	0	0	0	0	0	0	0	7.318
C’	0	0	0	0	0	0	0	0	6.727

[a]Water activity; [b]temperature; [c]$CuSO_4$ concentration; [d]$(NH_4)_2SO_4$ concentration; [e]KH_2PO_4 concentration; [f]asparagine concentration; [g]yeast extract concentration; [h]C and C’ represent the duplicates of the intermediate level.

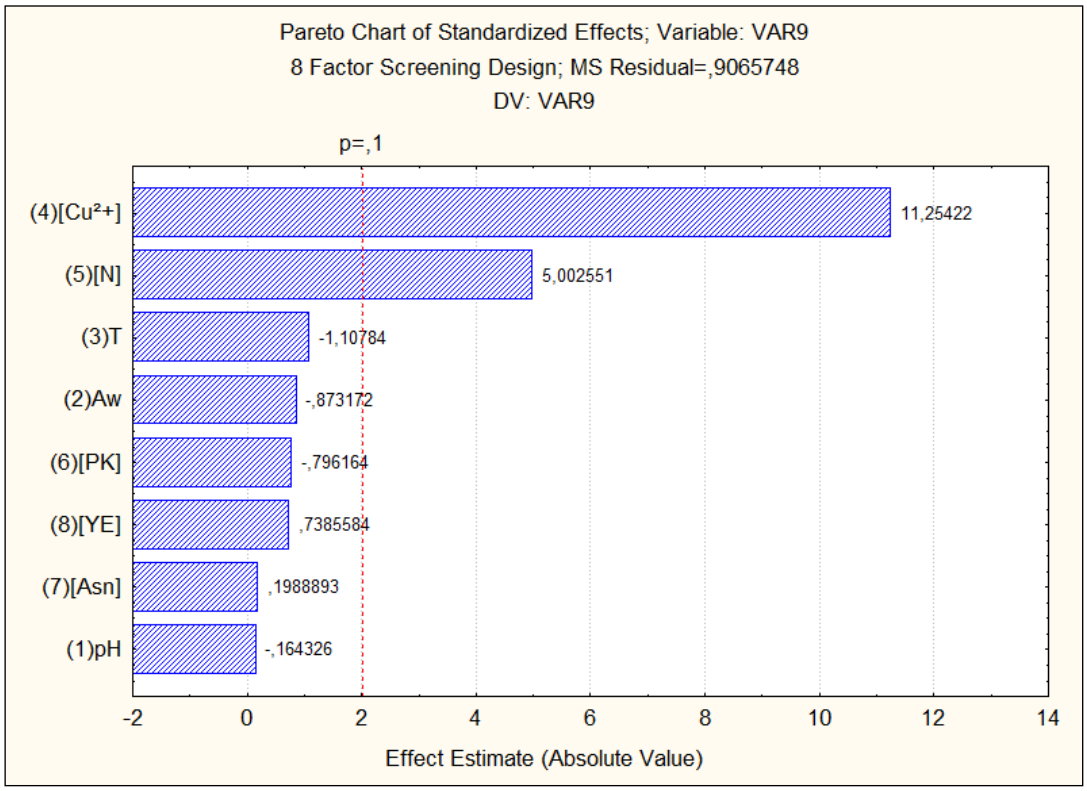

Figure 3.1. Pareto chart of standardized effects of the variables (1) pH, (2) water activity, (3) temperature, (4) $CuSO_4$ concentration, (5) $(NH_4)_2SO_4$ concentration, (6) KH_2PO_4 concentration, (7) asparagine concentration and (8) yeast extract concentration on laccase activity, at the confidence level of 90% (p = 0.1), R^2 = 0.96873.

The addition of copper as an inducer of laccase production has been reported in literature. Different studies have shown that laccase production is regulated by metal ions such as Cu^{2+} and Fe^{3+} by gene expression induction or through translational or post-translational regulation (Fonseca et al., 2010). Palmieri et al. (2000) demonstrated that the addition of copper sulfate 150 μM to a *P. ostreatus* liquid culture medium caused a 30-fold increase in total laccase activity, and Hou et al. (2004) reported a 4.5-fold increase in laccase activity in *P. ostreatus* when Cu^{2+} 1 mM was added to the liquid culture medium. Baldrian and Gabriel (2002) concluded that Cu^{2+} not only induces laccase by the expression of laccase genes in *P. ostreatus*, but it also positively affects activity and stability of the enzyme.

The production of ligninolytic enzymes has been associated with the secondary metabolism and with conditions of limited nitrogen for many white rot fungi, including the model organism for laccase production and lignin degradation *Phanerochaete chrysosporium* (Kaal et al., 1995). For *P. ostreatus*, however, a higher concentration of nitrogen in the medium did not repress but rather slightly stimulated mineralization of lignin, as reported by Stajic et al. (2006). Kaal et al. (1995) also suggested that several white rot fungi strains, including *P. ostreatus*, produce higher ligninolytic enzyme activities in response to a nitrogen-rich medium.

3.3.2. Comparison between organic and inorganic nitrogen sources and evaluation of ferulic acid as inducer of laccase activity production

The comparison between the laccase activities obtained when different sources of nitrogen were used and when ferulic acid was added is presented in Table 3.4. Yeast extract (7.5 g/L, containing 7% total nitrogen) showed an increase of 5.7 fold in laccase production in relation to ammonium sulfate (2.5 g/L, containing 21% nitrogen), for the same concentration of total nitrogen. Also ferulic acid enhanced laccase production (2.0 fold) in the presence of copper sulfate.

Table 3.4 – Effect of inorganic and organic nitrogen sources and different inducers – Cu^{2+} and ferulic acid (Fer) – on the level of laccase activity produced by the strain *P. ostreatus* 22 Em after 5 days of fermentation on sugarcane bagasse.

N source (g/L)	Inducer	U/g dry substrate
Ammonium sulfate		
2.5	$CuSO_4$ 150 μM	9.942 ± 1.97
Yeast extract		
2.5	0	2.970 ± 0.651
2.5	$CuSO_4$ 150 μM	44.23 ± 2.44
2.5	$CuSO_4$ 150 μM + Fer 2 mM	89.18 ± 3.95
7.5	$CuSO_4$ 150 μM	56.25 ± 5.08

These results are in accordance with Hou et al. (2004), who demonstrated that the most suitable nitrogen sources for laccase production by *P. ostreatus* were peptone and yeast extract, in comparison with urea, ammonium sulfate and ammonium tartarate. Mishra and Kumar (2007) also demonstrated that regarding enhancement of laccase production in solid state fermentation by *P. ostreatus*, yeast extract was preferred to inorganic nitrogen sources, reaching 23 U/gds, which may be attributed to the presence of some additives (nutrients/activators) and favorable C:N ratio.

Ferulic acid is also a known inducer of laccase production. The structure of this organic acid is similar to that of coniferyl alcohol, the most abundant monolignol of the three lignin precursors (Zucca et al., 2011). Vanhulle et al. (2007) reported a positive effect of ferulic acid 0.5 mM on laccase production by *P. ostreatus* in submerged fermentation with glucose and lactose as substrates. A peak of laccase activity (around 7,500 U/L) was observed at the 15^{th} day of fermentation (3-fold increase when compared to control). Ferulic acid was also shown to be the best inducer of laccase activity in *Pleurotus sajor-caju* (Zucca et al., 2011). Meza et al. (2007) reported a laccase activity of approximately 70 U/g of sugarcane bagasse in the presence of ferulic acid 10 mM, produced by *Pycnoporus cinnabarinus* after 10 days, in contrast with around 10 U/g without inducers.

3.3.3 Determination of the mathematical model of laccase production through the response surface methodology – Central Composite Design

Table 3.5 presents the results of 16 experiments to evaluate the effect of yeast extract, copper sulfate and ferulic acid concentrations on laccase production by solid state fermentation of sugarcane bagasse. Response surfaces described by the mathematical model are shown in Figures 3.2, 3.3 and 3.4.

Table 3.5 – Results of laccase activity obtained for the Central Composite Design experiments after 5 days of solid state fermentation on sugarcane bagasse.

	Variables			Response
	Yeast extract g/L	$CuSO_4$ µM	Ferulic acid mM	Activity U/g
1	4	75	1	61.30
2	10	75	1	51.02
3	4	225	1	98.12
4	10	225	1	58.80
5	4	75	3	50.90
6	10	75	3	46.60
7	4	225	3	68.80
8	10	225	3	65.60
9	1.96	150	2	82.19
10	12.04	150	2	52.75
11	7	24	2	62.33
12	7	276	2	35.91
13	7	150	0.32	48.03
14	7	150	3.68	89.43
15	7	150	2	158.8
16	7	150	2	149.4

Note: Enzyme activities in units per gram of dry substrate.

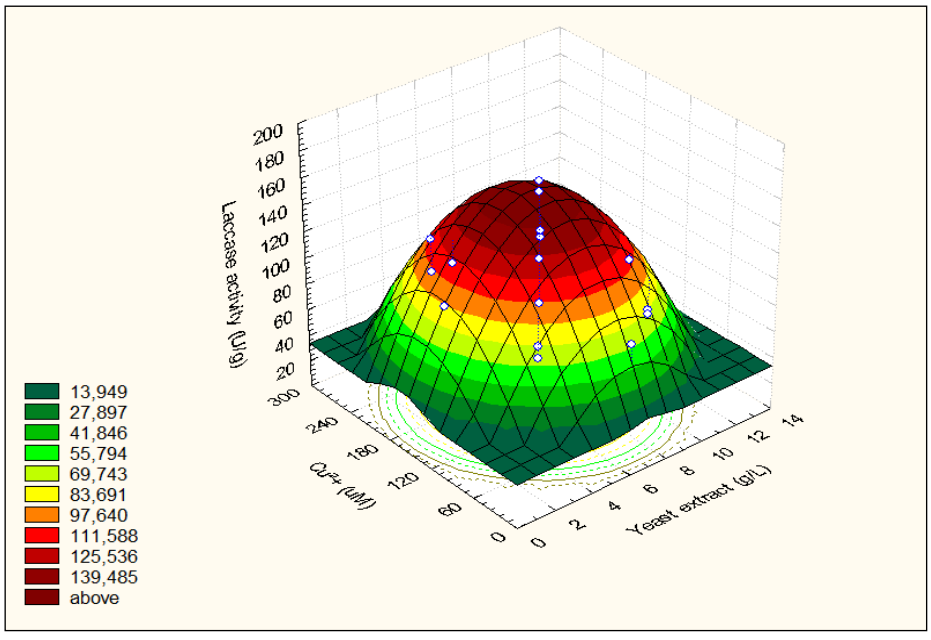

Figure 3.2. Response surface plot described by the model, showing the effect of yeast extract concentration (g/L) and $CuSO_4$ concentration (µM) on laccase activity (U/g).

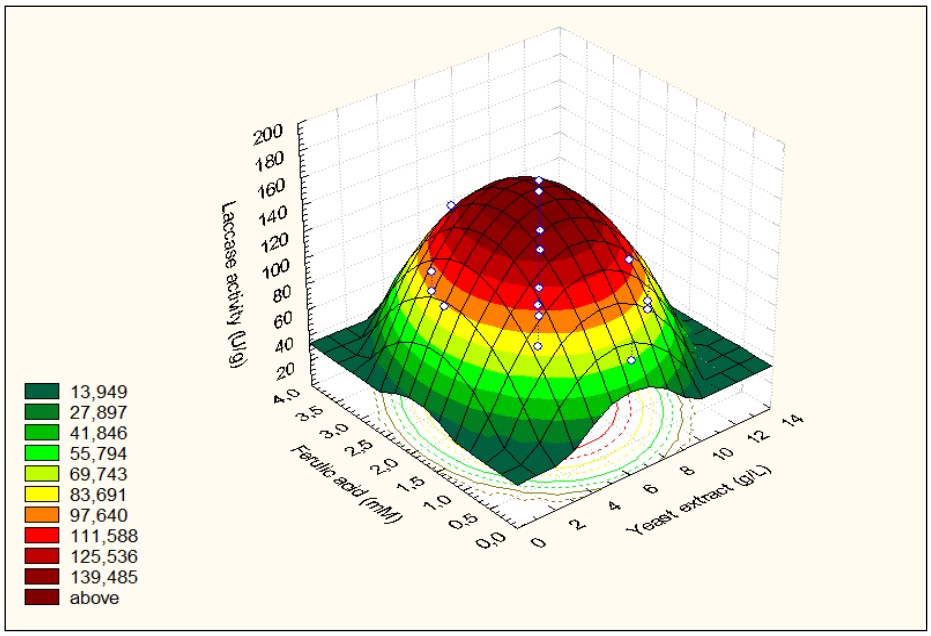

Figure 3.3. Response surface plot described by the model, showing the effect of yeast extract concentration (g/L) and ferulic acid concentration (mM) on laccase activity (U/g).

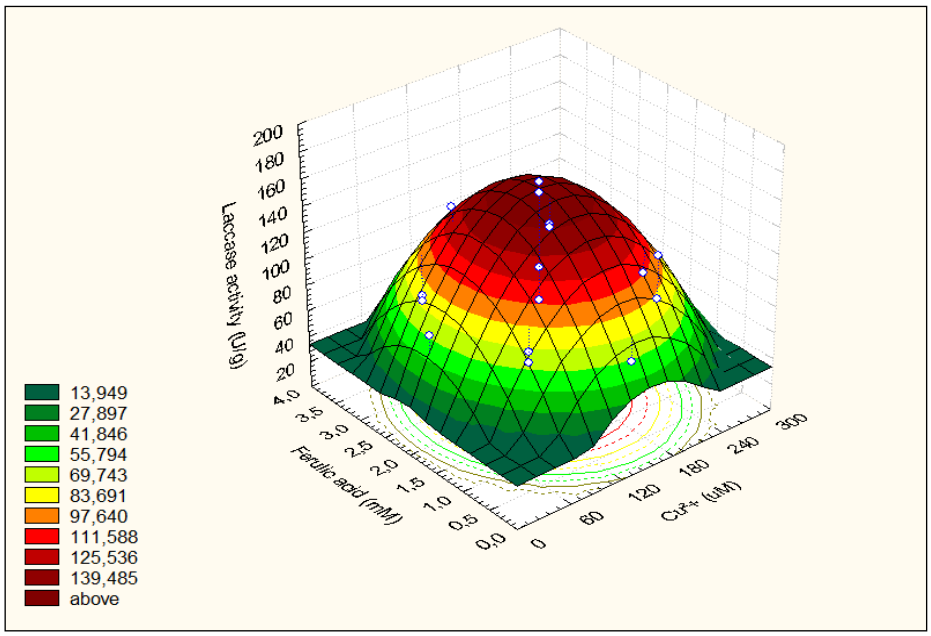

Figure 3.4. Response surface plot described by the model, showing the effect of $CuSO_4$ concentration (μM) and ferulic acid concentration (mM) on laccase activity (U/g).

Table 3.6 – Regression coefficients and identification of significant variables ($p < 0.05$) for laccase production using Central Composite Design, $R^2 = 0.8753$.

Factor	Coefficients	Standard error	*t*-value	*p*-value
Intercept	-249.9	80.11	-3.120	0.02059
Yeast extract (L[a])	41.53	12.19	3.405	0.01440
Yeast extract (Q[b])	-3.236	0.7220	-4.482	0.004182
Cu^{2+} (L)	2.071	0.4551	4.550	0.003890
Cu^{2+} (Q)	-0.0060	0.00116	-5.483	0.001539
Ferulic acid (L)	106.7	34.13	3.127	0.02040
Ferulic acid (Q)	-28.68	6.498	-4.414	0.004501

[a]Linear; [b]Quadratic

According to Table 3.6, the linear and quadratic terms of all three variables significantly affected the response. Interaction effects (not shown) were not significant. The mathematical model of laccase production can be given by the following equation:

$$\text{Laccase activity (U/g)} = -249.9 + 41.53[YE] - 3.236[YE]^2 + 2.071[Cu^{2+}] - 0.0060[Cu^{2+}]^2 + 106.7[Fer] - 28.68[Fer]^2$$

where concentrations of yeast extract (YE), copper (Cu^{2+}) and ferulic acid (Fer) are given in g/L, μM and mM, respectively.

The predicted model indicated that the maximum laccase activity (161.3 U/g) would be obtained at the following conditions: yeast extract 6.417 g/L, Cu^{2+} 172.6 μM and ferulic acid 1.860 mM.

Experiments for the verification of the predicted model (Table 3.7) revealed a correlation coefficient (R^2) of 0.8963, the most significant differences being obtained at the lowest and highest levels (24 and 44%, respectively). However, when these points were not considered, the model described the laccase production as a function of yeast extract, $CuSO_4$ and ferulic acid concentrations – within the range of 5.5 to 8.5 g/L, 112.5 to 187.5 μM and 1.5 to 2.5 mM, respectively – with a correlation coefficient of 0.9798.

Table 3.7 – Experiments for the verification of the predicted model of laccase production.

Yeast extract (g/L)	$CuSO_4$ (μM)	Ferulic acid (mM)	Predicted (U/g)	Experimental (U/g)
4.0	75	1.0	64.04	79.74 ± 7.94
5.5	112.5	1.5	133.2	114.7 ± 7.17
6.4	172.6	1.86	161.3	155.3 ± 5.92
7.0	150	2.0	156.6	149.2 ± 4.95
8.5	187.5	2.5	134.2	122.9 ± 5.12
10	225	3.0	66.01	95.17 ± 7.75

Other values reported in literature for laccase production by *P. ostreatus* in solid state fermentation are 65.42 U/g with copper as inducer (Mishra and Kumar, 2007); 9 U/g without inducers (Iandolo et al., 2011[a]); 36 U/g without inducers (Iandolo et al., 2011[b]). In liquid culture, *P. ostreatus* produced a maximum activity of 0.147 U/mL after 4 days of growth on tomato pomace (Freixo et al., 2011).

3.3.4. Kinetics of laccase production under optimized conditions

Figure 3.5 presents the kinetics of laccase production under optimized conditions. The peak of laccase activity (151.6 U/g) was obtained between the 4th and the 5th day of solid state fermentation. Other values of laccase productivity reported in literature are 80 U/mL after 12 days in liquid culture of *P. ostreatus* without exogenous inducers (Lettera et al., 2011); 12.2 U/mL after 18 days of liquid fermentation by *P. ostreatus* (Tlecuitl-Beristain et al., 2008); 90 U/g of sugarcane bagasse produced by *Pycnoporus cinnabarinus* after 14 days of solid state fermentation in columns, and activities near 80 U/g after 6 days (Meza et al., 2005); 70 U/g of sugarcane bagasse, produced by *Pycnoporus cinnabarinus* after 10 days (Meza et al., 2007).

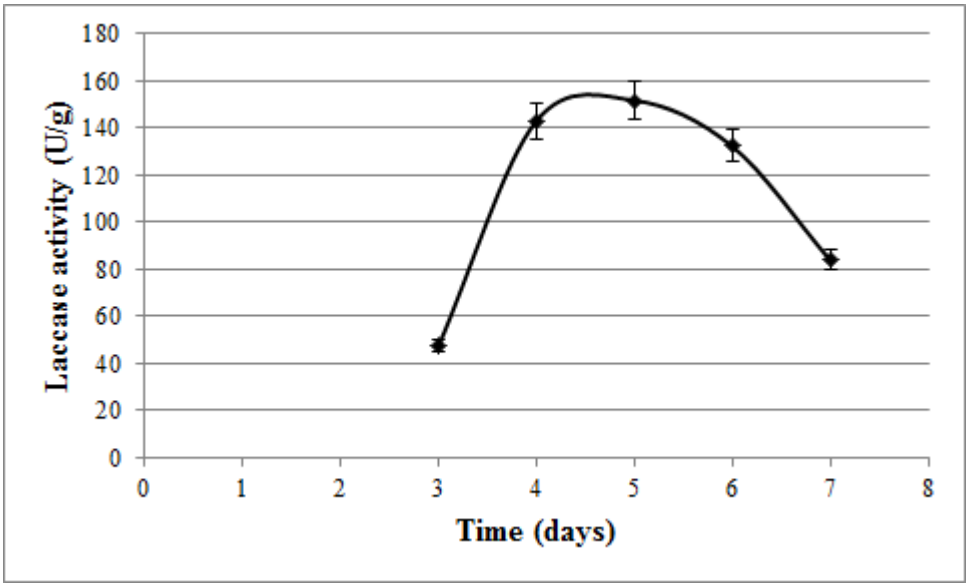

Figure 3.5. Kinetics of laccase production on solid state fermentation of sugarcane bagasse by *P. ostreatus* 22 Em under optimized conditions: 6.4 g/L yeast extract, 172.6 μM $CuSO_4$ and 1.86 mM ferulic acid.

3.4. Conclusions

The level of laccase activity produced by *P. ostreatus* 22 Em in solid state fermentation of sugarcane bagasse was significantly affected by the concentrations of nitrogen source, copper sulfate and ferulic acid. The use of an organic nitrogen source (yeast extract) provided and increase of 5.7 fold in laccase production, in comparison with the inorganic source (ammonium sulfate). The response surface methodology was applied to determine the mathematical model of laccase production as a function of yeast extract, copper sulfate and ferulic acid concentrations. The predicted model (R^2 0.8753) indicated that the maximum laccase activity (161.3 U/g of sugarcane bagasse) would be obtained at the following conditions: yeast extract 6.417 g/L, Cu^{2+} 172.6 μM

and ferulic acid 1.860 mM. Experimentally, the maximum laccase activity of 151.6 U/g was produced, under optimized conditions, at the 5th day of solid state fermentation.

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CHAPTER IV

Characterization of laccase isoforms produced by *Pleurotus ostreatus* in solid state fermentation of sugarcane bagasse

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Abstract

Laccases are oxidative enzymes linked to biological degradation of lignin. The aim of this work was to evaluate the effect of inducers and different concentrations of nitrogen on production level of total laccase activity and pattern of laccase isoforms, produced in solid state fermentation of sugarcane bagasse by a selected strain of *Pleurotus ostreatus*. The addition of yeast extract 5 g/L, copper sulfate 150 μM and ferulic acid 2 mM provided highest enzymatic activity (167 U/g) and zymograms indicated the presence of six laccase isoforms (POXA1b, POXA3, POXC and three other isoforms). Results of protein identification by mass spectrometry confirmed the presence of POXC and POXA3 as the main isoenzymes, and also identified a glyoxal oxidase and three galactose oxidases. The fact that the isoenzyme POXA1b was not identified in the analyzed samples can be possibly explained by its sensitivity to protease degradation.

Keywords: Laccase isoforms, sugarcane bagasse, solid state fermentation, *Pleurotus ostreatus*, zymogram

4.1. Introduction

The valorization of agro-residues by biological routes, especially solid state fermentation, is a key technology that contributes to the development of sustainable processes and the generation of value-added products. Brazilian economy is one of the most important agricultural-based economies in the world, the most important products being coffee, sugarcane, beans, soybean, cassava and fruits, and the residues generated by this intense agricultural activity represent potential feedstock that could be inserted in diverse production chains instead of being discarded (Singhania et al., 2009; Soccol and Vandenberghe, 2003).

Sugarcane bagasse is an important agro-residue generated in high amount (186 million tons / year) by the sugar and alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane, and is composed of 19-24% lignin, 27-32% hemicellulose, 32-44% cellulose and 4.5-9% ashes (Soccol et al., 2011). The utilization of sugarcane bagasse as feedstock has been limited because of its lignocellulosic structure – presence of lignin and hemicelluloses – that restrain the efficient hydrolysis of cellulose, unless the material is previously treated. Pretreatment is one of the most expensive and least technologically mature steps in the process of converting biomass to fermentable sugars (Binod et al., 2012).

The biological degradation of lignin is an oxidative process that can be carried out by white-rot fungi. These microorganisms produce several ligninolytic enzymes (laccases, manganese peroxidases and lignin peroxidases) that catalyze one-electron oxidation of lignin units, producing aromatic radicals (Giardina et al., 2000). Laccases (E.C. 1.10.3.2, p-diphenol: dioxygen oxidoreductases) are blue multicopper oxidases

able to oxidize a variety of phenolic compounds including polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds, with concomitant reduction of molecular oxygen to water (Autore et al., 2009; Dwivedi et al., 2011). These enzymes are secreted in multiple isoforms depending on the fungal species and the environmental conditions, and this variety is related to the diversity of their roles: lignin degradation/synthesis, fruiting bodies development, pigment production, cell detoxification (Piscitelli et al., 2011). The biochemical diversity of laccase isoenzymes appears to be due to the multiplicity of laccase genes; however, regulation of their expression can be substantially diverse between fungal species (Palmieri et al., 2003).

Pleurotus ostreatus belongs to a class of white-rot fungi that produces laccases, manganese peroxidases but not lignin peroxidases (Giardina et al., 2000). The sequencing and annotation of the *P. ostreatus* PC 15 genome version 2.0 (Joint Genome Institute, 2011) indicates the presence of at least twelve genes of multicopper oxidases. Some of the corresponding enzymes have been purified and characterized, and these include POXA1b (Giardina et al., 1999), POXA1w and POXA2 (Palmieri et al., 1997), POXA3 (Palmieri et al., 2003) and POXC, previously named POX2 (Giardina et al., 1996) where POX means phenol oxidase. Other isoenzymes whose sequences have been determined are POX1 (Giardina et al., 1995), POX3 and POX4 (Joint Genome Institute, 2011). POXC is the most abundantly produced under all growth conditions examined according to Giardina et al. (1999).

The objective of these studies was to evaluate the effect of known inducers of laccase expression (copper sulfate and ferulic acid) and two levels of organic nitrogen concentration in the form of yeast extract, on the production level of total laccase

activity and laccase isoforms – identified by zymograms and mass spectrometry – produced in solid state fermentation, utilizing the sugarcane bagasse as substrate and a selected strain of *Pleurotus ostreatus* (coded Pl 22 Em).

4.2. Materials and Methods

4.2.1. Solid state fermentation

Glass flasks containing 1 g of sugarcane bagasse were autoclaved and humidified with a saline solution (sterilized by filtration, 15 mL/g bagasse) presenting the following constant composition: $MgSO_4.7H_2O$ (0.3 g/L), $FeSO_4.7H_2O$ (0.005 g/L), $MnSO_4.H_2O$ (0.00156 g/L), $ZnSO_4.7H_2O$ (0.0014 g/L), $CaCl_2$ (0.3 g/L), $CoCl_2$ (0.002 g/L), KH_2PO_4 (1.5 g/L), pH 5.5, with some differences regarding the following variables: yeast extract (2 or 5 g/L, sterilized by autoclaving at 121ºC and 1 atm); $CuSO_4$ (0 or 150 μM, sterilized by filtration), ferulic acid (2mM, sterilized by filtration and added after 48h of fermentation). The strains *Pleurotus ostreatus* 22 Em (Pl 22 Em – selected strain) and *Pleurotus ostreatus* ATCC MYA-2306 (Pl ATCC – standard strain for laccase production in liquid culture) were reactivated in PDA dishes and after 7 days of growth, 4 disks of 7 mm diameter were transferred to Czapek liquid medium containing the antibiotic streptomycin sulfate (0.1 g/L). After 5 days of growth at 28ºC and 125 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a homogenizer (IKA T-10 basic Ultra-Turrax) and resuspended in the residual medium to a lower final volume (10% of the initial volume). 0.2 mL of the homogenized mycelium was transferred to the fermentation flasks containing the

previously prepared sugarcane bagasse. Substrate and inoculum were manually homogenized and incubated at 28ºC for different times.

4.2.2. Extraction of the enzymes

Enzymes produced by solid fermentation were extracted by solid-liquid extraction using sodium phosphate buffer as solvent ($NaH_2PO_4.H_2O$, 50 mM, pH 7.0) (Iandolo et al., 2011[b]). The fermented material was manually homogenized, weighed (around 1 g) and the extraction buffer was added in the proportion of 1:10 (w/w). A protease inhibitor (phenylmethylsulfonyl fluoride, PMSF 1mM) was added to the extraction mixture. The mixture was homogenized in vortex for 1 min and centrifuged for 7,500 g, 4ºC, 45 min. The supernatant was separated and submitted to analyses.

4.2.3. Liquid fermentation

Liquid fermentation was conducted in YPD medium (potato dextrose 24 g/L and yeast extract 5 g/L) containing Cu^{2+} 150 μM, added after autoclaving, in flasks of 500 mL filled with 250 mL (final volume). The strains Pl 22 Em and Pl ATCC were reactivated in PDA dishes and after 7 days of growth, 4 disks of 7 mm diameter were transferred to Czapek liquid medium containing the antibiotic streptomycin sulfate (0.1 g/L). After 5 days of growth at 28ºC and 125 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a homogenizer (IKA T-10 basic Ultra-Turrax) and resuspended in the residual medium to a lower final volume (10% of the

initial volume). 2.5 mL of the homogenized mycelium were transferred to the fermentation flasks, which were incubated at 28ºC and 125 rpm.

4.2.4. Laccase activity and protein concentration assays

The enzymatic activity of laccases was assayed by the oxidation of ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). The reaction mixture contained 100 μL of ABTS 20mM (in sodium citrate buffer 0.1M, pH 3.0), sample (usually 20-50 μL) and sodium citrate buffer ($C_6H_8O_7.H_2O$ 0.1M, pH 3.0) up to 1 mL. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}). The enzyme activity was expressed in International Units (U), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.

Protein concentration was determined using the BioRad Protein Assay based on the method developed by Bradford (1976), with bovine serum albumin as a standard.

4.2.5. Enzyme preparations

Enzyme solutions were concentrated by ultrafiltration and dialyzed in sodium phosphate buffer (50 mM, pH 7.0). Samples were centrifuged at 8,000 g, 4ºC in Amicon tubes (Amicon Ultra centrifugal filters 0.5 mL, Ultracel 30 kDa membrane - Millipore).

4.2.6. Zymograms of native PAGE

Polyacrylamide gel electrophoresis (PAGE) was performed at an alkaline pH under non-denaturing conditions. The separating and stacking gels contained, respectively, 9 and 4% acrylamide and 50 mM Tris-HCl (pH 9.5) and 18 mM Tris-HCl (pH 7.5) as buffers. The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8.4). Visualization of the bands was achieved by the addition of ABTS 20 mM to the gel submerged in sodium citrate buffer (0.1M, pH 3.0) after electrophoresis.

4.2.7. Preparation of enzymes for protein identification

Enzymes from the samples selected for enzyme identification were extracted according to item 4.2.2 and concentrated by ammonium sulfate precipitation (80% saturation, 4ºC, overnight) followed by centrifugation (8,000 g, 4ºC,40 min). Protein precipitate was suspended in 1 mL sodium phosphate buffer (50 mM, pH 6.5) and extensively dialyzed against the same buffer in a 12-14,000 Daltons membrane (Delchimica dialysis tubing – visking, code DTV 12000), until the ammonium sulfate was completely removed. Activity and protein concentration in the enzyme suspension were determined according to item 4.2.4.

Semi-denaturating SDS-PAGE was performed by loading the protein suspension in 0.1% SDS buffer not containing 2-mercaptoethanol and without boiling. The separating and stacking gels contained, respectively, 12.5 and 4% acrylamide and 375 mM Tris-HCl plus 0.1% SDS (pH 8.8) and 125 mM Tris-HCl plus 0.1% SDS (pH 6.8) as buffers. The electrode reservoir solution contained 25 mM Tris, 190 mM glycine and

3.5 mM SDS (pH 8.4). Visualization of the bands was achieved by Coomassie brilliant blue staining.

4.2.8. Protein identification

Coomassie blue-stained protein bands were excised from the gels and washed first with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubating the bands with 10 mM dithiothreitol (DTT) for 45 min at 56°C. Cysteines were alkylated by incubation in 5 mM iodoacetamide for 15 min at room temperature in the dark. The bands were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestion was carried out with trypsin (12.5 ng/µL) in 50 mM ammonium bicarbonate buffer, pH 8.5. Gel particles were incubated at 4°C for 2h, in order to allow the enzyme to enter the gel. The buffer solution was then removed and a new aliquot of buffer solution was added for 18 h at 37°C. A minimum reaction volume, enough for completing gel rehydration was used. Peptides were then extracted by washing the gel particles with 0.1% formic acid in 50% acetonitrile at room temperature and lyophilized.

Peptide mixtures were analyzed by LC-MS/MS, using a HPLC-Chip LC system (Agilent 1200) connected to a Q-TOF 6520 (Agilent Technologies). Lyophilized samples were resuspended in 10 µL of 0.1% formic acid. After loading, the peptide mixtures were concentrated and washed at 4 µL/min in a 40 nL enrichment column with 0.2% formic acid in 2% acetonitrile. Fractionation was carried out on a C-18 reverse phase column (75 µm x 43 mm) at a flow rate of 0.4 µL/min with a linear gradient of

eluent B (95% acetonitrile and 0.2% formic acid) in eluent A (2% acetonitrile and 0.1% formic acid) from 7% to 80% in 51 min.

Mass spectrometry analyses were performed using data dependent acquisition MS scans (mass range 300-2400 m/z), followed by MS/MS scans (mass range 100-2000 m/z) of the 4 most intense ions of a chromatographic peak.

Raw data from LC-MS/MS were converted to m/z data, and searched against the PleosPC15 database available at the Joint Genome Institute's website (http://genome.jgi-psf.org/PleosPC15_1) using the licensed version of Mascot 2.1 (Matrix Science). The Mascot search parameters were: allowed number of missed cleavages 2; enzyme trypsin; variable post-translational modifications, methionine oxidation, pyro-glu N-term Q; peptide tolerance 10 ppm and MS/MS tolerance 0.6 Da; peptide charge, from +2 to +3.

4.3. Results and Discussion

The following experiments evaluated the effect of organic nitrogen concentration, in the form of yeast extract, together with the presence of two known inducers of laccase expression, copper sulfate and ferulic acid, on the production of laccases (total activity) and the expression pattern of laccase isoforms by *P. ostreatus* 22 Em during solid state fermentation on sugarcane bagasse.

4.3.1. Evaluation of the effect of copper sulfate and ferulic acid addition on laccase activity production level and laccase isoenzymes pattern

The effect of the variables $CuSO_4$ (0 or 150 μM) and ferulic acid (0 or 2 mM), which are known inducers of laccase expression, on the level of total laccase production by *P. ostreatus* 22 Em was evaluated during fungal solid state fermentation using sugarcane bagasse at two values (2 and 5 g/L) of yeast extract (YE) concentration.

The results in Table 4.1 showed a peak of activity at the 5th day, highest values being obtained with the combination of copper sulfate and ferulic acid: 167 U/g with 5 g/L YE and 86.8 U/g with 2 g/L YE. Without the addition of ferulic acid, the highest value achieved was 51.7 U/g dry substrate, and without any inducer the maximum activity of 14.6 U/g was achieved at the 8th day. Other values reported in literature for laccase production by *P. ostreatus* in solid state fermentation are 65.42 U/g with copper as inducer (Mishra and Kumar, 2007); 9 U/g without inducers (Iandolo et al., 2011[a]); 36 U/g without inducers (Iandolo et al., 2011[b]).

Table 4.1 – Maximum level of laccase activity produced by the strain *P. ostreatus* 22 Em on sugarcane bagasse in different conditions of yeast extract (YE) concentrations and inducers – Cu^{2+} and ferulic acid (Fer).

YE (g/L)	Inducer	Time (days)	U/g dry substrate
2	0	5	1.06
		8	14.6
		10	11.6
2	Cu^{2+} 150 μM	5	40.5
		10	22.3
		12	18.0
2	Cu^{2+} 150 μM + Fer 2 mM	5	86.8
		10	32.2
		12	17.6
5	Cu^{2+} 150 μM	5	51.7
		10	21.4
		12	10.7
5	Cu^{2+} 150 μM + Fer 2 mM	5	167
		10	57.1
		12	37.6

The effect of the variables $CuSO_4$ (0 or 150 μM) and ferulic acid (0 or 2 mM), shown to be inducers of laccase production by *P. ostreatus* 22 Em through the above experiments, was also evaluated on the pattern of laccase isoenzymes secreted at the 5^{th}, 8^{th} and 10^{th} days of solid state fermentation on sugarcane bagasse. The concentration of yeast extract was kept constant (2 g/L), and the behavior of the fungal isoenzymes on native PAGE was compared to that of the laccase isoenzymes purified from the dikaryotic strain Pl ATCC, as standards (Figure 4.1). According to the visual analysis of the native PAGE zymogram, the isoenzyme POXC was the most abundantly produced under all conditions evaluated, which is in accordance with Giardina et al. (1996; 1999). The expression of the isoenzyme POXA3 (Palmieri et al., 2003) was induced by copper sulfate and even more by the combination of copper sulfate and ferulic acid, when comparing the three samples obtained at the 8^{th} day. However, samples of the 10^{th} day presented a more intense POXA3 band, indicating an increase of this isoenzyme production. The band corresponding to the isoenzyme POXA1b (Giardina et al., 1999)

could be visualized only in those samples containing ferulic acid. Besides the three known isoforms POXA1b, POXA3 and POXC, three more bands were visualized. However, an isoenzyme with electrophoretic mobility similar to that of the isoform 3 had been already identified by Lettera et al. (2010) as LACC12. Moreover, isoforms with similar electrophoretic mobility to that of the isoforms 1 and 2 had been already detected during solid state fermentation of *P. ostreatus* ATCC on tomato pomace (Iandolo et al., 2011[b]).

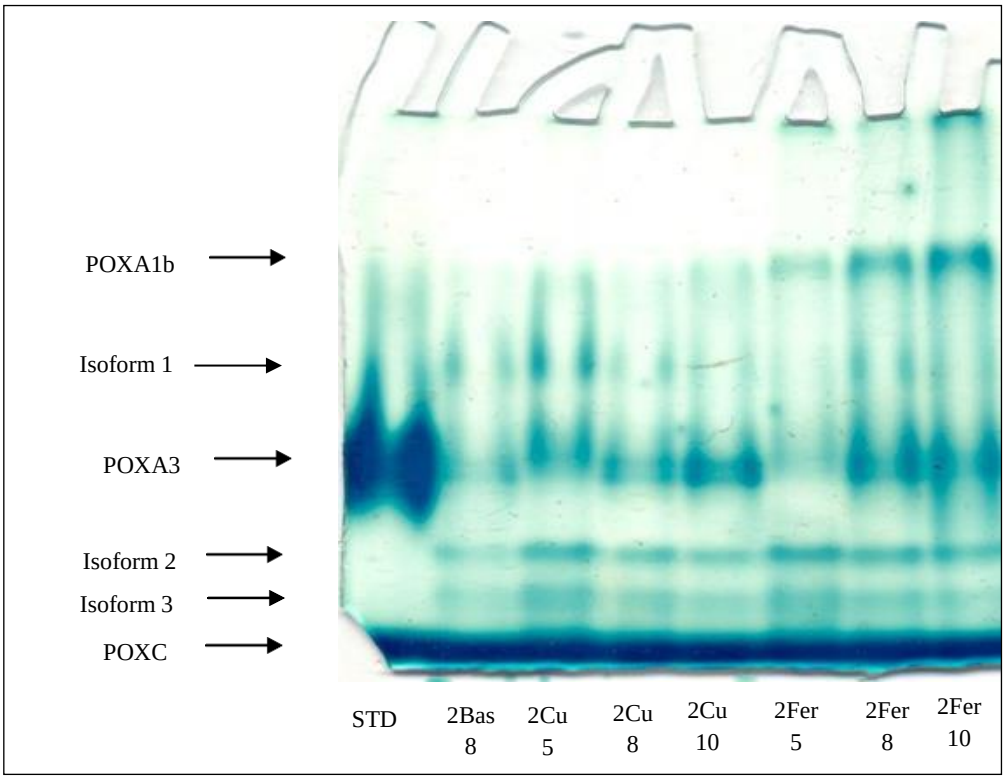

Figure 4.1. Zymogram of laccases produced under different conditions of solid state fermentation by the strain *P. ostreatus* 22 Em, with 2 g/L yeast extract. STD – standards (POXA1b, POXA3 and POXC secreted by Pl ATCC); Bas – Basal medium (without inducers); Cu – with Cu^{2+} 150 μM; Fer – with Cu^{2+} 150 μM and ferulic acid 2 mM; numbers below represent the time of fermentation (5, 8 and 10 days). All enzymes were loaded with an activity of 0.0075 U.

It has been previously demonstrated that the addition of copper sulfate 150 μM to a *P. ostreatus* liquid culture medium causes a 30-fold increase in total laccase activity and induces the isoenzymes POXC and POX A1b in *P.ostreatus* at the level of gene transcription (Palmieri et al., 2000). Analysis of the *poxc* and *poxa1b* promoter regions revealed the presence of multiple putative metal responsive elements (Faraco et al., 2002). In fact, in this experiment, the total laccase activity obtained in the presence of copper was 38 fold higher in comparison with the basal medium when analyzing the activities obtained at the 5^{th} day at the same concentration of yeast extract (Table 4.1). Hou et al. (2004) also reported a 4.5-fold increase in laccase activity in *P. ostreatus* when Cu^{2+} 1 mM was added to the liquid culture medium. Copper sulfate has been reported to be a good inducer of laccase expression not only in *P. ostreatus* but also in the most extensively studied ligninolytic fungus *Phanerochaete chrysosporium*, however, for this last one, it showed no influence on the pattern of the two isoforms produced (Gnanamani et al., 2006).

Different studies have shown that laccase production is regulated by metal ions such as Cu^{2+} and Fe^{3+} by gene expression induction or through translational or post-translational regulation (Fonseca et al., 2010). Baldrian and Gabriel (2002) concluded that Cu^{2+} not only induces laccase by the expression of laccase genes in *P. ostreatus*, but it also positively affects activity and stability of the enzyme.

Ferulic acid was shown to be the best inducer of laccase activity in *Pleurotus sajor-caju*. The structure of ferulic acid is similar to that of coniferyl alcohol, the most abundant monolignol of the three lignin precursors (Zucca et al., 2011).

A more deepened analysis of modulation of isoenzymes production profile secreted by *P. ostreatus* 22 Em during solid state fermentation on sugarcane bagasse

was performed, in duplicates, analyzing the isoenzyme pattern at the 5th, 8th, 10th, 12th and 15th day at different concentrations of yeast extract (2 and 5 g/L), together with presence or absence of 150 μM $CuSO_4$ with or without 2 mM ferulic acid.

According to the zymogram reported in Figure 4.2, the intensity of the band related to POXC was apparently not affected by different concentrations of yeast extract and the presence of both inducers, and its intensity decreased only after 15 days (sample 5Fer15). The expression of the isoenzyme POXA3 was induced by ferulic acid, in accordance with the previous results, for both concentrations of organic nitrogen, and the expression kinetics was dependent on the concentration of yeast extract. For the isoenzyme POXA1b, the higher concentration of organic nitrogen had an apparent negative effect. It is important to remark that this isoenzyme is very sensitive to protease degradation (Palmieri et al., 2000), so this effect could be possibly explained by the relation between the presence of proteases and yeast extract concentration. Ferulic acid did not significantly induce the expression of POXA1b from the 10th day on, in comparison with copper sulfate alone, when the concentration of yeast extract was 2 g/L, however for the concentration of 5 g/L this effect was observed from the 12th day on.

The new band 1 was more clearly visualized when the higher concentration of yeast extract was used (5 g/L), and increased with fermentation time in the presence of ferulic acid. However, in the absence of ferulic acid, the most intense band was obtained at the 10th day of fermentation. Vanhulle et al. (2007) also reported a positive effect of ferulic acid 0.5 mM on laccase production by *P. ostreatus*, and a peak of laccase activity (around 7,500 U/L) at the 15th day of fermentation (3-fold increase when compared to control). The isoforms 2 and 3 were also more intense in the presence of higher

concentration of yeast extract, and ferulic acid showed an observable positive effect on their expression only at this condition. Also the reference strain *P. ostreatus* ATCC produced three new bands on solid state fermentation of sugarcane bagasse, indicating that this pattern is more related to the fermentation conditions than to the strain in this case.

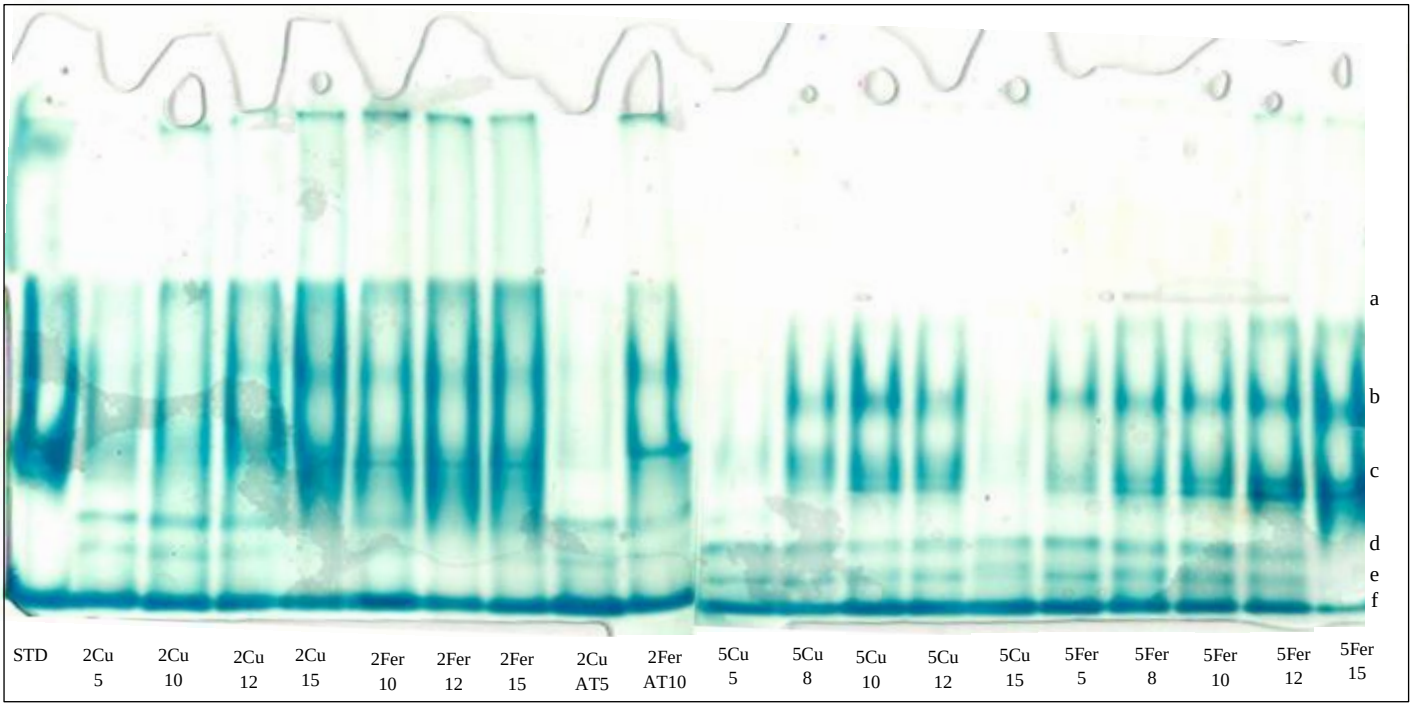

Figure 4.2. Zymogram of laccases produced under different conditions of solid state fermentation by the strain *P. ostreatus* 22 Em. STD – standards ([a]POXA1b, [c]POXA3 and [f]POXC); [b]Isoform 1; [d]Isoform 2; [e]Isoform 3; 2Cu – with yeast extract 2 g/L and Cu^{2+} 150 μM; 2Fer – with yeast extract 2 g/L, Cu^{2+} 150 μM and ferulic acid 2mM; 5Cu – with yeast extract 5 g/L and Cu^{2+} 150 μM; 5Fer – with yeast extract 5 g/L, Cu^{2+} 150 μM and ferulic acid 2mM; AT represents that the strain *P. ostreatus* ATCC was used for comparison; numbers below represent the time of fermentation (5, 8, 10, 12 and 15 days). All enzymes were loaded with an activity of 0.015 U.

Although ligninolytic enzymes have been considered as products of the secondary metabolism produced under conditions of limited nitrogen for most of the white rot fungi (Kaal et al., 1995), in *P. ostreatus* a higher concentration of nitrogen in the medium did not repress but rather slightly stimulated mineralization of lignin, as reported by Stajic et al. (2006). Kaal et al. (1995) also suggested that several white rot fungi strains, including *P. ostreatus*, produce higher ligninolytic enzyme activities in response to a nitrogen-rich medium, in contrast to the aforementioned physiological model proposed for *Phanerochaete chrysosporium*. Mishra and Kumar (2007) demonstrated that regarding enhancement of laccase production in solid state fermentation by *P. ostreatus*, yeast extract was preferred to inorganic nitrogen sources, reaching 23 U/gds, which may be attributed to the presence of some additives (nutrients/activators) and favorable C:N ratio. Hou et al. (2004) reported an opposite effect on laccase activity regarding nitrogen concentration in the form of urea, for a strain of *P. ostreatus*. The higher concentration of urea (5 g/L) inhibited laccase activity in relation to the lower concentration (0.5 g/L), however, the most suitable nitrogen sources were shown to be peptone and yeast extract, in comparison with urea, ammonium sulfate and ammonium tartarate.

4.3.2. Comparison of *P. ostreatus* 22 Em with the standard strain *P. ostreatus* ATCC in liquid culture

Figure 4.3 represents the zymogram of laccases produced in liquid culture by the strains *P. ostreatus* 22 Em and *P. ostreatus* ATCC. The new bands revealed during solid state fermentation were not observed, corroborating the previous hypothesis that the

expression pattern of laccases is related to the fermentation conditions. Also the isoenzyme POXA1b was not detected in the medium fermented by *P. ostreatus* 22 Em. It is worth noting that *P. ostreatus* 22 Em produces an isoenzyme very close to the band corresponding to the isoenzyme POXC, with similar electrophoretic mobility to one reported by Lettera et al. (2010) in different growth conditions.

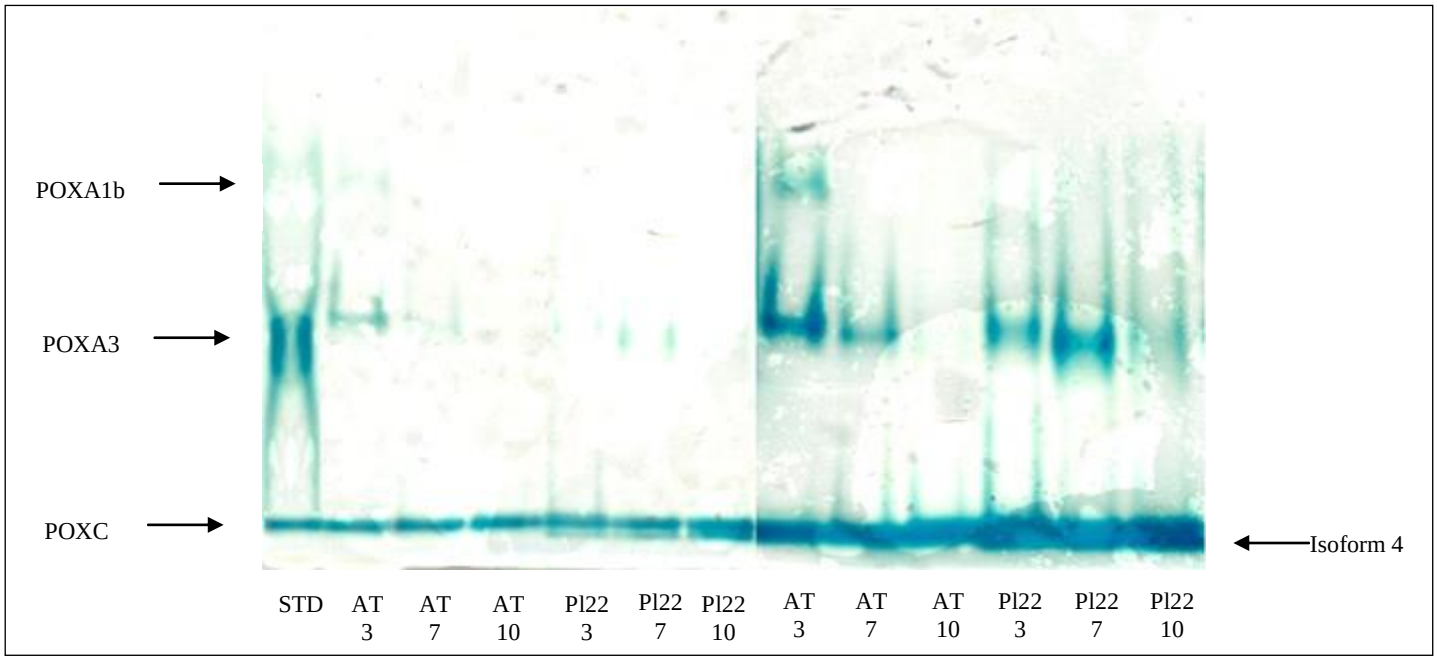

Figure 4.3. Zymogram of laccases (same gel at two times of staining) produced in liquid culture (PDY medium with Cu^{2+} 150 μM) by the strains *P. ostreatus* 22 Em and *P. ostreatus* ATCC. STD – standards (POXA1b, POXA3 and POXC); AT – strain *P. ostreatus* ATCC; Pl22 – strain *P. ostreatus* 22 Em; numbers below represent the time of fermentation (3, 7 and 10 days). All enzymes were loaded with an activity of 0.015 U.

4.3.3. Protein identification

A sample containing the six isoforms of laccases (condition: 5 g/L yeast extract, with $CuSO_4$ 150 μM and ferulic acid 2 mM, after 10 days) was selected to get an enzyme preparation to perform identification by mass spectrometry.

Proteomic analysis confidently assessed the presence of the isoenzymes POXA3 and POXC. Interestingly a glyoxal oxidase and three galactose oxidases, which are enzymes linked to lignin degradation, were also identified (Table 4.2). No POXA1b isoenzyme could be identified; its absence can be possibly explained by its sensitivity to extracellular protease degradation, as already reported for *P. ostreatus* (Palmieri et al., 2000).

Table 4.2 – Protein identification by searching PleosPC15 genome database with MS/MS ion search Mascot software (Matrix Science), oxidation on Met, cyclization of Gln at N-terminus of the peptides to Pyro-Glu, as variable modifications.

Protein name (Accession Number)	**Number of peptides**	**Sequence coverage**
Glyoxal oxidase (52532)	11	26%
Laccase POXA3 (32778)	8	15%
Galactose oxidase (24058)	5	9%
Galactose oxidase (28647)	6	14%
Galactose oxidase (174951)	6	7%
Laccase POXC (36257)	2	2%

Note: Only proteins identified with at least two peptides were considered as significant. Peptides with individual ion scores > 20 were considered.

4.4. Conclusions

The highest values of laccase activities were produced by the strain *P. ostreatus* 22 Em at the 5th day of solid state fermentation on sugarcane bagasse: 167 U/g with 5 g/L yeast extract and ferulic acid 2 mM and 86.8 U/g with 2 g/L yeast extract and ferulic acid 2mM. Without the addition of ferulic acid, the highest value was 51.7 U/g with Cu^{2+} 150 μM and without any inducer the maximum activity of 14.6 U/g was achieved at the 8th day. Six isoforms of laccases (POXA1b, POXA3, POXC and three other isoforms) were observed in the native PAGE zymograms of the strain *P. ostreatus*

22 Em grown in solid state fermentation of sugarcane bagasse. The production of five of these isoforms was also demonstrated by the strain *P. ostreatus* ATCC in solid state fermentation, indicating that this pattern is more related to fermentation conditions than to the strain in this case. Copper had a positive effect on enzymatic activity for being an inducer of laccase expression, so it was added to all experiments. The presence of ferulic acid induced the expression of POXA3 and POXA1b, and the expression of POXC was apparently not affected by nitrogen concentration and inducers. Higher nitrogen concentration together with ferulic acid induced the expression of the isoforms 1, 2 and 3. In liquid culture the strain *P. ostreatus* 22 Em produced an isoenzyme very close to the band corresponding to the isoenzyme POXC, with similar electrophoretic mobility to one reported by Lettera et al. (2010) in different growth conditions. Mass spectrometry results confirmed the presence of POXC and POXA3, and also identified a glyoxal oxidase and three galactose oxidases, which are enzymes linked to lignin degradation. The absence of the isoenzyme POXA1b in the analyzed samples can be possibly explained by its sensitivity to protease degradation.

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CHAPTER V

Biological delignification and recovery of laccases from solid-state fermented sugarcane bagasse

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Abstract

Delignification is a necessary pretreatment step in the process of converting plant biomass into fermentable sugars. Sugarcane bagasse, an agro-residue generated by the sugar and alcohol industry in Brazil, may present a lignin content of almost one third of its mass. The objective of this work was to evaluate the kinetics of biological delignification of sugarcane bagasse by *Pleurotus ostreatus* in solid state fermentation, under optimized conditions for laccase production. Laccases were recovered by solid-liquid extraction, concentrated and used in the process of enzymatic delignification of sugarcane bagasse. Highest laccase activity (152 U/g) was obtained at the 5th day of fermentation. Lignin content was reduced from 31.89% to 26.36% after 5 days and to 20.79% after 15 days by the biological treatment of solid state fermentation. Enzymatic hydrolysis reduced the lignin content from 31.89% to 14.98% in 12h.

Keywords: Biological delignification, solid state fermentation, enzymatic delignification, sugarcane bagasse

5.1. Introduction

One of the main challenges in the utilization of lignocellulosic biomass in fermentative processes is the transformation of the complex polysaccharides into simple sugars that can be assimilated by microorganisms. This can be achieved by chemical or enzymatic hydrolysis, preceded by appropriate pretreatments that enhance the efficiency of hydrolysis. The aim of the pretreatment is to separate lignin and break the structure of lignocellulose, and it is one of the most expensive and least technologically mature steps in the process of converting biomass to fermentable sugars (Binod et al., 2012).

Sugarcane bagasse is an important lignocellulosic agro-residue generated in high amount (186 million tons / year) by the sugar and alcohol industry in Brazil. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane, and is composed of 19-24% lignin, 27-32% hemicellulose, 32-44% cellulose and 4.5-9% ashes (Soccol et al., 2011). The utilization of sugarcane bagasse as feedstock has been limited because of its lignocellulosic structure – presence of lignin and hemicelluloses – that restrain the efficient hydrolysis of cellulose, unless the material is previously treated.

Delignification can be performed by thermo-chemical processes or by the biological route, using enzymes or microorganisms. The advantages of biological delignification over the thermo-chemical methods may include mild reaction conditions, higher product yields and fewer side reactions, less energy demand and less reactor resistance to pressure and corrosion (Lee, 1997).

Lignin decomposition in nature is primarily attributed to the metabolism of microorganisms. Among all other organisms, white-rot basidiomycetes degrade lignin more rapidly and extensively than other groups (Falcón et al., 1995). These microorganisms produce several ligninolytic enzymes (laccases, manganese peroxidases and lignin peroxidases) that catalyze one-electron oxidation of lignin units, producing aromatic radicals (Giardina et al., 2000).

Some white-rot fungi preferentially attack lignin more readily than hemicellulose and cellulose. *Ceriporiopsis subvermispora*, *Phellimus pini*, *Phlebia* spp. and *Pleurotus* spp. belong to this group. Many white-rot fungi, however, exhibit a pattern of simultaneous decay characterized by degradation of all cell wall components. Examples of this group include *Trametes versicolor*, *Heterobasidium annosum* and *Irpex lacteus* (Wong, 2009).

Solid state fermentation is an interesting process to perform biological delignification because it mimics the natural environment of lignin-degrading fungi. The advantages of the solid state fermentation process over submerged fermentation include: smaller fermenter volume; lower sterilization energy costs; easier aeration; reduced or eliminated costs for stirring and effluent treatment; lower costs for product recovery and drying; less favorable environment for many bacteria, lowering the risk of contamination (Lee, 1997; Soccol and Vandenberghe, 2003).

One of the main disadvantages of the biological delignification by fermentation is the long incubation time, usually many days or weeks. In comparison with solid state fermentation, enzymatic delignification processes performed in enzymatic reactors demand less incubation time. Kuila et al. (2011) reported a maximum delignification of *Bambusa bambos* of 84% using laccase from *Pleurotus* sp. at 400 U/mL, after 8h of

incubation. Recently, Moniruzzaman and Ono (2012) described an enhanced method to promote enzymatic delignification of wood chips, using ionic liquid swollen biomass in ionic liquid aqueous systems. Enzymatic treatment resulted in around 50% delignification of wood biomass after 24 h in the presence of laccase.

The aim of this work was to evaluate the kinetics of biological delignification of sugarcane bagasse by *Pleurotus ostreatus* in solid state fermentation, under conditions which were previously optimized. Laccases produced by this process were recovered, concentrated and employed to perform the enzymatic treatment of sugarcane bagasse.

5.2. Materials and Methods

5.2.1. Characterization of the sugarcane bagasse

The sugarcane bagasse was provided by the private company Ourofino Agronegócio, located in the region of Ribeirão Preto, São Paulo, Brazil. The bagasse was previously washed with water, dried at 60°C and the portion presenting particle sizes greater than 2 mm was grinded in a knife mill. Separation of the fractions presenting different particle sizes was performed by sieving. Contents of lignin, total extractives, ashes and moisture were determined according to the TAPPI norms T222, T264, T413 and T264, respectively. Holocellulose content was calculated by difference.

5.2.2. Solid state fermentation

Erlenmeyer flasks containing 3 g of sugarcane bagasse (particle size between 0.8 and 2 mm 50% and < 0.8 mm 50%) were autoclaved and humidified with a saline solution (sterilized by filtration, 10 mL/g bagasse) presenting the following constant composition: $MgSO_4.7H_2O$ (0.3 g/L), $FeSO_4.7H_2O$ (0.005 g/L), $MnSO_4.H_2O$ (0.00156 g/L), $ZnSO_4.7H_2O$ (0.0014 g/L), $CaCl_2$ (0.3 g/L), $CoCl_2$ (0.002 g/L), KH_2PO_4 (1.5 g/L), pH 5.5. Yeast extract (sterilized by autoclaving at 121ºC, 1 atm, 15 min) and copper sulfate (sterilized by filtration) were added to the saline solution to reach final concentrations of 6.4 g/L and 173 μM, respectively. Ferulic acid (sterilized by filtration) was added after 48h of fermentation to a final concentration of 1.86 mM.

The strain of *P. ostreatus* (coded Pl 22 Em) was reactivated in PDA dishes and after 7 days of growth, 4 disks of 7 mm diameter were transferred to Czapek liquid medium containing the antibiotic cephalexin (0.08 g/L). After 5 days of growth at 30ºC and 120 rpm, the mycelium was separated from the residual medium by a sieve, homogenized with a spatula and resuspended in the residual medium to a lower final volume (10% of the initial volume). 0.2 mL of the homogenized mycelium (containing 4% of dry biomass) was transferred to the fermentation flasks containing the previously prepared sugarcane bagasse. The substrate and the inoculum were manually homogenized and incubated at 29ºC.

5.2.3. Extraction of the enzymes

Enzymes produced by solid fermentation were extracted by solid-liquid extraction using sodium phosphate buffer as solvent ($NaH_2PO_4.H_2O$, 50 mM, pH 7.0) (Iandolo et al., 2011[b]). The fermented material was manually homogenized, weighed (around 1 g) and the extraction buffer was added in the proportion of 1:10 (w/w). A protease inhibitor (phenylmethylsulfonyl fluoride, PMSF 1mM) was added to the extraction mixture. The mixture was homogenized in vortex for 1 min and centrifuged for 7,500 g, 4ºC, 45 min. The supernatant was separated and submitted to analyses.

5.2.4. Laccase activity and protein concentration assays

The enzymatic activity of laccases was assayed by the oxidation of ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). The reaction mixture contained 100 μL of ABTS 20mM (in sodium citrate buffer 0.1M, pH 3.0), sample (usually 20-50 μL) and sodium citrate buffer ($C_6H_8O_7.H_2O$ 0.1M, pH 3.0) up to 1 mL. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}). The enzyme activity was expressed in International Units (U), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate in 1 min.
Protein concentration was determined using the BioRad Protein Assay based on the method developed by Bradford (1976), with bovine serum albumin as a standard.

5.2.5. Concentration of the enzymes

Enzyme solutions were concentrated by ultrafiltration and dialyzed in sodium phosphate buffer (50 mM, pH 7.0). Samples were centrifuged at 8,000 g, 4ºC in Amicon tubes (Amicon Ultra centrifugal filters, Ultracel 30 kDa membrane - Millipore).

Enzyme solutions extracted according to item 5.2.3 were also concentrated by ammonium sulfate precipitation (80% saturation, 4ºC, overnight) followed by centrifugation (8,000 g, 4ºC,40 min). Protein precipitate was resuspended in 1 mL sodium phosphate buffer (50 mM, pH 6.5) and extensively dialyzed against the same buffer in a 12-14,000 Daltons membrane (Delchimica dialysis tubing – visking, code DTV 12000), until the ammonium sulfate was completely removed. Activity and protein concentration in the enzyme suspension were determined according to item 5.2.4.

5.2.6. Enzymatic hydrolysis of sugarcane bagasse

Glass flasks containing 1 g of sugarcane bagasse were humidified with sodium citrate buffer (0.1M, pH 3.0) to a solid:liquid ratio of 1:20. Laccases, extracted according to item 5.2.3 (initial activity 2 U/mL) and concentrated by ultrafiltration according to item 5.2.5 (final activity 60 U/mL), were added to reach an enzyme concentration of 180 U/g bagasse. This value was selected according to some literature reports (Bajpai et al., 2006; Kuila et al., 2011; Thakur et al., 2012) and also in order to have a higher enzyme activity, in units per gram, than that obtained by solid state

fermentation. Flasks were incubated at room temperature and 120 rpm, and samples of 0h, 4h, 8h, 12h and 16h were taken for lignin quantification.

5.3. Results and Discussion

5.3.1. Characterization of the sugarcane bagasse and kinetics of biological delignification

The sugarcane bagasse generated by the sugar and alcohol industry presented the following particle size distribution: 21.2% between 0.8 and 2.0 mm, 22.8% < 0.8 mm and 56% > 2.0 mm. After grinding and classification, the particle size distribution changed to 42.4% between 0.8 and 2.0 mm and 57.6% < 0.8 mm. The physicochemical composition of sugarcane bagasse before and after biotreatment is presented in Table 5.1.

Table 5.1 – Physicochemical composition of sugarcane bagasse – in natura and after biotreatment.

	Bagasse in natura	Bagasse prepared for biotreatment	Biotreated bagasse 5 days	Biotreated bagasse 10 days	Biotreated bagasse 15 days
Lignin (%)	31.89	28.48	26.36	22.37	20.79
Holocellulose (%)	63.36	63.30	64.38	67.84	69.12
Extractives (%)	2.15	5.88	5.57	6.05	6.17
Ashes (%)	2.60	2.34	3.69	3.74	3.92
Moisture (%)	7.57	91.6	88.28	87.71	85.91

Note: Percentages of lignin, holocellulose, extractives and ashes are in moisture free basis. Average standard deviations were 0.367 for lignin, 0.165 for extractives, 0.233 for ashes and 0.0587 for moisture.

Reduction of lignin content was of 5.53% after 5 days and of 11.1% after 15 days of solid state fermentation, and the highest laccase activity was obtained at the 5th

day (152 U/g). There are several reports on biological treatments to remove lignin requiring long incubation times. The process developed by Pellinen et al. (1989) to delignify kraft pulp and chemithermo-mechanical pulp (CTMP) using *Phanerochaete chrysosporium* presented delignification times of around two weeks, the kappa number (residual lignin) being reduced from 33 to less than 10 for the kraft pulp and the lignin content decreasing from 26.5% to 21.3% for the CTMP. Delignification of sugarcane bagasse by *Ceriporiopsis subvermispora* during 30 days resulted in a pulp yield of 46-54% (Costa et al., 2005). Meza et al. (2006) presented a process for biological delignification of sugarcane bagasse and simultaneous production of laccases that yielded a laccase activity of 80 U/g and an energy economy of 50% during pulping and refining, after 28 days of fungal treatment.

5.3.2. Strategies for laccase recovery and concentration

Table 5.2 presents the results of laccase activity obtained after concentration of the crude extract by ultrafiltration and precipitation. There was a high recovery yield of 96.25% after concentration by ultrafiltration. After precipitation with ammonium sulfate, there was a loss of activity of 55.66% after resuspension of the pellet. After 48h of re-solubilization, however, the activity was found to be 83.68% of the initial value, indicating that the denaturation caused by precipitation was reversible. After this time, activity decreased possibly because of protease degradation or denaturation.

Table 5.2 – Results of laccase activity obtained after concentration by ultrafiltration and precipitation.

	Laccase activity (U/mL)	Specific activity (U/mg protein)	Recovery yield (%)
Crude extract*	0.5099	2.091	100
Ultrafiltration	15.09	6.277	96.25
Precipitation and dialysis			
0h	7.228	11.19	44.34
24h	10.60	12.71	65.03
48h	13.64	14.59	83.68
72h	9.720	12.02	59.63

*Crude extract of a sample containing the six isoforms of laccases produced by *P. ostreatus* 22 Em on solid state fermentation of sugarcane bagasse.

Litthauer et al. (2007) reported a specific activity of 32.9 U/mg for a laccase produced by *Pycnoporus sanguineus* on molasses, after purification by ammonium sulfate precipitation, ion exchange and dye affinity chromatography. Purification yields reported by Liu et al. (2009) for a laccase produced by *P. ostreatus* in liquid culture were 87% after ammonium sulfate precipitation, 46% after ion exchange chromatography and 34% after gel filtration chromatography.

5.3.3. Evaluation of laccase stability

In order to evaluate the laccase stability to storage, an experiment was performed to compare the behavior of the enzymes produced by *P. ostreatus* 22 Em and the standard strain for laccase production in liquid culture *P. ostreatus* ATCC MYA-2306, both cultivated in the same conditions of solid state fermentation on sugarcane bagasse. According to the results presented in Figure 5.1, enzymes produced by both strains could not be stored at any temperature (4, -20 or -80ºC) in the form of crude extract, without losing significantly their activities. It is known that one of the laccase

isoenzymes produced by both strains (POXA1b) should be stored at -80ºC in order to avoid degradation. Since the highest losses of activity were obtained for the frozen samples, it is supposed that these enzymes produced by solid state fermentation, differently from those produced by submerged fermentation, could lose activity during the process of defreezing. For the enzyme preparations concentrated from 5.5 to 8-fold by ultrafiltration and dialysis, however, the loss of activity was significantly reduced, the best temperature for storage being -80ºC.

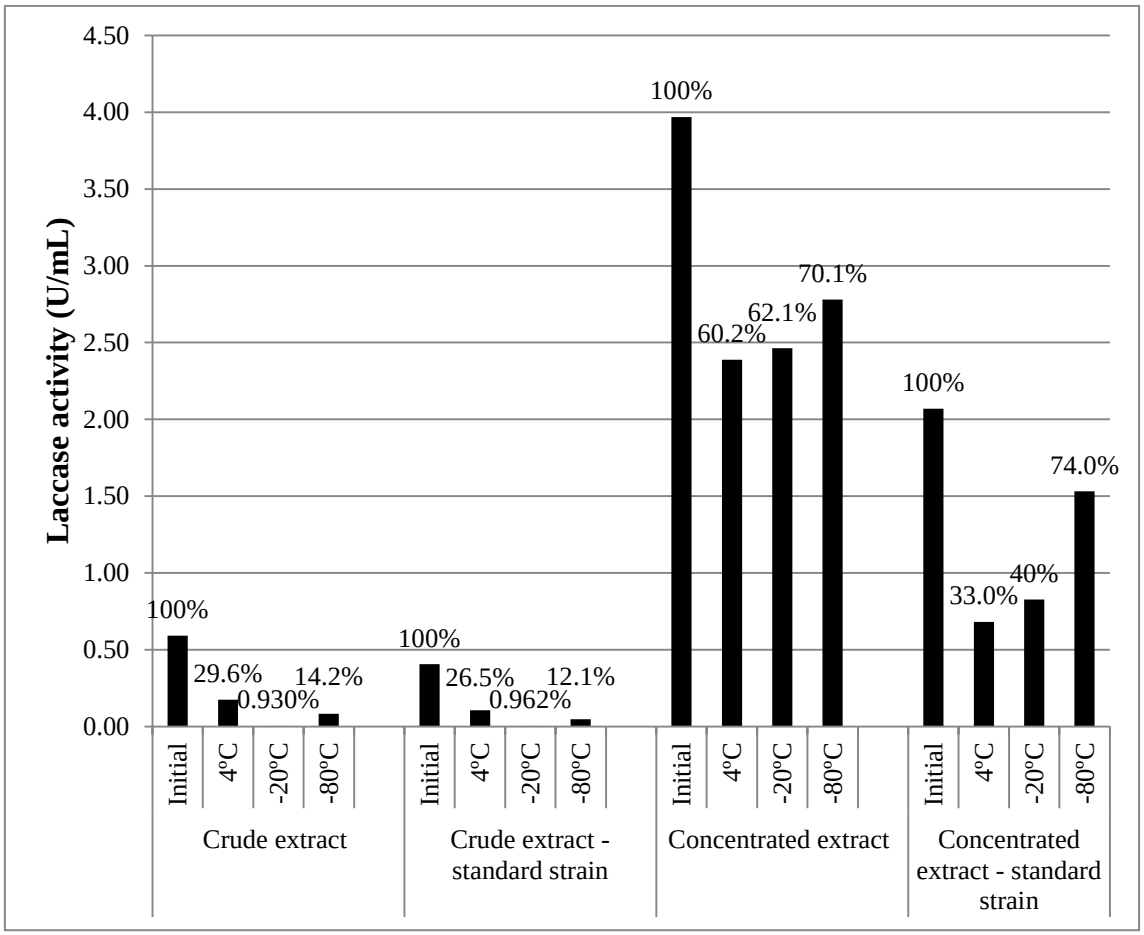

Figure 5.1. Laccase activities obtained after 20 days of storage at different temperatures.

5.3.4. Kinetics of enzymatic delignification of sugarcane bagasse

Figure 5.2 shows the kinetics of enzymatic delignification of sugarcane bagasse. Lignin content was reduced from 31.89% to 14.98%, which represents 53% of delignification after 12h of incubation. Moniruzzaman and Ono (2012) reported the enzymatic delignification of wood biomass in ionic liquid aqueous media containing 5% ionic liquid, which resulted in around 50% delignification after 24 h in the presence of laccase, whereas only 10% delignification was obtained when original wood chips (without ionic liquid pretreatment) were used. In the process of kraft pulp delignification, the utilization of laccase and ABTS reduced the lignin content in 40% using residence times, temperatures and pressures similar to those used in kraft bleaching processes (Bourbonnais and Paice, 1996). Enzymatic treatment demonstrated to be a more appropriate method for biological delignification in relation to solid state fermentation, when considering industrial applications that require high efficiency.

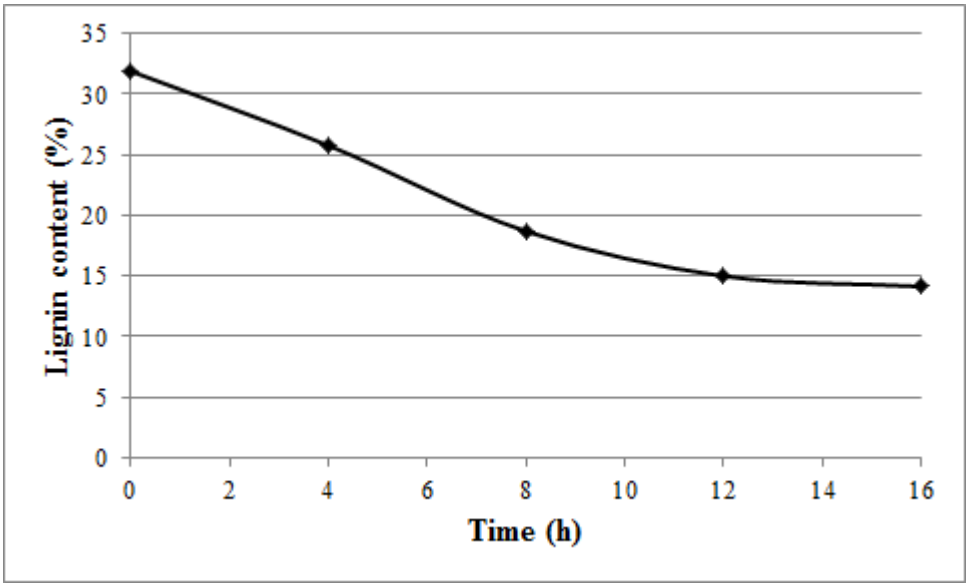

Figure 5.2. Kinetics of enzymatic delignification of sugarcane bagasse.

5.4. Conclusions

The process of biological delignification of sugarcane bagasse by *P. ostreatus* reduced the lignin content from 31.89% to 26.36% after 5 days and to 20.79% after 15 days of solid state fermentation. Laccases were recovered from the fermented bagasse at the 5th day, presenting an activity of 152 U/g of dry bagasse, and were concentrated by ultrafiltration prior to enzymatic hydrolysis. Enzymatic treatment reduced the lignin content from 31.89% to 14.98% after 12h of incubation.

Acknowledgements

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CONCLUSIONS

Among 45 strains of basidiomycetes available at the culture collection of the Department of Bioprocess Engineering and Biotechnology, Federal University of Paraná (DEBB-UFPR), one of *Pleurotus* sp. (coded Pl 22 Em) presented a laccase activity on sugarcane bagasse significantly higher than all the other strains, i.e. 183 U/L after 5 days of growth in semi-solid condition. Direct sequencing of the ITS amplicon and its sequence analysis revealed high sequence identity (99%, E-value 0.0) with *nuc*ITS from *Pleurotus ostreatus* (GenBank accession number AY854077, AFTOL-ID 564). The sequence containing ITS1-5.8rDNA-ITS2 is available at the NCBI GenBank (accession code JQ316531). Cultivation of the fungus in solid state fermentation on sugarcane bagasse resulted in a maximum laccase activity of 2049 U/kg after 5 days of fermentation.

The level of laccase activity produced by *P. ostreatus* 22 Em in solid state fermentation of sugarcane bagasse was significantly affected by the concentrations of nitrogen source, copper sulfate and ferulic acid. The use of an organic nitrogen source (yeast extract) provided and increase of 5.7 fold in laccase production, in comparison with the inorganic source (ammonium sulfate). The response surface methodology was applied to determine the mathematical model of laccase production as a function of yeast extract, copper sulfate and ferulic acid concentrations. The mathematical model of laccase production can be given by the following equation:

$$\text{Laccase activity (U/g)} = -249.9 + 41.53[YE] - 3.236[YE]^2 + 2.071[Cu^{2+}] - 0.0060[Cu^{2+}]^2 + 106.7[Fer] - 28.68[Fer]^2$$

where concentrations of yeast extract (YE), copper (Cu^{2+}) and ferulic acid (Fer) are given in g/L, μM and mM, respectively. The predicted model (R^2 0.8753) indicated that

the maximum laccase activity (161.3 U/g) would be obtained at the following conditions: yeast extract 6.417 g/L, Cu^{2+} 172.6 μM and ferulic acid 1.860 mM. Experimentally, the maximum laccase activity of 151.6 U/g was produced, under optimized conditions, at the 5th day of solid state fermentation.

Six isoforms of laccases (POXA1b, POXA3, POXC and three other isoforms) were observed in the native PAGE zymograms of the strain *P. ostreatus* 22 Em grown in solid state fermentation of sugarcane bagasse. The production of five of these isoforms was also demonstrated by the strain *P. ostreatus* ATCC in solid state fermentation, indicating that this pattern is more related to fermentation conditions than to the strain in this case. The presence of ferulic acid induced the expression of POXA3 and POXA1b, and the expression of POXC was apparently not affected by nitrogen concentration and inducers. Higher nitrogen concentration together with ferulic acid induced the expression of the isoforms 1, 2 and 3. In liquid culture the strain *P. ostreatus* 22 Em produced an isoenzyme very close to the band corresponding to the isoenzyme POXC, with similar electrophoretic mobility to one reported by Lettera et al. (2010) in different growth conditions. Mass spectrometry results confirmed the presence of POXC and POXA3, and also identified a glyoxal oxidase and three galactose oxidases, which are enzymes linked to lignin degradation. The absence of the isoenzyme POXA1b in the analyzed samples can be possibly explained by its sensitivity to protease degradation.

The process of biological delignification of sugarcane bagasse by *P. ostreatus* reduced the lignin content from 31.89% to 26.36% after 5 days and to 20.79% after 15 days of solid state fermentation. Laccases were recovered from the fermented bagasse at the 5th day, presenting an activity of 152 U/g, and were concentrated by ultrafiltration

prior to enzymatic hydrolysis. Enzymatic treatment reduced the lignin content from 31.89% to 14.98% after 12h of incubation.

SUGGESTIONS FOR FUTURE RESEARCH

The process of solid state fermentation of sugarcane bagasse to produce laccases should be scaled-up in a solid state fermenter with the aim of improving mass and (especially) heat transfer. These are important parameters that could enhance laccase production. The methods for laccase purification are well known and include ammonium sulfate precipitation and chromatography. However, purification of enzymes produced by solid state fermentation of agroindustrial wastes need further studies to improve economic feasibility. Also the enzymatic hydrolysis of the sugarcane bagasse should be optimized in a pilot scale reactor. The economic analysis of the overall process could be performed once the process is well established at pilot scale.

Future projects of the Bioprocess Engineering and Biotechnology Department (Federal University of Paraná) in partnership with the University of Naples "Federico II" include the cloning of the cDNA from different laccase isoforms produced by the selected strain *P. ostreatus* 22 Em (preliminary results are presented in Annex A) in order to study their characteristics, especially focusing on the maintenance of stability after extraction from the solid medium. These projects also include further development of downstream methods to purify lignocellulolytic enzymes produced from agroindustrial wastes.

ANNEX A

Experiments to prepare the material for cDNA cloning of laccase genes

1. Objectives

The objectives of these experiments were to extract the RNA from fungal mycelium, from a sample containing the six laccase isoforms (POXA1b, POXA3, POXC and Isoforms 1, 2 and 3); to synthesize the cDNA from the extracted RNA; to check the quality of cDNA and confirm the presence of the aforementioned isoforms for posterior cloning.

2. Materials and Methods

2.1. Preparation of fungal mycelium

The sample consisted of mycelium of *P. ostreatus* 22 Em grown on sugarcane bagasse in the presence of yeast extract (5g/L), copper sulfate (150 μM) and ferulic acid (2 mM), after 10 days of fermentation, and was prepared according to item 4.2.1. Different amounts of mycelium (approximately 0.1, 0.5, 1.0, 3.0 and 10 mg) were collected with a platinum wire loop and transferred to sterile and previously weighed microtubes. Mycelium samples were washed 4 times with DEPC-treated water (diethyl pyrocarbonate 97%, Sigma, 1:1000 v/v, agitated overnight and then removed by autoclaving – 121ºC, 1 atm, 15 min) and were then stored at -80ºC and lyophilized. All

procedures were performed in a sterile cabin treated with RNase inhibitor (RNase Exitus Plus, Appli Chem).

2.2. RNA extraction

RNA was extracted from all samples (containing 0.1, 0.7, 1.3, 2.8 and 12.2 mg of mycelium) using the RNeasy Mini Kit (Qiagen). Lyophilized mycelium samples, stored at -20ºC, were grinded with pestles (treated with NaOH 0.1M and SDS 1% for 2h and abundantly rinsed with DEPC-treated water) inside the microtubes in the presence of liquid nitrogen prior to the addition of RLC-buffer (600 µL) containing β-mercaptoethanol (Annex B, protocol page 53). The following extraction steps were performed according to the Qiagen protocol (Annex B, protocol pages 53-55). The extracted RNA was eluted in 40 µL of RNase-free water and stored at -20ºC. All procedures were performed in a sterile cabin treated with RNase inhibitor.

2.3. RNA electrophoresis

Electrophoresis to check the quality of RNA was performed in agarose gel 1% (w/v) in TAE buffer (Tris-acetate 40 mM, EDTA 1 mM, pH 8.0), containing ethidium bromide (5µg/100mL). Electrophoretic run was performed in TAE buffer, 100V, for 30 min. Lambda-DNA Eco RI Hind III (0.1 µg/µL) was used as a marker, and the nucleic acids were visualized by UV exposure. All materials were treated with NaOH 0.1M and SDS 1% for 2h and abundantly rinsed with DEPC-treated water.

2.4. Synthesis of total cDNA

Reverse transcriptase polymerase chain reaction (RT-PCR, Applied Biosystems) was performed to synthesize the total cDNA from the extracted RNA. Reagents were added according to Table 1. Preparation of the RT-PCR mixes was performed in a sterile cabin treated with RNase inhibitor.

Table 1 – Composition of the RT-PCR mixes.

	Reagent	Volume
Mix 1	Oligonucleotide d(T) 50 μM	5 μL
	dNTP 10 mM	40 μL
	RNA	10 μL*
	DEPC-H_2O	8.5 μL
Mix 2	Buffer 10x	10 μL
	$MgCl_2$ 25 mM	22 μL
	Ribonuclease inhibitor 20 U/μL	2 μL
	Multiscribe enzyme 50 U/μL	2.5 μL
Total		100 μL

*corresponding to 0.2-2 μg

The RT-PCR was conducted in a thermal-cycler according to the following steps:

Addition of mix 1
65ºC, 5 min for denaturation of the secondary structure
Addition of mix 2
25ºC, 10 min for hybridization
48ºC, 60 min for polymerization
95ºC, 5 min for enzyme denaturation
4ºC, forever

2.5. Quality control of the cDNA

The quality of the synthesized cDNA was checked through the amplification of specific regions within different laccase genes. The PCR mix was prepared according to Table 2.

Table 2 – Composition of the PCR mix.

Reagent	Positive control	Negative control
cDNA	4 μL	0
Oligonucleotide (forward primer) 10 μM	5 μL	5 μL
Oligonucleotide (reverse primer) 10 μM	5 μL	5 μL
Buffer 5x GoTaq (Promega)	10 μL	10 μL
$MgCl_2$ 25 mM (Promega)	3.5 μL	3.5 μL
dNTP mix 10 mM (Promega)	4 μL	4 μL
Taq polymerase GoTaq 5 U/μL (Promega)	0.3 μL	0.3 μL
Sterile H_2O	18.2 μL	22.2 μL
Total	50 μL	50 μL

The PCR was conducted in a thermal-cycler according to the following steps:

94ºC, 2 min for denaturation

30 cycles:
94ºC, 30 s for denaturation
58-64ºC, 1 min for hybridization
72ºC, 1 min for polymerization

72ºC, 10 min
4ºC, forever

Amplified fragments were separated by electrophoresis (agarose gel 1% w/v in TAE buffer containing ethidium bromide 50μg/100mL, 100V, 20 min) and visualized by UV exposure.

2.6. Sequences of the oligonucleotides

POXC 5’

POXC fw Eco-Hind (Tm 96ºC)

5’ TTTGAATTCAACGTTATGTTTCCAGG 3’

POXC rev Kpn (Tm 60ºC)

5’ GGACGATATGGTACCAATCC 3’

Expected fragment size: 560 bp

POXC 3’

POXC fw Eco-RV (calculated Tm 66ºC)

5’AGCCGATATCAACATCAATCTCG 3’

LacC term (calculated Tm 64ºC)

5’ CATCTATAACGCTTTGAGCGAC 3’

Expected fragment size: 514 bp

POXA1b 3’

POXA1b fw Bgl II (calculated Tm 58ºC)

5’ TGTTGCAGATCTTGTCGG 3’

POXA1b rev Bam HI (calculated Tm 40ºC)

5’ TAAGGATCCGAATTCTTATAATCATGCTTTC 3’

Expected fragment size: 491 bp

POXA3 3’

POXA3 fw Bam HI (calculated Tm 56ºC)

5’ CTACTGGATCCGTGCGC 3’

POXA3 rev Hind III (calculated Tm 62ºC)

5’ ATTTTAAGCTTACTGGAACTCGGGAGCGAG 3’

Expected fragment size: 727 bp

3. Initial results

The presence of fungal ribosomal RNA was detected in one sample that contained 12.2 mg of mycelium as starting material, so this sample was selected for the synthesis of cDNA.

3.1. Amplification of POXC 5’

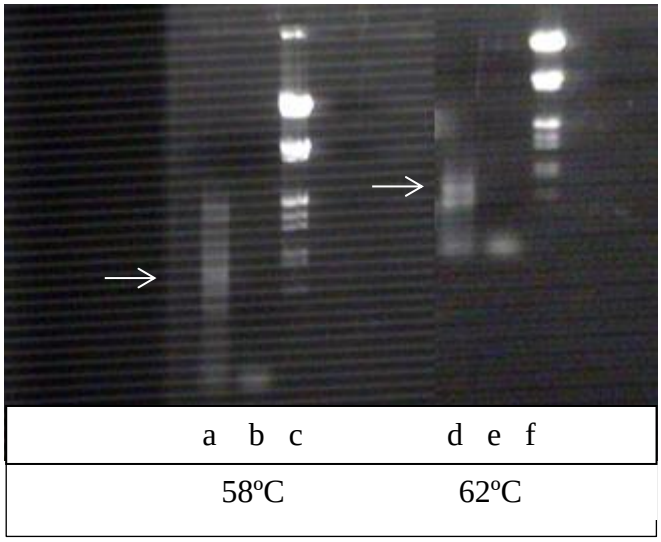

Figure 1. Amplified fragments of cDNA using POXC fw Eco-Hind and POXC rev Kpn primers, at two different temperatures. a, d – positive controls; b, e – negative controls; c, f – λDNA marker. The arrow indicates the position of the expected fragment.

Figure 1 presents the results of POXC cDNA amplification from the 5' extremity, at hybridization temperatures of 58ºC and 62ºC. The higher temperature resulted in a more intense band with reduced smear. This was expected since the higher temperature improves specificity of hybridization.

3.2. Amplification of POXC, POXA1b and POXA3 3'

The following amplification experiments were performed using primers that align at the 3' extremity, considering that the cDNA was synthesized with oligonucleotide (dT) that aligns at the poly-A tail (at the 3' extremity). Results are presented in Figure 2.

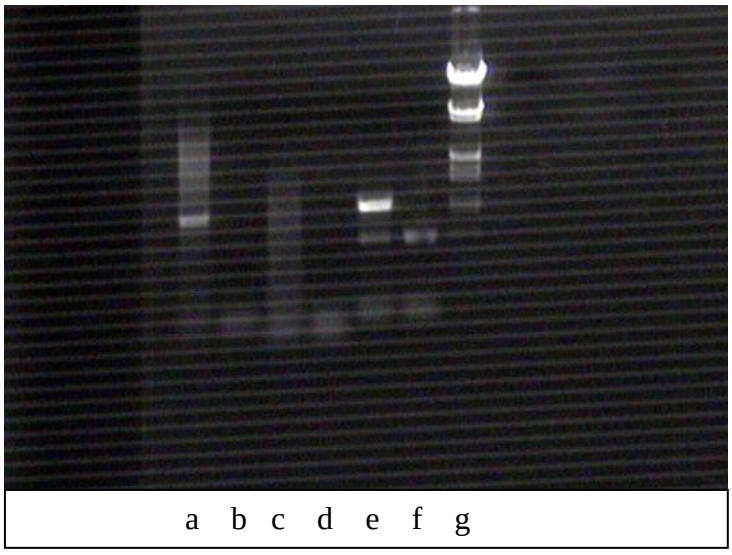

Figure 2. Amplified fragments of cDNA using primers POXA1b fw Bgl II and POXA1b rev Bam HI (a), POXA3 fw BamHI and POXA3 rev Hind III (c) and POXC fw Eco RV and LacC term (e); temperatures were 64ºC for POXC and 58ºC for POXA1b and POXA3; b, d and f are the negative controls; g is the λDNA marker.

The sizes of the PCR amplicons indicated that genomic DNA was amplified. The alternatives to solve this problem could be the treatment of the RNA sample with ribonuclease-free DNase, a procedure that can degrade the RNA, or the utilization of primers that do not amplify genomic DNA, aligning with regions that flank at last one intron. The latter procedure was chosen.

3.3. Design of primers

The primers were designed according to the following pre-requisites:

- Alignment position between two exons
- Forward direction
- Amplified fragment of around 500 bp
- Approximate size of 20 nucleotides
- GC content higher than 50%

Based on these pre-requisites, the following oligonucleotides were designed:

POXA1b fw (Figure 3)

5’ CCTCTGCAGGTCGATCAAGTG 3’

POXA3 fw (Figure 4)

5’ GCCGAGATGCACCCAATTGCTC 3’

POXC fw (Figure 5)

5’ ACCATCAACGGTGTTCCTTTCC 3’

POX1 fw (Figure 6)

5’ ACCATCAACGGCTCCCCCTTC 3’

The expected fragments were not observed after amplification and electrophoresis, so the extraction of RNA should be repeated.

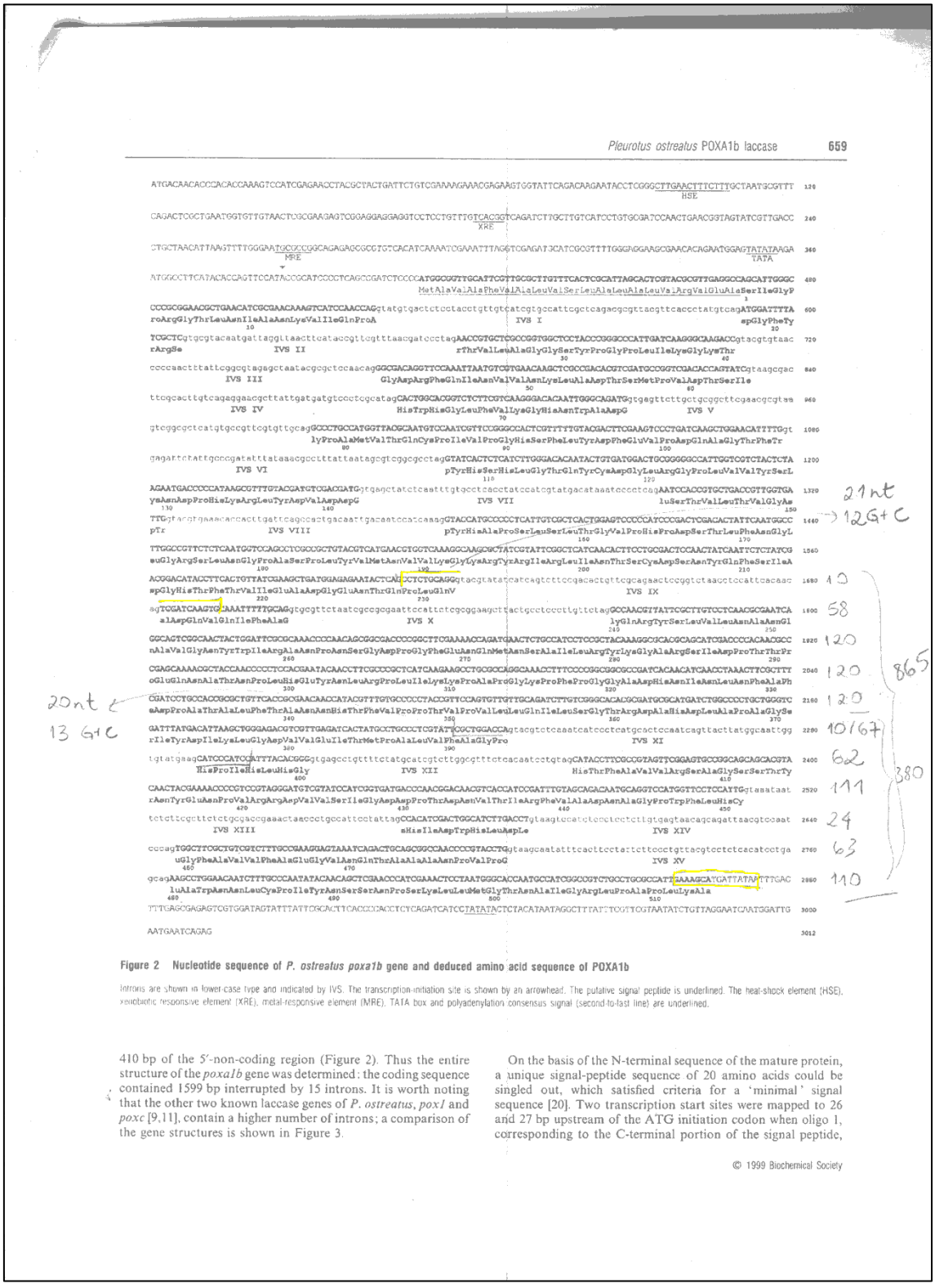

Figure 3. Genomic sequence of laccase POXA1b gene. Alignment position of primers is marked in yellow.

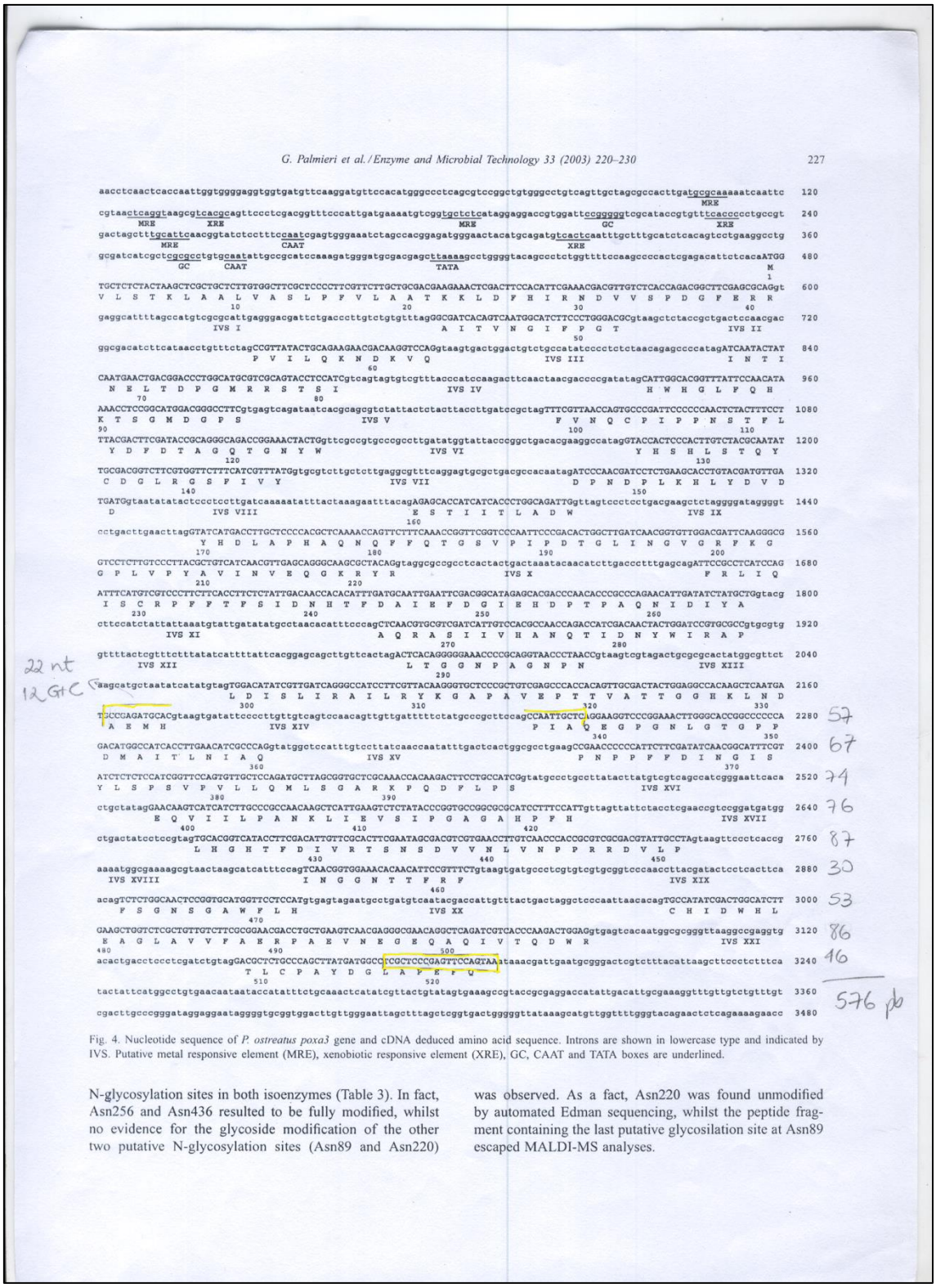

Fig. 4. Nucleotide sequence of *P. ostreatus poxa3* gene and cDNA deduced amino acid sequence. Introns are shown in lowercase type and indicated by IVS. Putative metal responsive element (MRE), xenobiotic responsive element (XRE), GC, CAAT and TATA boxes are underlined.

N-glycosylation sites in both isoenzymes (Table 3). In fact, Asn256 and Asn436 resulted to be fully modified, whilst no evidence for the glycoside modification of the other two putative N-glycosylation sites (Asn89 and Asn220) was observed. As a fact, Asn220 was found unmodified by automated Edman sequencing, whilst the peptide fragment containing the last putative glycosilation site at Asn89 escaped MALDI-MS analyses.

Figure 4. Genomic sequence of laccase POXA3 gene. Alignment position of primers is marked in yellow.

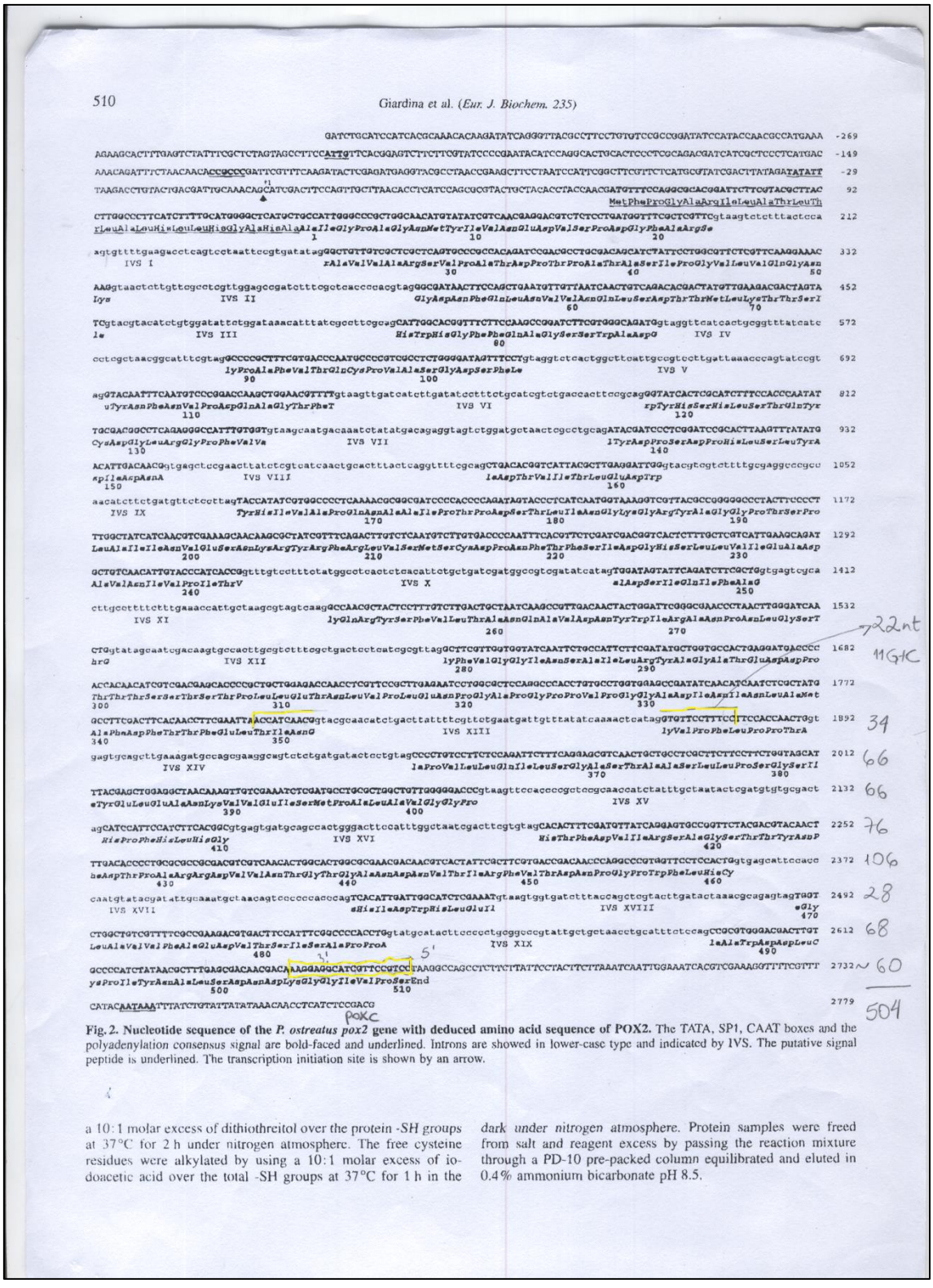

Fig. 2. Nucleotide sequence of the *P. ostreatus pox2* gene with deduced amino acid sequence of POX2. The TATA, SP1, CAAT boxes and the polyadenylation consensus signal are bold-faced and underlined. Introns are showed in lower-case type and indicated by IVS. The putative signal peptide is underlined. The transcription initiation site is shown by an arrow.

a 10:1 molar excess of dithiothreitol over the protein -SH groups at 37 °C for 2 h under nitrogen atmosphere. The free cysteine residues were alkylated by using a 10:1 molar excess of iodoacetic acid over the total -SH groups at 37 °C for 1 h in the dark under nitrogen atmosphere. Protein samples were freed from salt and reagent excess by passing the reaction mixture through a PD-10 pre-packed column equilibrated and eluted in 0.4% ammonium bicarbonate pH 8.5.

Figure 5. Genomic sequence of laccase POXC gene. Alignment position of primers is marked in yellow.

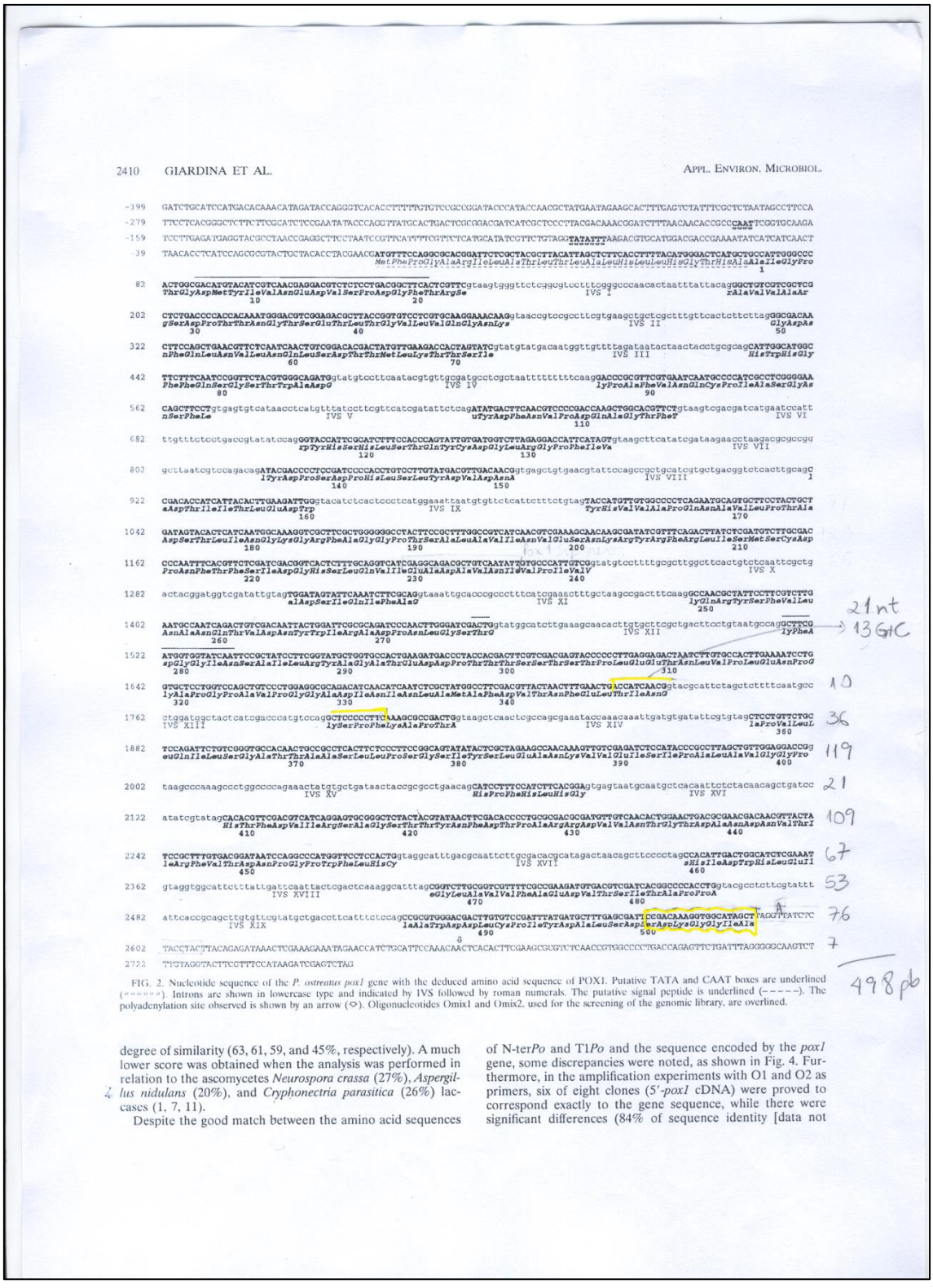

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FIG. 2. Nucleotide sequence of the *P. ostreatus pox1* gene with the deduced amino acid sequence of POX1. Putative TATA and CAAT boxes are underlined (------). Introns are shown in lowercase type and indicated by IVS followed by roman numerals. The putative signal peptide is underlined (-----). The polyadenylation site observed is shown by an arrow (⟲). Oligonucleotides Omix1 and Omix2, used for the screening of the genomic library, are overlined.

degree of similarity (63, 61, 59, and 45%, respectively). A much lower score was obtained when the analysis was performed in relation to the ascomycetes *Neurospora crassa* (27%), *Aspergillus nidulans* (20%), and *Cryphonectria parasitica* (26%) laccases (1, 7, 11).

Despite the good match between the amino acid sequences of N-ter*Po* and T1*Po* and the sequence encoded by the *pox1* gene, some discrepancies were noted, as shown in Fig. 4. Furthermore, in the amplification experiments with O1 and O2 as primers, six of eight clones (5′-*pox1* cDNA) were proved to correspond exactly to the gene sequence, while there were significant differences (84% of sequence identity [data not

Figure 6. Genomic sequence of laccase POX1 gene. Alignment position of primers is marked in yellow.

ANNEX B

RNA extraction protocol (RNeasy, Qiagen)

Protocol: Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi

This protocol requires the RNeasy Plant Mini Kit.

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 100 mg plant material or 1 x 10^7 cells can generally be processed. For most plant materials, the RNA binding capacity of the RNeasy spin column and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Average RNA yields from various plant materials are given in Table 2 (page 19).

If there is no information about the nature of your starting material, we recommend starting with no more than 50 mg plant material or 3–4 x 10^6 cells. Depending on RNA yield and purity, it may be possible to use up to 100 mg plant material or up to 1 x 10^7 cells in subsequent preparations.

Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.

Counting cells or weighing tissue is the most accurate way to quantitate the amount of starting material. As a guide, a 1.5 cm diameter leaf disc weighs 25–75 mg.

Important points before starting

- If using the RNeasy Plant Mini Kit for the first time, read "Important Notes" (page 18).
- If working with RNA for the first time, read Appendix A (page 63).
- Fresh or frozen tissues can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 4 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 5. Avoid prolonged incubation, which may compromise RNA integrity.
- The RNeasy Plant Mini Kit provides a choice of lysis buffers: Buffer RLT and Buffer RLC, which contain guanidine thiocyanate and guanidine hydrochloride, respectively. In most cases, Buffer RLT is the lysis buffer of choice due to the greater cell disruption and denaturation properties of guanidine thiocyanate. However, depending on the amount and type of secondary metabolites in some tissues (such as milky endosperm of maize or mycelia of filamentous fungi), guanidine thiocyanate can cause solidification of the sample, making extraction of RNA impossible. In these cases, Buffer RLC should be used.

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- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Buffer RLT, Buffer RLC, and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 8 for safety information.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT or Buffer RLC before use. Add 10 µl β-ME per 1 ml Buffer RLT or Buffer RLC. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT or Buffer RLC containing β-ME can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix D (page 69).

Procedure

1. **Determine the amount of plant material. Do not use more than 100 mg.**

   Weighing tissue is the most accurate way to determine the amount.

2. **Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Proceed immediately to step 3.**

   RNA in plant tissues is not protected until the tissues are flash-frozen in liquid nitrogen. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

3. **Add 450 µl Buffer RLT or Buffer RLC (see "Important points before starting") to a maximum of 100 mg tissue powder. Vortex vigorously.**

   A short 1–3 min incubation at 56°C may help to disrupt the tissue. However, do not incubate samples with a high starch content at elevated temperatures, otherwise swelling of the sample will occur.

Note: Ensure that β-ME is added to Buffer RLT or Buffer RLC before use (see "Things to do before starting").

4. **Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Carefully transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps.**

   It may be necessary to cut off the end of the pipet tip to facilitate pipetting of the lysate into the QIAshredder spin column. Centrifugation through the QIAshredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet when transferring the lysate to the new microcentrifuge tube.

5. **Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.**

   Note: The volume of lysate may be less than 450 µl due to loss during homogenization.

   Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. **Transfer the sample (usually 650 µl), including any precipitate that may have formed, to an RNeasy spin column (pink) placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.***

   Reuse the collection tube in step 7.

   If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.*

   Optional: If performing optional on-column DNase digestion (see "Eliminating genomic DNA contamination", page 23), follow steps D1–D4 (page 69) after performing this step.

7. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.***

   Reuse the collection tube in step 8.

* Flow-through contains Buffer RLT, Buffer RLC, or Buffer RW1 and is therefore not compatible with bleach. See page 8 for safety information.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

Skip this step if performing optional on-column DNase digestion (page 69).

8. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

   Reuse the collection tube in step 9.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

9. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane.**

   The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.**

12. **If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.**

    If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.