UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
ÁREA DE CONCENTRAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

TATIANE BRUGNARI

APLICAÇÃO DE LACASES IMOBILIZADAS NA DEGRADAÇÃO DE BISFENOL A

MARINGÁ
2018

Avenida Colombo, 5790 Bloco I-89 Sala 002 ● Campus Universitário ● CEP 87.020-900 ● Maringá-PR.
Fone: (44) 3011-4908- http://www.pbc.uem.br

TATIANE BRUGNARI

APLICAÇÃO DE LACASES IMOBILIZADAS NA DEGRADAÇÃO DE BISFENOL A

Tese apresentada ao Programa de Pós - Graduação em Ciências Biológicas (área de concentração – Biologia Celular e Molecular), da Universidade Estadual de Maringá para obtenção do grau de Doutora em Ciências Biológicas.

Orientadora: Dra. Rosane Marina Peralta

MARINGÁ
2018

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR, Brasil)

Brugnari, Tatiane
B891a Aplicação de lacases imobilizadas na degradação de bisfenol A/ Tatiane Brugnari. -- Maringá, PR, 2018.
ix, 42 f.: il. color.

Orientadora: Profª. Drª. Rosane Marina Peralta.
Tese (doutorado) - Universidade Estadual de Maringá, Centro de Ciências Biológicas, Programa de Pós-Graduação em Ciências Biológicas, 2018.

1. Enzimas (Lacases). 2. Lacases - Imobilização. 3. Basidiomicetos. 4. Biorremediação. 5. Biodegradação. I. Peralta, Rosane Marina, orient. II. Universidade Estadual de Maringá. Centro de Ciências Biológicas. Programa de Pós-Graduação em Ciências Biológicas. III. Título.

CDD 23.ed. 660.634
Márcia Regina Paiva de Brito – CRB-9/1267

TATIANE BRUGNARI

APLICAÇÃO DE LACASES IMOBILIZADAS NA DEGRADAÇÃO DE BISFENOL A

Tese apresentada ao Programa de Pós - Graduação em Ciências Biológicas (área de concentração – Biologia Celular e Molecular), da Universidade Estadual de Maringá para obtenção do grau de Doutora em Ciências Biológicas.

Aprovado em: 23/02/2018

Banca Examinadora

Profa. Dra. Rosane Marina Peralta
Universidade Estadual de Maringá

Profa. Dra. Cristina Giatti Marques de Souza
Universidade Estadual de Maringá

Profa. Dra. Ione Parra Barbosa Tessmann
Universidade Estadual de Maringá

Profa. Dra. Gisella Maria Zanin
Universidade Estadual de Maringá

Pra. Dra. Roselene Ferreira Oliveira
Instituto Federal de Mato Grosso do Sul

BIOGRAFIA

Tatiane Brugnari nasceu em Jacarezinho/PR em 15/07/1986. Possui graduação em Ciências Biológicas pelas Faculdades Integradas Espírita (2008) e Mestrado em Ciências Biológicas – Biologia Celular e Molecular (2015) pela Universidade Estadual de Maringá. Tem experiência nas áreas de Biologia Celular atuando principalmente com cultivo de células animais no desenvolvimento de vacinas e kits de diagnósticos e em Bioquímica de Microrganismos com ênfase em pesquisa de compostos bioativos de fungos basidiomicetos, produção, caracterização e imobilização de enzimas e biodegradação de xenobióticos.

"Cada pessoa deve trabalhar para o seu aperfeiçoamento e, ao mesmo tempo, participar da responsabilidade coletiva por toda a humanidade."

Marie Curie

Dedico

Aos meus pais Luiz e Rosângela

À minha irmã Lidiane

AGRADECIMENTOS

À minha orientadora Profª Dra. Rosane Marina Peralta, pela oportunidade, pelos melhores e mais úteis ensinamentos que permitiram o desenvolvimento dessa tese e também pela dedicação demonstrada na orientação deste trabalho.

Aos professores do Programa de Pós Graduação em Ciências Biológicas da Universidade Estadual de Maringá por todos os ensinamentos. Em especial à Profª Dra. Cristina Giatti Marques de Souza pelo constante apoio e pelos conhecimentos generosamente compartilhados.

À professora Dra. Maria de Lourdes Teixeira de Moraes Polizeli, do Departamento de Biologia, da Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, pelo suporte e apoio nas análises.

À Maria Aparecida Ferreira Costa e à Alvina Chaves técnicas do laboratório de Bioquímica e Fisiologia de Microrganismos da UEM pela gentil colaboração.

A todos os amigos do laboratório de Bioquímica de Microrganismos e Alimentos da UEM que direta ou indiretamente me ajudaram a chegar até aqui. A amizade de vocês me fez sentir acolhida e foi de suma importância para que eu me mantivesse firme em meu propósito.

A todos os amigos e colegas que estão longe, mas que sempre estarão pertos. Em especial Elton, Leonardo, Izis, Débora, Jhony, Marina e Ana Carolina, por fazerem dessa etapa mais leve a cada sorriso dado com vocês.

Ao meu primo Marcos Vinícius Brunhari, minha inspiração nessa caminhada da pós-graduação. Com você que aprendi que com esforço e dedicação somos capazes de ir muito além do que até nós mesmos conseguimos imaginar. Quero agradecer também aos primos Raphael Cunha e Bruna Andrade Pereira pelos momentos de descontração e incentivo.

Ao meu amigo André Nigro, por todo apoio recebido. A sua importância nesse trajeto é imensurável. Obrigado por ter estado sempre que precisei ao meu lado e por sempre me lembrar de que independente do destino o legal é apreciar o caminho.

Aos meus pais Luiz e Rosângela e à minha irmã Lidiane, vocês são a razão do meu viver e nada disso faria sentido se vocês não estivessem ao meu lado. Obrigada pelo carinho, paciência, por sempre estimularem meu crescimento pessoal e intelectual. Por me darem amor, força e estarem sempre ao meu lado em todos os momentos da minha vida. Nenhum agradecimento feito por mim aqui estaria à altura do que vocês fizeram e fazem por mim.

APRESENTAÇÃO

Este trabalho foi realizado no Laboratório de Bioquímica de Microrganismos e Alimentos da Universidade Estadual de Maringá. Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas a presente tese está apresentada na forma de dois artigos científicos e os mesmos foram redigidos de acordo com as normas dos periódicos correspondentes.

Brugnari T, Pereira MG, Bubna GA, Freitas EN, Contato AG, Correa RC, Castoldi R, Polizeli MLT, Bracht A, Peralta RM. A highly reusable MANAE-agarose-immobilized *Pleurotus ostreatus* laccase for degradation of bisphenol A. *Science of the Total Environment*. (submetido). Fator de impacto JCR: 4,900

Brugnari T, Pereira MG, Moryama C, Bubna GA, Freitas, EN, Buzzo AJ, Polizeli MLT, Bracht A, Peralta RM. Laccase immobilization on activated agarose supports and application on bisphenol A degradation. *International Biodeterioration and Biodegradation.* Fator de impacto JCR: 2,962

RESUMO GERAL

INTRODUÇÃO - Lacases (benzenediol: oxigênio-oxidorredutase, EC 1.10.3.2) são oxidases multi-cobre que por transferência de elétrons catalisam a oxidação de fenóis, aminas aromáticas e outros substratos ricos em elétrons com uma redução concomitante de O_2 a H_2O. O crescente interesse em aplicações práticas da lacase baseia-se no seu excelente potencial biotecnológico e versatilidade para oxidar uma grande variedade de substratos. Nos últimos anos, as lacases foram utilizadas para a degradação de uma ampla gama de compostos xenobióticos, incluindo corantes sintéticos, hidrocarbonetos aromáticos policíclicos, pesticidas, produtos farmacêuticos, como o diclofenaco e produtos químicos disruptores endócrinos (CDE), como bisfenol A. Bisfenol A [2 ,2 -bis (4-hidroxifenil) propano, BPA] é um dos produtos químicos mais fabricados, utilizado em grande parte em plásticos de policarbonato e resinas epoxi para embalagens de alimentos. O BPA pode interferir no sistema endócrino e produzir efeitos adversos de desenvolvimento, reprodutivos, neurológicos, mutagênicos, carcinogênicos e imunes em humanos e animais selvagens. No entanto, a aplicação de lacases solúveis na biodegradação de BPA apresenta algumas desvantagens, como o alto custo de produção, perda de estabilidade e não reutilização. Esses fatores tornam o tratamento com lacases economicamente inaceitável. A imobilização de lacases pode superar, pelo menos em parte, as limitações para aplicação em grande escala. Com base nesta perspectiva, os objetivos do presente estudo foram imobilizar as lacases de *Ganoderma lucidum*, *Pleurotus ostreatus* e *Pleurotus pulmonarius*, a fim de avaliar a influência desse fenômeno nas propriedades catalíticas das enzimas e no desempenho das lacases imobilizadas na eliminação contínua de bisfenol A.

MÉTODOS - Para a obtenção das lacases, os basidiomicetos *G. lucidum*, *P. ostreatus* e *P. pulmonarius* foram cultivados em culturas de estado sólido usando casca de laranja como substrato por 14 dias. Para a imobilização, três substratos foram testados, MANAE-agarose (mono-aminoetil-N-etil-agarose), agarose-epóxido e glioxil-agarose. A imobilização foi realizada na proporção de 1 g de cada suporte para 10 mL de extrato enzimático bruto. Utilizou-se ABTS (2,2'-azino bis (etilbenzotiazolina-6-sulfônico) como substrato nos testes de

estabilidade das enzimas livres e imobilizadas e para a determinação dos parâmetros cinéticos. Para a avaliação da eficiência na degradação de BPA, as lacases livres e imobilizadas foram incubadas com 100 mg/L de solução de BPA durante 60 minutos. Os experimentos foram conduzidos a 28 °C em tampão de acetato (pH 5,0) e com agitação a 120 rpm. O BPA residual foi quantificado por cromatografia líquida de alta eficiência. A possibilidade de reutilização da enzima imobilizada foi analisada por repetições consecutivas do experimento nas mesmas condições mencionadas acima.

RESULTADOS E DISCUSSÃO – A lacase foi a principal enzima encontrada nos extratos enzimáticos brutos de *G. lucidum*, *P. ostreatus* e *P. pulmonarius* cultivados em condições de estado sólido usando resíduo de laranja como substrato. A imobilização das lacases no suporte MANAE-agarose foi altamente eficiente (100% da imobilização), enquanto 68% e 15% de imobilização foram obtidos nos suportes de agarose-epóxido e glioxil-agarose, respectivamente. As lacases permaneceram ligadas (100%) ao suporte após a adição de até 100 mmol/L de NaCl, ocorrendo desprendimento em concentrações mais elevadas do sal. Na avaliação da estabilidade, observou-se que, em altas temperaturas, o comportamento da enzima imobilizada de *P. ostreatus* foi modificado quando comparado à enzima livre, com aumento de atividade entre 40 e 65 °C. Para as lacases dos outros fungos, a temperatura ideal das enzimas livres e imobilizadas permaneceu na faixa de 60-65 °C. Houve uma redução na sensibilidade enzimática ao pH após a imobilização. As enzimas livres e imobilizadas apresentaram atividade ideal em pH 5,0. Em pH 10, a enzima livre de *G. lucidum* mostrou atividade residual de apenas 6,37% e as enzimas dos outros dois fungos estavam completamente inativas. As enzimas imobilizadas de *G. lucidum*, *P. ostreatus* e *P. pulmonarius*, no entanto, ainda apresentaram 54,35%, 44,25% e 39,23%, respectivamente, de sua atividade máxima em pH 10. Pode-se concluir que a imobilização tornou a enzima mais estável a altos valores de pH. Após 30 min a 55 °C, as enzimas livres mantiveram em média 20% das suas atividades iniciais, enquanto as imobilizadas mantiveram 70%. A análise cinética revelou um comportamento Michaeliano para as três lacases. As lacases livres foram eficientes na degradação da molécula de BPA, alcançando 90,26% de degradação com a lacase de *P.pulmonarius*. A imobilização

melhorou claramente a atividade das enzimas de todos os fungos no processo de degradação ($p < 0,05$). O maior aumento, 97,33%, foi apresentado pela enzima de *P. ostreatus*. Uma perspectiva altamente positiva deste estudo é a possibilidade de reutilização dos derivados imobilizados, uma vez que se descobriu que a atividade das lacases imobilizadas permaneceu alta após 15 ciclos.

CONCLUSÃO - Os dados obtidos neste estudo mostraram que a imobilização da lacase em suporte de MANAE-agarose foi satisfatória e melhorou a estabilidade da enzima em relação às mudanças no pH e na temperatura. A imobilização também proporcionou melhores resultados de degradação do BPA. Esses resultados são importantes porque as enzimas imobilizadas são de grande interesse industrial, uma vez que a ligação a um suporte permite reutilização, o que diminui o custo operacional do processo.

Palavras-chaves: Lacase, imobilização enzimática, MANAE-agarose, bisfenol A, basidiomicetos, biorremediação.

GENERAL ABSTRACT

INTRODUCTION - Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are copper oxidases that catalyse the one-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates with a concomitant reduction of O_2 to H_2O. The growing interest in practical aplications of laccase is based on its great biotechnological potential and versatility to oxidize a wide variety of substrates. In recent years laccases have been used for the degradation of a wide range of xenobiotic compounds including synthetic dyes, polycyclic aromatic hydrocarbons, pesticides, pharmaceuticals, such as diclofenac, and endocrine disrupting chemicals (EDC), such as bisphenol A. Bisphenol A [2, 2-bis (4-hydroxyphenyl) propane, BPA] is one of the most widely manufactured chemicals, largely used in polycarbonate plastics and epoxy resins for food packaging. BPA can interfere with the endocrine system and produce adverse developmental, reproductive, neurological, mutagenic, carcinogenic and immune effects in both humans and wildlife. However, the application of soluble laccases in the biodegradation of BPA presents some disadvantages such as the high cost of production, loss of stability and non-reusability. These factors make the laccase treatment economically unacceptable. The immobilization of laccases may overcome, at least in part, the limitations for large scale application. Based on this perspective, the objectives of the present study were to immobilize the laccases from *Ganoderma lucidum*, *Pleurotus ostreatus* and *Pleurotus pulmonarius,* in order to evaluate the influence of this phenomenon on the catalytic properties of the enzymes and on the performance of the immobilized laccases in the continuous elimination of bisphenol A.

METHODS - For the obtainment of the laccases, the basidiomycetes *G. lucidum*, *P. ostreatus* and *P. pulmonarius* were cultivated in solid state cultures using orange peel as substrate for 14 days. For immobilization three substrates were tested, MANAE-agarose (mono-aminoethyl-N-ethyl-agarose), agarose-epoxide and glyoxyl-agarose. The immobilization was carried out with a ratio of 1 g of the each support to 10 mL of crude enzymatic extract. ABTS (2,2'-azino bis (e-ethylbenzthiazoline-6-sulphonic acid) was used as the substrate in the stability

tests of the free and immobilized enzymes and for the determination of the kinetic parameters. For evaluating the efficiency in the degradation of BPA, free and immobilized laccases were incubated with 100 mg/L BPA solution for 60 minutes. The experiments were conducted at 28 °C in acetate buffer (pH 5.0) and with stirring at 120 rpm. Residual BPA was quantified by high performance liquid chromatography. The possibility of reusing the immobilized enzyme was analyzed by consecutive repetitions of the experiment under the same conditions mentioned above.

RESULTS AND DISCUSSION - Laccase was the main enzyme found in the crude enzymatic extract of *G. lucidum*, *P. ostreatus* and *P. pulmonarius* cultivated on solid state conditions using orange waste as substrate. The immobilization of the laccases in the MANAE-agarose support was highly efficient (100% of immobilization), whereas 68% and 15% of immobilization was obtained in agarose-epoxide and glioxyl-agarose supports, respectively. The laccases remained bound (100%) to the support upon the addition of NaCl up to 100 mmol/L, a detachment occurring at higher concentrations of the salt. In the evaluation of stability, it was observed that at high temperatures, the behavior of the immobilized enzyme of *P. ostreatus* was modified when compared to the free enzyme, with an increase in activity between 40 and 65 °C. For the laccases of the other fungi, the ideal temperature of the free and immobilized enzymes remained in the range of 60-65 °C. There was a reduction in the enzymatic sensitivity to pH after immobilization. Free and immobilized enzymes presented optimal activity at pH 5.0. At pH 10 the free enzyme of *G. lucidum* showed a residual activity of only 6.37% and the enzymes of the other two fungi were completely inactive. The immobilized enzymes of *G. lucidum*, *P. ostreatus* and *P. pulmonarius*, however, still presented 54.35%, 44.25% and 39.23%, respectively, of its maximal activity at pH 10. It can be concluded that immobilization made the enzyme more stable at high pH values. After 30 min at 55 °C the free enzymes maintained 20% of their initial activities on average, whereas the immobilized ones maintained 70%. The kinetic analysis revealed a Michaelian behaviour for the three laccases. The free laccases were efficient in the degradation of the BPA molecule, the *P. pulmonarius* enzyme having reached 90.26% degradation. Immobilization clearly improved the activity of the enzymes of all fungi in the

degradation process ($p < 0.05$). The largest increase, 97.33%, was that one presented by the *P. ostreatus* enzyme. A highly positive perspective of this study is the possibility of reusing the immobilized derivatives as it was found that the activity of the immobilized laccases remained high after 15 cycles.

CONCLUSION - The data obtained in this study showed that the immobilization of the laccase in a support of MANAE-agarose was satisfactory and improved the stability of the enzyme in relation to changes in pH and temperature. Immobilization also provided better degradation results of BPA. These results are important because the immobilized enzymes are of great industrial interest and their binding to a support allows reuse, which reduces the operational cost of the process.

Keywords: Laccase, enzyme immobilization, MANAE-agarose, bisphenol A, basidiomycetes, bioremediation.

ARTICLE 1

A highly reusable MANAE-agarose-immobilized *Pleurotus ostreatus* laccase for degradation of bisphenol A

Tatiane Brugnari[a], Marita Gimenez Pereira[b], Gisele Adriana Bubna[a], Emanuelle Neiverth de Freitas[a], Alex Graça Contato[a], Rúbia Carvalho Gomes Correa[a], Rafael Castoldi[a], Maria de Lourdes Teixeira de Moraes Polizeli[b], Adelar Bracht[a], Rosane Marina Peralta[a*]

[a]Department of Biochemistry, Universidade Estadual de Maringá, 87020900, Brasil.
[b]Department of Biology, Universidade de São Paulo, Ribeirão Preto, São Paulo,14049-900, Brasil.

*Corresponding author: Rosane Marina Peralta. Department of Biochemistry, Universidade Estadual de Maringá, Av. Colombo, 87020900, Paraná, Brasil. Email-rosanemperalta@gmail.com; rmperalta@uem.br

Abstract

Bisphenol A (BPA) is an endocrine disruptor compound that is continuously released into the environment and is barely degraded in wastewater treatment plants. A previous study showed that free *Pleurotus ostreatus* laccase is efficient in degrading BPA producing less toxic metabolites. In the present study, the laccase was successfully immobilized onto MANAE-agarose, improving its efficiency in degrading BPA and its thermal and storage stabilities. In addition to this, the immobilized enzyme retained more than 90% of its initial capability to degrade BPA after 15 cycles of reuse. *P. ostreatus* laccase immobilized onto MANAE-agarose could be an economical alternative for large scale degradation of BPA in aqueous systems.

Keywords: immobilized laccase, bisphenol A, MANAE agarose, *Pleurotus ostreatus*.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are copper oxidases that catalyze the one-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates with a concomitant reduction of O_2 to H_2O. The enzyme is widely distributed in fungi, plants, insects, bacteria and lichens (Guzik et al., 2014). The majority of laccases characterized so far have been derived from white rot fungi, which are efficient lignin degraders (Rivera-Hoyos et al. 2013).

The growing interest in laccase applications is based on its great biotechnological potential and versatility to oxidize a wide variety of substrates. In recent years laccases have been used for the degradation of a wide range of xenobiotic compounds including synthetic dyes, polycyclic aromatic hydrocarbons, pesticides, pharmaceuticals such as diclofenac and endocrine disrupting chemicals (EDC) (Fernández-Fernández et al., 2013; Taheran et al., 2017; Barrios-Estrada et al., 2018). The application of soluble laccases in the biodegradation of xenobiotics presents some disadvantages such as the high cost of production, loss of stability and non-reusability. These factors make the laccase treatment economically unacceptable. The immobilization of laccases may solve, at least in part, the limitation for large scale application (Ba et al., 2013; Fernández-Fernández et al., 2013). For this reason, considerable efforts have been done in the last years for obtaining immobilized laccases useful in bioremediation processes. The immobilization of laccase results in several improvements for its application, including increase in storage and operational stabilities, better control of the enzymatic reaction in aqueous solution, and possibility of repeated use (Dai et al., 2016)

Bisphenol A [2, 2-bis (4-hydroxyphenyl) propane, BPA] is one of the most widely manufactured chemicals, largely used in polycarbonate plastics and epoxy resins for food packaging (Michalowicz, 2014). BPA has been identified as an EDC by the US Environmental Protection Agency (EPA) and the World Wide Fund for Nature (WWF) and has been declared as a social, environmental and global issue (Mohapatra et al., 2011).EDCs are synthetic chemicals that may interfere with the endocrine system and produce adverse developmental, reproductive, neurological, mutagenic, carcinogenic and immune effects in both humans and wildlife (Mueller and Heger, 2013). Several free and immobilized

laccases have been considered as useful for biological degradation of BPA (Upadhyay et al., 2016, Couto et al., 2006, Ba et al., 2013). Recently, our group has described a laccase from *Pleurotus ostreatus* that efficiently degrades BPA reducing the acute toxicity from 85% to less than 5% (Freitas et al. 2017). Different aromatic and aliphatic BPA metabolites were identified including *p*-isopropenylphenol, methylpent-3-oic acid, ethyl-3-ethoxy propanoate, and 4-ethyl-2-methoxyphenol. Taking these previous results in consideration, the objectives of the present study were to immobilize the laccase from *P. ostreatus* on anionically activated agarose support (monoaminoethyl-N-aminoethyl agarose gel) and to determine the effect of this phenomenon on the catalytic properties of the enzyme. Especial emphasis was placed on the performance of the immobilized laccase in the continuous elimination of bisphenol A.

2. Materials and Methods

2.1. Materials

ABTS (2,2-azino-bis-(3-ethyl-benzothiazoline-6-sulphonic acid), bisphenol A (4,4-isopropylidenediphenol, EDA (ethylenediamine), glycydol (2,3-epoxy-1-propanol) and sodium borohydride were purchased from Sigma Chemical Co (Jurubatuba, SP, Brazil). DEAE-Sepharose 4B was purchased from GE-Healthcare Bio-Sci AB (Uppsala, Sweden). Sodium periodate was purchased from Fluka (Neu Ulm, Germany). All other chemicals were of the highest purity and of analytical grade. A laccase from *Pleurotus ostreatus* with molecular weight of 60 kDa was produced in our laboratory (Freitas et al., 2017). The culture supernatant filtered at 0.22 mm containing 12 U mL^{-1} of laccase activity was used for immobilization.

2.2. Determination of laccase activity

Laccase activity was measured with 2,2'-azino bis (e-ethylbenzthiazoline-6-sulphonic acid, ABTS) in 50 mM sodium acetate buffer (pH 5.0) and temperature of 40 °C. Oxidation of ABTS was determined by the increase in $A_{420\ nm}$ (ε=36 $mM^{-1}cm^{-1}$) (Mota et al., 2015). One activity unit of laccase was defined as the amount of enzyme required to catalyse 1µmol of substrate per minute.

2.3. Enzyme immobilization by ionic adsorption

Monoaminoethyl-N-aminoethyl-agarose (MANAE–agarose), used as hydrophilic support, was prepared as described previously (Fernandez-Lafuente et al., 1998, Mateo et al., 2007). Briefly, immobilization procedure consisted of the addition of 1 g of the support to 10 mL of laccase solution (1.8 mg protein.mL^{-1}, in sodium phosphate buffer 25 mmol.L^{-1} pH 7.0). The material was gently shaken at 4 °C for 24 h. After that, the preparation was washed five times with 2 volumes of the same adsorption buffer and, finally, twice with 2 volumes of 25 mmolL^{-1} sodium phosphate buffer, pH 7.0. Protein was determined by the dye-binding method of Bradford using BSA as standard (Bradford, 1976).

2.4. Evaluation of parameters of immobilization

The efficiency of the immobilization procedure was expressed as immobilization yield (IY), remaining activity (RA), and loading, using the following equations:

$IY(\%)=[(U_A-U_E)/U_A]\times100$

$RA(\%)=[U_H/(U_A-U_E)]\times100$

Loading [U/g]=[activity of immobilized enzyme (U)]/[support mass (g)]

Loading [mg/g]= [amount of immobilized enzyme (mg)]/[support mass (g)]

where: U_A = added units or units of activity offered for immobilization; U_E = output units or units of activity in the solution after the immobilization procedure; U_H = immobilized units.

2.5. Desorption of laccase adsorbed on the support

In the desorption process, 10 mg of MANAE-agarose derivative was suspended in 1 mL of 50 mmolL^{-1} sodium acetate buffer pH 5.0. Increasing amounts of NaCl (from 50 mmolL^{-1} to 500mmolL^{-1}) were added and maintained under agitation for 60 min. Aliquots were drawn from both the supernatant and the suspension of immobilized material for determination of laccase activity.

2.6. Characterization of free and immobilized laccase

The effect of pH and temperature on the activity of free and immobilized

enzymes was determined at different pH values (3.0–10.0) and temperatures (30-90 °C). The pH conditions were fixed with a citric acid-phosphate buffer (100 mmolL^{-1}). Thermal stability tests of the free and immobilized enzymes were performed by incubating enzyme samples at 40 and 55 °C at pH 5.0 (determined by pH variation test) for up to 3 h. To determine the long-term stability (storage stability), both free and immobilized enzymes were stored in 50 mM acetate buffer, pH 5.0, in refrigerator (4-8 °C) for up 6 months. For all stability tests, the enzyme activity was determined with the ABTS assay under standard conditions.

2.7. Application and reusability of laccase in the degradation of bisphenol A

To evaluate the efficiency of laccase on the degradation of BPA, 5 UmL^{-1} of both free and immobilized enzymes were incubated with 100 mg/L^{-1} BPA in 50 mmol.L^{-1}acetate buffer (pH 5.0) for up to 60 min at 28 °C and under agitation of 120 rpm. The residual amounts of BPA in the samples were analysed by the methodology previously described (Freitas et al., 2017) using a high efficiency liquid chromatography system (Shimadzu, Japan) equipped with a quaternary pump and diode array detector (DAD) and an Ascentis® C18 column (4.6 mm× 250 mm, 5 µm) thermostated at 40 °C. The mobile phase was water: acetonitrile (40:60, v/v) at a flow rate of 1 mL/min for 15 min. Spectrophotometric detection was performed at 277 nm. The reuse of the enzyme immobilized on the MANAE-agarose support was analyzed by consecutively repeating the experiment under the same conditions mentioned above. After each cycle the derivatives were washed five times with sodium acetate buffer pH 5.0, centrifuged and filtered. As control of the reaction, an activated MANAE-agarose support without immobilized enzyme was used.

2.8. Statistical analysis

All analyses were performed in triplicate. The data were expressed as means ± SD and Student's *t* test was used to compare two means; $p \leq 0.05$ was adopted as a criterion of significance.

3. Results and Discussion

3.1. Immobilization of laccase enzyme

Immobilization of laccase through adsorption to the ionic support MANAE-agarose was quite efficient. The procedure resulted in an immobilization yield (IY) of 100% with an enzyme loading of 120±6.0 U/g support, corresponding to 18.0±0.5 mg protein/g. The activity retention value was 138%, indicating that the immobilization of the enzyme produced an over activated form or that it improved the catalytic properties with respect to the corresponding free form (Mateo et al.,2007). MANAE agarose gel contains primary amino groups which are very suitable supports for immobilization of enzymes whose surface contains carboxyl groups still ionized even at acid pH values (Pesic et al., 2012). This is the case of aspartic and glutamic acid residues, which usually constitute one of the major fractions of the surface groups in proteins (Fernandez-Lafuente et al., 1998).

3.2. Desorption in NaCl

No significant desorption of the enzyme was observed by the addition of NaCl up to 100 $mmolL^{-1}$. Substantial desorption (≈60%), however, was obtained by the addition of 200 $mmolL^{-1}$NaCl.This is a relatively high concentration and suggests that salts at the concentrations normally found in the environment will not cause significant desorption. This has important industrial implications considering that the enzyme should not be eluted from the support under gentle reaction conditions. On the other hand, the possibility of promoting the complete desorption of the enzyme from the support when it is no longer catalytically active, makes it possible to reuse the support for immobilizing a newand fully active enzyme, with the consequent reduction of costs (Mateo et al., 2007, Fuentes et al., 2004, Vieira et al., 2011).

3.3. Comparison of stabilities of free and immobilized laccases

The effects of pH on the activity of the free and immobilized laccases were studied in the pH range from 3.0 to 10.0. Fig. 1 shows that the optimum pH value was 5.0 for both free and immobilized laccases. For the free laccase, the activity was strongly reduced in the alkaline pH range. At pH 8.0, only 20% of its maximal activity was maintained. The immobilized laccase retained almost 60% of its

maximal activity at pH 8.0 ($p \leq 0.05$).

The effects of temperature on the activity of the free and immobilized laccases were also studied in the temperature range of 30–90 °C and the data are shown in Fig. 2. The optimal catalytic temperature of immobilized laccase was slightly higher than that of the free form with a shift from 50 to 55 °C. On the other hand, the thermal stability of immobilized laccase was significantly higher ($p \leq 0.05$) than that of the free enzyme, with half-lives 2.3- and 7.0-fold higher at the temperatures of 40 and 55 °C, respectively (Fig.3).

The thermal stability of an immobilized enzyme is one of the most important criteria of its application. Binding of the enzyme to the matrix makes it more resistant to denaturing agents and heat. The increase in stability may occur due to the stabilization of the protein structure or simply because immobilized enzymes are less accessible to denaturants (Guzick et al. 2014). Such stability is governed by the number and nature of the bonds formed between the enzyme and the carrier, by the degree of confinement of the enzyme to the support, by the micro-environment formed between the enzyme and the support and by the conditions of immobilization (Cao, 2005).

The storage stability of free and immobilized laccases was evaluated at 4°C for up to 6 months (Fig. 4). The free enzyme retained 50 and 40% of its initial activity after 20 and 40 days of storage, respectively. On the other hand, the immobilized enzyme maintained 80 and 70% of its original activity after 40 and 170 days of storage, respectively ($p \leq 0.05$). Evidently, the immobilized laccase presented a much better storage stability than its free form.

3.4. Evaluation of free and immobilized laccase efficiency in the degradation of bisphenol A and reuse of the MANAE-agarose derivative

The capability of free and immobilized laccases to degrade BPA is shown in Fig. 5. Immobilized laccase was slightly more efficient than free laccase in the biodegradation of BPA: after 1 h, the immobilized laccase degraded 100% of BPA at the initial concentration of 100 mgL^{-1}, while the free laccase removed 93% (Fig. 5A). As the reusability is an important parameter to be considered in a large scale application, the reuse of immobilized laccase was examined by applying consecutive cycles of BPA degradation. As can be seen in Fig. 5B, the immobilized enzyme retained more than 90% of its initial capability to degrade

BPA after 15 cycles of reuse.

The potential use of laccases for bioremediation and detoxification of endocrine-disrupting chemicals including BPA from wastewater and soils has been explored for several years. Free and immobilized laccases from *Trametes versicolor* have been more frequently used for these purposes (Fernando-Bautista et al., 2010, Xu et al., 2015, Dai et al., 2016, Nicollucci et al., 2011, Dodor et al., 2004). The preference for using this laccase is because it is amply available. In fact, several studies were undertaken using the enzyme sold by Sigma-Aldrich (Yang et al., 2017). Immobilization of *P. ostreatus* laccase has been conducted with different purposes such as fruit juice clarification (Lettera et al., 2016), decolorization of azo dyes (Pezzella et al., 2014, Dai et al., 2016) and continuous elimination of phenolic pollutants (Hublik and Schimmer, 2000). The free laccase from *P. ostreatus* has also been demonstrated to be efficient in the degradation of BPA (Freitas et al., 2017, Macellaro et al., 2014, Zhang et al., 2015). Until now, however, no study has been done using immobilized *P. ostreatus* laccase for the continuous degradation of BPA. The present study, to our knowledge, is the first attempt at using this immobilized enzyme as a tool for degrading BPA.

The BPA concentration used in the present work is much higher than those generally found in contaminated environments. BPA has been detected at concentrations of approximately 21 µg/L in rivers (Belfroid et al., 2002), 17.2 µg/L in landfill lea chates (Yamamoto et al., 2001), and 150 µg/L in industrial wastewaters (Lee and Peart, 2000).Considering its efficiency in removing BPA at high concentrations the use of the immobilized *P. ostreatus* laccase in situations of real envinronmental contamination should prove equally or perhaps even more efficient.

4. **Conclusion**

In the present study, for the first time, a laccase from *P. ostreatus* was successfully immobilized onto MANAE-agarose with an enzyme loading of 12U/g of support. Compared to free laccase, thermal and storage stabilities of the immobilized laccase were improved. The immobilized laccase was more efficient in the degradation of BPA than free laccase. In addition to this, the immobilized enzyme retained more than 90% of its initial capability to degrade BPA after 15

cycles of reuse. *P. ostreatus* laccase onto MANAE-agarose could be an economic alternative for large scale degradation of bisphenol A in aqueous systems.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Proc. 3079/2015-8) for funding this study. Author T. Brugnari, E.N. Freitas, thanks Coordenação de Aperfeiçoamento do Pessoal do Ensino Superior (CAPES) for the financial support provided for their post-graduate studies in Universidade Estadual de Maringá. G. A. Bubna was a post-doc research grant recipient of CAPES. A. Bracht, M.L.T.M. Polizeli and R.M. Peralta are research grant recipients of CNPq.

References

Ba, S., Arsenault, A., Hassani, T., Peter Jones, J., Cabana, H., 2013. Lacase immobilization and insolubilization: from fundamentals to application for the elimination of emerging contaminants in wastewater treatment. Crit. Rev. Biotechnol., 33(8), 1809-1825.

Barrios-Estrada, C., Rostro-Alanis, M. de J., Muñoz-Guttiérrez, B.D., Iqbal, H.M.N., Kannan, S., Parra-Saldívar, R., 2018, Emergent contaminants: endocrine disruptors and their laccase-assisted degradation – A review. Sci. Total Environ., 612, 1516-1531.

Belfroid, A. van Velzen, M., van der Horst, B., Vethaak, D., 2002. Occurrence of bisphenol A in surface water and uptake in fish: evaluation of field measurements, Chemosphere, 49, 97–103.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem., 72(1-2), 248–254.

Cao L. 2005. Immobilized enzymes: science or art?, Curr.Opin. Chem. Biol., 9, 217-226.

Couto, S.R., Herrera, J.L.T., 2006, Industrial and biotechnological applications of

laccases: a review. Biotechnol. Adv., 24, 500-513.

Dai, J., Wang, H., Chi, H., Wang, Y., Zhao, J., 2016, Immobilization of laccase from *Pleurotus ostreatus* on magnetic separable SiO_2 support and excellent activity towards azo dye decolorization, J. Environ. Chem. Eng, 4, 2585-2591

Dodor, |D.E., Hwang, H.M., Ekunwe, S., 2004. Oxidation of anthracene and benzo[a] pyrene by immobilized laccase from *Trametes versicolor*. Enzyme Microb. Technol., 35, 210-217.

Fernández-Fernández, M., Sanromán, M.A., Moldes, D., 2013. Recent developments and applications of immobilized laccase. Biotech. Adv., 31(8), 1808-1825.

Fernandez-Lafuente, R., Rodriguez, V., Guisán, J.M.,1998. The coimmobilization of D-aminoacid oxidase and catalase enables the quantitative transformation of D-amino acid (phenylalanine) into alpha-keto acids (phenylpyruvic acid),1998. Enzyme Microb. Technol., 23, 28-33.

Fernando-Bautista, L., Morales, G., Sanz, R., 2010, Immobilization strategies for laccase from *Trametes versicolor* on meso-structured silica materials and the application to the degradation of naphthalene. Bioresour. Technol., 101, 8541-8548.

Freitas, E.M., Bubna, G.A., Brugnari, T., Kato, C.G., Nolli, M., .Rauen, T.G., Moreira, R.F.P.M., Peralta, R.A., Bracht, A., Souza, C.G.M., Peralta, R.M. 2017.Removal of bisphenol A by laccases from Pleurotus ostreatus and Pleurotus pulmonarius and evaluation of ecotoxicity of degradation products. Chem. Eng. J. 330, 1361-1369.

Fuentes, M., Pessela, B.C.C., Maquiese, J.V., Ortiz, C., Segura, R.L., Palomo, J.M., Abian, O., Torres, R., Mateo, C., Fernández-Lafuente, R., Guisán, J.M., 2004. Reversible and strong immobilization of proteins by ionic exchange on supports coated with sulfate-dextran, Biotechnol. Prog., 20, 1134-1139.

Guzik, U., Hupert-Kocurek, K., Wojcieszynska, D., 2014. Immobilization as a strategy for improving enzyme properties-application to oxidoreductases. Molecules, 19, 8995-9018.

Hublik, G., Schinner, F., 2000. Characterization and immobilization of the laccase from *Pleurotus ostreatus* and its use for the continuous elimination of phenolic pollutants. Enzyme Microb. Technol., 27, 330-336.

Lee, H.B., Peart, T.E., 2000, Bisphenol A contamination in Canadian municipal

and industrial wastewater and sludge samples, Water Pollut. Res. J. Can., 35, 283–289.

Lettera, V., Pezzella, C., Cicatiello, P., Piscitelli, A., Giacobelli, V.G., Galano, E., Amoresano, A., Sannia, A., 2016, Efficient immobilization of a fungal laccase and its exploration in fruit juice clarification, Food Chem., 196, 1272-1278.

Macellaro, G., Pezzella, C.,Cicatiello, P., Sannia, G., Piscitelli, A.,2014,Fungal laccases degradation of endocrine disrupting compounds. Biomed Res Int., Article ID 614038, 8 pages.

Mateo, C., Palomo, J.M.G., Fernandez-Lorente, G., Guisán, J.M., Fernandez-Lafuente, R.,2007,Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb. Technol., 40, 1451-1463.

Michalowicz, J., 2014, Bisphenol A – sources, toxicity and biotransformation, Environ.Toxicol. Pharmacol. 37, 738–758.

Mohapatra, D.P.,Brar, S.K.,Tyagi, R.D., Surampalli, R.Y., 2011, Concomitant degradation of bisphenol A during ultrasonication and Fenton oxidation and production of biofertilizer from wastewater sludge, Ultrason. Sonochem. 18(5), 1018–1027.

Mota, T.R., Kato, C.G.K., Peralta, R.A., Bracht, A., Morais, G.R., Baesso, M.L., Souza, C.G.M., Peralta, R.M., 2015, Decolourization of congo red by *Ganoderma lucidum* laccase: evaluation of degradation products and toxicity. Water Air. Soil Pollut., 226, 351-362.

Mueller, J.K., Heger, S., 2013, Endocrine disrupting chemicals affect the Gonadotropin releasing hormone neuronal network, Reprod. Toxicol. 44, 73–84.

Nicollucci, C., Rossi, S., Menale, C., Godjevargova, T., Ivanov, Y., Bianco, M., Mita, L., Bencivenga, V., Mita, D.G., Diano, N., 2011, Biodegradation of bisphenols with immobilized laccase or tyrosinase on polyacrylonitrile beads, Biodegradation, 22, 673-683.

Pesic, M., Lopes, C., Álvaro, G., López-Santin, J., 2012, A novel immobilized chloroperoxidase biocatalyst with improved stability for the oxidation of amino alcohols to amino aldehydes. J. Mol. Catal. B Enzym., 84, 144-151.

Pezzella, C., Russo, M.E., Marzocchella, A., Salatino, P., Sannia, G., 2014. Immobilization of a *Pleurotus ostreatus* laccase mixture on perlite and its

application to dye decolourisation, Biomed Res. Int., article ID 308613, 11 pages, DOI: 10.1155/2014/308613.

Rivera-Hoyos, C.M., Morales-Álvarez, E.D., Poutou-Piñales, R.A., Pedrosa-Rodriguez, A.M., Rodriguez-Vásquez, R., Delgado-Bocada, J.M., 2013, Fungal laccases. Fungal Biol. Rev.,27, 67-82.

Taheran, M, Naghdi, M., Brar, S.K., Knystautas, E. J., Verma, M., Surampalli, R. Y., 2017, Degradation of chlortetracycline using immobilized laccase on polyacrylonitrile-biochar composite nanofibrous membrane. Sci. Total Environ., 605-606, 315-321.

Upadhyay, P., Shrivaslava, R., Agrawal, P.K., 2016, Bioprospecting and biotechnological applications of fungal laccase. Biotech, 6, 15-27.

Vieira, M.F., Vieira, A.M.S., Zanin, G.M., Tardioli, P.W., Mateo, C., Guisán, J.M., 2011, β-glucosidase immobilized and stability on agarose matrix functionalized with distinct reactive groups. J. Mol. Catal. B Enzym., 69, 47-53.

Xu, R., Tang, R., Zhou, Q., Li, F., Zhang, B., 2015. Enhancement of catalytic activity of immobilized laccase for diclofenac biodegradation by carbono nanotubes. Chem. Eng. J., 262, 88-93.

Yamamoto, T.,Yasuhara, A., Shiraishi, H., Nakasugi, O., 2001, Bisphenol A in hazardous waste landfill lea chates, Chemosphere, 42, 415–418.

Yang, J., Li, W., Ng, T.B., Deng, X., Lin, J., Ye, X., 2017. Laccases: production, expression regulation, and applications in pharmaceutical biodegradation, Front. Microbiol., 8, 832-856.

Zhang, C., Li, M., Chen, X., Li, M., 2015. Edible fungus degrade bisphenol A with no harmfull effect on its fatty acid composition. Ecotoxicol. Environ. Saf., 118, 126-132.

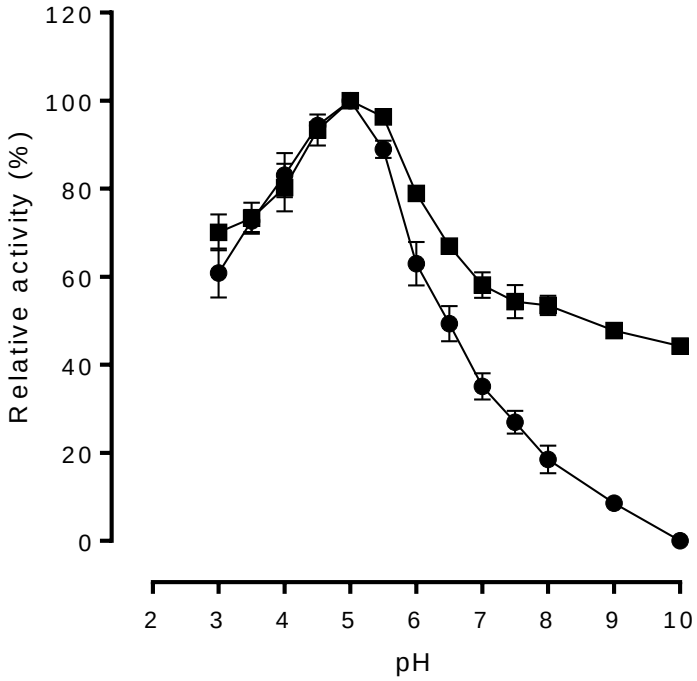

Figure 1. Effect of pH on the activity of *P. ostreatus* laccase. Free laccase (●); immobilized laccase on MANAE-agarose (■). Data are presented as mean ± SD (n=3).

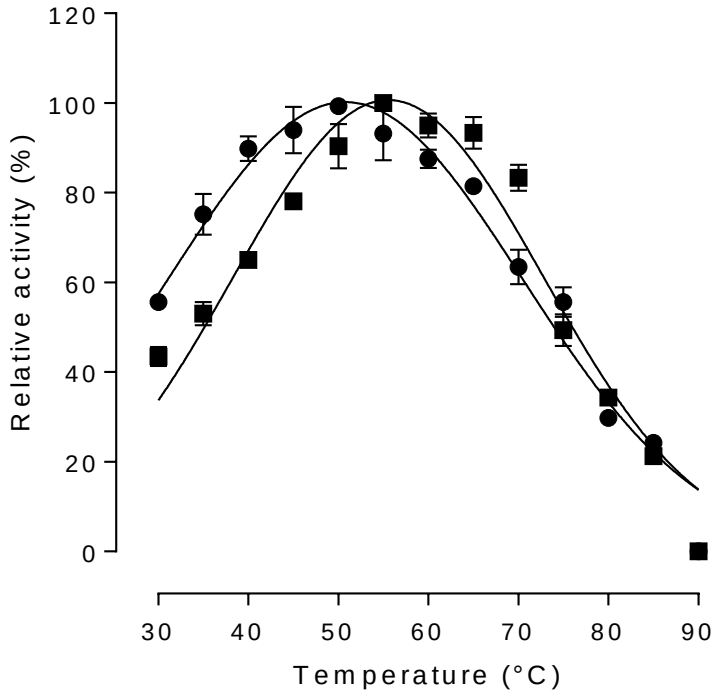

Figure 2. Effect of temperature on the activity of *P. ostreatus* laccase. Free laccase (●); immobilized laccase on MANAE-agarose (■).Data are presented as mean ± SD (n=3).

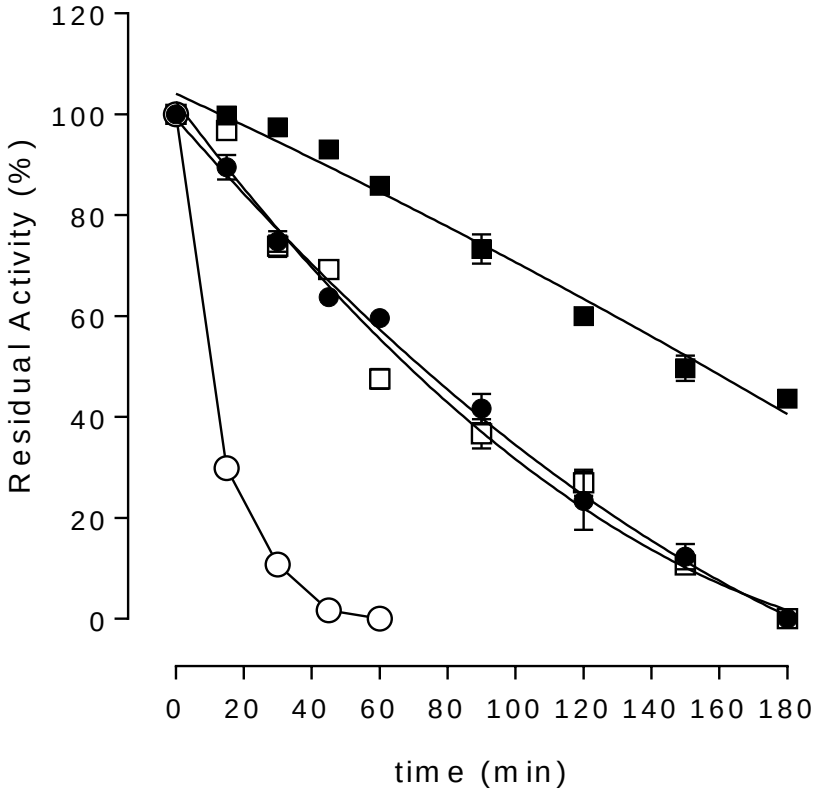

Figure 3. Thermostability of free and immobilized laccases. Free laccase at 40°C (□) and 55 °C (○); immobilized laccase at 40°C (■) and 55°C (●).Data are presented as mean ± SD (n = 3).

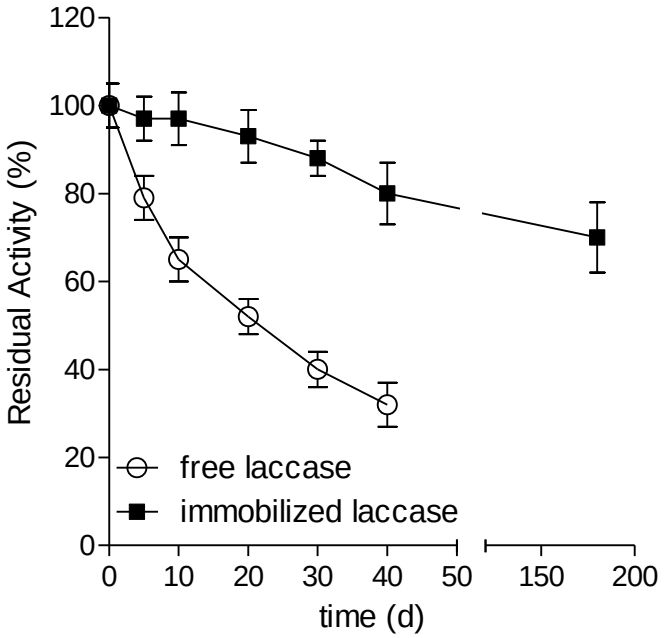

Figure 4. Storage stability of the free (○) and immobilized (■) laccases at 4-8 °C.

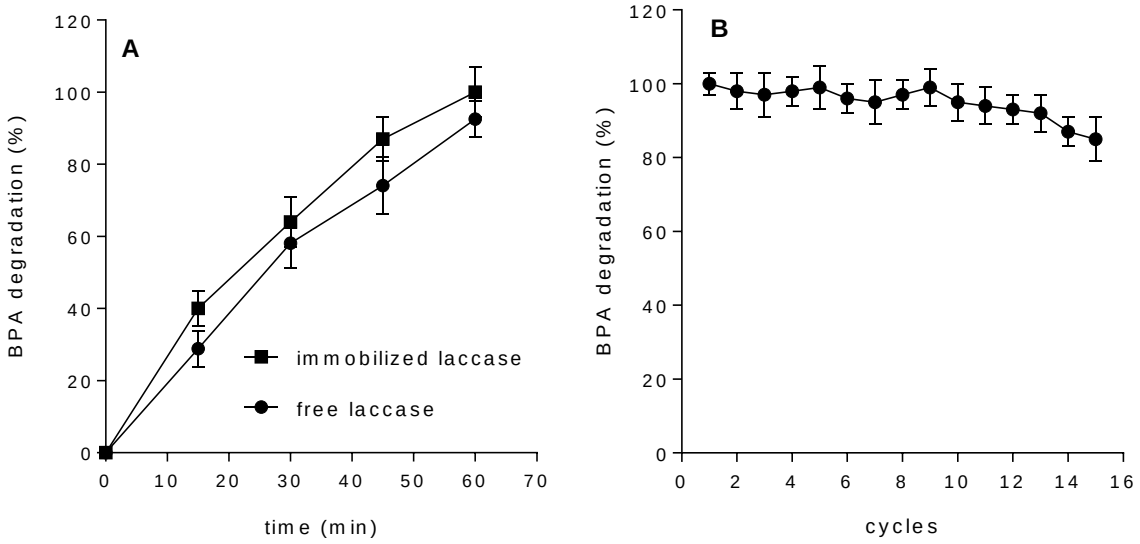

Figure 5. Degradation and reusability potential of immobilized laccases in cycles of BPA degradation. Data are presented as mean ± SD (n = 3).

ARTICLE 2

Laccase immobilization on activated agarose supports and application on bisphenol A degradation.

Tatiane Brugnari[a], Marita Gimenez Pereira[b], Emanuelle N. de Freitas[a], Caio Moriyama[a], Gisele Adriana Bubna[a], Ana Julia Buzzo[a], Maria de Lourdes T.M. Polizeli[b], Adelar Bracht[a], Rosane Marina Peralta[a*]

[a]Department of Biochemistry - Universidade Estadual de Maringá, 87020900, Maringá, Paraná, Brazil.
[b]Department of Biology - Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil

*__Corresponding author__: Rosane Marina Peralta. Departmentof Biochemistry. Laboratory of Biochemistry of Microorganisms and Food Science. Universidade Estadual de Maringá, Av. Colombo, 87020900, Paraná, Brasil.
Email-rosanemperalta@gmail.com

Abstract

Laccases have a broad range of industrial applications. The objective of this study was to immobilize the laccases of the white-rot fungi *Ganoderma lucidum* and *Pleurotus pulmonarius* on activated agarose substrates to overcome problems associated with stability and reusability of the free enzyme. Three types of supports, agarose-epoxide, glyoxyl-agarose, and MANAE-agarose were tested, the latter being the most efficient. No significant alterations due to immobilization were observed in the optima pH and temperature. However, after immobilization, both laccases presented higher thermostability than the free forms. Kinetic studies revealed that free and immobilized *G. lucidum* and *P. pulmonarius* laccases obeyed the Michaelis-Menten equation. Immobilization increased K_M and V_{max} of both enzymes indicating a higher catalytic efficiency at high substrate concentrations. However, the V_{max}/K_M ratios were decreased by immobilization, indicating a diminished catalytic efficiency at low substrate concentrations. The immobilized enzymes retained more than 80% of their initial

capability to degrade BPA after 15 cycles of reuse.

Key words: bisphenol A, laccase, enzyme immobilization.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are copper oxidases that catalyze the one-electron oxidation of phenolics, aromatic amines, and other electron rich substrates with a concomitant reduction of O_2 to H_2O (Brijwani et al., 2010; Aghaie-Khouzani et al., 2012). These enzymes have a high biotechnological potential as they are used in various applications such as biotransformation of xenobiotics and industrial effluents, decolorization of synthetic dyes, bioremediation of contaminated soils, production of biofuels, clarification of wines and teas and the production of biosensors (Mate and Alcade, 2017; Kordon et al., 2010). In the last years, laccases from different microorganisms have been considered as an option for degrading bisphenol A (BPA), one of the most widely manufactured chemical in the world which unfortunately has also been identified as an endocrine disrupting chemical (EDC) (Barrios-Estrada et al., 2018; Daâssi et al., 2016). EDCs are synthetic chemicals that may interfere with the body′s endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife (Mueller and Heger, 2013).

The application of soluble laccases presents some disadvantages such as the high cost of production, loss of stability and non-reusability. These factors make the laccase treatment economically unacceptable. The immobilization of laccases may solve, at least in part, the limitations for large scale application (Ba et al., 2013; Fernández-Fernández et al., 2013). For this reason, considerable efforts have been done in the last years for obtaining immobilized laccases, what could result in several improvements for their application, including increase in storage and operational stabilities, better control of the enzymatic reaction in aqueous solution, and possibility of repeated use (Dai et al., 2016). This paper reports the immobilization of laccases from two white-rot fungi, *Pleurotus pulmonarius* and *Ganoderma lucidum* on agarose supports activated ionic and covalently to improve their stabilities and to evaluate the

perspectives of repeated use in the degradation of BPA.

2. Materials and methods

2.1. Materials

ABTS (2,2-azino-bis-(3-ethyl-benzothiazoline-6-sulphonic acid), EDA (ethylenediamine), glycydol (2,3-epoxy-1-propanol), and sodium borohydride were purchased from Sigma Chemical Co (Jurubatuba, SP, Brazil). DEAE-Sepharose 4B was purchased from GE-Healthcare Bio-Sci AB (Uppsala, Sweden). Sodium periodate Fluka (Neu Ulm, Germany). All other chemicals were of the highest purity and of analytical grade.

2.2. Production of laccases

Pleurotus pulmonarius (Fr.) CCB19 (obtained from culture collection of the Institute of Botany of São Paulo, Brazil) and *Ganoderma lucidum* (isolated and identified at the State University of Maringá) fungi were cultivated as described previously using orange fruit waste as substrate for 2 weeks (Inácio et al., 2015). Crude enzymatic extracts were obtained by adding water (20 mL to 5 g wet solid-state culture).The mixtures were agitated for 30 min at 8 °C. Mycelium debris were removed by centrifugation (8000 rpm for 12 min) and the water extracts were dialyzed against deionized water (molecular mass 12,000 to 16,000 Da cut-off). The dialyzed protein extracts were concentrated by freeze-drying and stored at −20 °C until use.

2.3. Enzyme Assay

Ligninolytic enzyme activities were measured as described previously (Mota et al., 2015). The substrate used for the determination of laccase was 2,2′-azino bis (3-ethylbenzothiazoline-6-sulphonic acid; ABTS) in 50 mmol/L sodium acetate buffer (pH 5.0). Oxidation of ABTS was determined as the increase in absorbance at 420 nm (ε=36 mM^{-1} cm^{-1}). The Mn peroxidase activity (MnP; EC 1.11.1.13) was assayed spectrophotometrically by following the oxidation of 1 mmol/L $MnSO_4$ in 50 mmol/L sodium malonate, pH 4.5, in the presence of 0.1 mmol/L H_2O_2. Manganic ions, Mn^{3+}, form a complex with malonate, which absorbs at 270 nm (ε = 11.59 mM^{-1} cm^{-1}). The lignin peroxidase activity (EC

1.11.1.14) was determined by spectrophotometric measurement at 310 nm of the H_2O_2-dependent veratraldehyde formation (ε = 9.3 mM^{-1} cm^{-1}) from veratryl alcohol. The enzyme activities were determined at 40 °C and expressed in (U) international enzymatic units (mol × 10^{-6} min^{-1}).

2.4. Laccase zymogram

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described (Laemmli, 1970). However, the sample buffer was prepared without addition of β-mercaptoethanol (non-reducing condition) and the samples were not heated before running. After electrophoresis, SDS was removed by washing the gel at room temperature firstly with acetate buffer (pH 5.0) containing 25% isopropanol for 30 min and thereafter with acetate buffer for additional 30 min. The gel was then transferred onto a glass plate and a layer of ABTS-agar (0.02 g of ABTS, 0.4 g of agar, 40 mL of water; heated to dissolve agar) was placed on the gel. These layers were incubated at 25 °C until the green bands' appearance. The molecular masses of the laccases were determined by comparison of their electrophoretic mobilities with those of standard protein markers. The following Mr standards were used: bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). The standard protein bands were visualized by silver staining.

2.5. Enzyme immobilization

For the immobilization of laccase in MANAE-agarose support, first, mono-aminoethyl-N-ethyl-agarose (MANAE-agarose) was ionically activated (Fernandez-Lafuente et al., 1998). Agarose-epoxide supports and glyoxyl-agarose, agarose commercial BCL 4 were covalently activated (Bolivar et al., 2010). The immobilization was carried out with 1 g of the corresponding support (MANAE-agarose, agarose-epoxide and glyoxyl- agarose) with 10 mL of the enzyme solutions (1.8 mg protein/mL extract in sodium phosphate buffer 25 mmol/L, pH 7.0, for MANAE- agarose support, pH 8.0 for agarose-epoxide and pH 10.0 for the glyoxyl-agarose). The different suspensions were gently stirred for 24 h at 4°C. After that, the preparations were washed five times with 2 volumes of the same adsorption buffer and, finally, twice with 2 volumes of 25

mM sodium phosphate buffer pH 7.0. The derivates glyoxyl-agarose were reduced with sodium borohydride (1mg/mL) for 30 min, and then washed with distilled water and buffer. Protein was determined by the dye-binding method of Bradford using BSA as standard (Bradford, 1976). As indicators of immobilization the following parameters were defined: (a) % immobilization (IM), which is the ratio of the amount of immobilized enzyme to the amount of enzyme offered to immobilization; (b) activity recovery (AR), which is the ratio of the measured derivative activity to the theoretical immobilized activity (difference between the initial activity and the activity measured in the final supernatant – hyperactivation or hypoactivation).

2.6. Enzyme desorption

To analyze the effect of the sodium chloride salt on the desorption of the immobilized enzyme, the MANAE-agarose derivative was suspended at the ratio 1/100 (w/v) in 50 mmol/L sodium acetate buffer pH 5.0 added with different concentrations of NaCl (50-2000 mm/L). After 60 min of reaction, aliquots were removed from the supernatant and the suspension containing the derivative in order to measure the corresponding laccase activities.

2.7. *Studies of laccase stability*

The optimum temperature of each enzyme was determined at pH 5.0 by measuring the activity, using ABTS as substrate, at different temperatures in the range of 30-90 °C. The optimum pH was determined at 40 °C using 100 mM citric acid-phosphate buffer at different pH ranges (3.0-10.0). Thermostability of the enzymes was determined by measuring the residual activity after incubation of the enzyme diluted in the absence of substrate at 40 and 55°C in 50 mM sodium acetate buffer pH 5.0 for 60 minutes.The reusability was explored using ABTS as the substrate. At the end of each cycle, the immobilized laccase was recovered by centrifugation and washed with deionized water for two times to remove the residual ABTS. The recycling efficiency was defined as the ratio of the activity of the immobilized laccase in each cycle to the initial activity.

2.8. Determination of kinetic parameters

Determination of the kinetic constants V_{max} and K_M was carried out at 40 °C in 50 mM sodium acetate buffer (pH = 5.0) using ABTS as the substrate, with the substrate concentration varying from 0.01 mM to 2.0 mM. The kinetic parameters were calculated using the Graph Pad Prism software for fitting the Michelis-Menten equation to the experimental data, v_0 = (Vmax[S])/Km + [S], where v_0 is the initial velocity obtained at a given substrate concentration [S].

2.9. Application and reusability of laccase in the degradation of bisphenol A

To evaluate the efficiency of laccases in the degradation of BPA, 5 U mL^{-1} of both free and immobilized enzymes, they were incubated with 100 mg L^{-1} BPA in 50 mmol/L acetate buffer (pH 5.0) for 60 min at 28 °C and under agitation of 120 rpm. The residual amounts of BPA in the samples were analysed by the methodology previously described (Freitas et al., 2017) using a high efficiency liquid chromatography system (Shimadzu, Japan) equipped with a quaternary pump and diode array detector (DAD) and an Ascentis® C18 column (4.6 mm× 250 mm, 5 µm) thermostated at 40 °C. The mobile phase was water: acetonitrile (40:60, v/v) at a flow rate of 1 mL/min for 15 min. Spectrophotometric detection was performed at 277 nm. The reuse of the enzyme immobilized on the MANAE-agarose support was analyzed by consecutively repeating the experiment under the same conditions mentioned above. After each cycle the derivatives were washed five times with sodium acetate buffer pH 5.0, centrifuged and filtered. As control of the reaction, an activated MANAE-agarose support without immobilized enzyme was used.

2.10. Statistical analysis

All analyses were performed in triplicate. The data were expressed as means ± SD and one-way analysis of variance (ANOVA). Differences between means at the 5% ($P< 0.05$) level were considered significant.

Results and discussion

3.1. Enzymes production by G. lucidum and P. pulmonarius in solid-state

cultivation

The solid state cultivation of the fungi *G.lucidum* and *P. pulmonarius* on orange residues was efficient for the obtainment of high laccase activities, higher than 12,000 U/L in all cultures as described previously (Inácio et al., 2015). The Mn peroxidase was produced in small amounts (less than 200 U/L), and no lignin peroxidase activity was detected in the culture filtrates. The analysis by nondenaturing SDS-PAGE followed by zymogram with ABTS allowed to identify a single laccase as the main ligninolytic enzyme in both extracts (Figure 1). The apparent molecular masses of laccases found in the culture extracts from *G. lucidum* and *P. pulmonarius* were 42 and 45 kDa, respectively. Similar results have already been reported in other studies of our group (Motta et al., 2015; Freitas et al., 2017; Zilly et al., 2011). However, multiple forms of *G. lucidum* laccase and *P. pulmonarius* have been reported by different authors, with molecular weights ranging from 38 to 150 kDa (Baldrian 2006; Souza et al., 2003).

3.2. Immobilization of laccases

The MANAE-agarose support was the most efficient one in the immobilization of the laccases of the two fungi with the obtainment of 100% retention. The agarose-epoxide and glyoxyl-agarose substrates showed a low immobilization rate (Table 1). These differences in the percentage of immobilization may be due to the nature of the bonds offered for immobilization. MANAE-agarose is a carrier where enzymes are immobilized by adsorption through ionic bonds. In the other substrates, the enzymes bind through the hydrophobic residues by means of covalent bonds. The results, thus, suggest that both enzymes display only few hydrophobic residues or, that they are not sufficiently exposed. All substrates showed hyperactivation of the enzymatic activity (Table 1), with the highest laccase activations found in the MANAE-agarose derivatives. Changes of some chemical groups during immobilization by covalent attachment of the laccase on the agarose-epoxide and glyoxyl-agarose substrates may interfere in the recovery of the enzymatic activity. A disadvantage of covalent binding is that the enzyme is chemically modified, since the amino groups of the enzyme are used for covalent immobilization (Hanefeld et al., 2009). Immobilization may result in

over-activated forms of the enzymes or even promote totally altered and even better catalytic properties than their soluble forms (Mateo et al., 2007).

3.4. Desorption in NaCl

In the desorption process, the laccases remained bound (100%) to the support until the addition of 100 mmol/L NaCl. The laccase of the fungus *G. lucidum* showed a lower desorption rate, 51.10% in 2000 mmol/L, when compared with the *P.pulmonarius* laccase that was the most strongly affected by salt addition, reaching a rate of 61.07% desorption at the addition of 2000 mmol/L NaCl. Studies related to the binding strength of the enzyme to the support are an important feature in the production of biocatalysts, since addition of salts may be present in many industrial processes.

3.5. Comparison of the stabilities of free and immobilized laccases

Figure 2 and 3 shows the effects of pH and temperature on the activity of free and immobilized enzymes. There was a reduction in enzyme sensitivity to pH after immobilization. At pH 5.0, both enzymes presented optimal activity. At pH 10 the free enzyme of *G. lucidum* presented only 6.37% residual activity whereas the and *P. pulmonarius* enzyme presented no activity at all. The immobilized enzymes of *G. lucidum* and *P. pulmonarius*, however, still retained 54.35% and 39.23%, respectively, of their activities at pH 10. At high temperatures the behavior of the immobilized enzyme was the same as that of the free enzyme for both laccases. The optimum temperature of both free and immobilized enzymes remained in the 60-65°C range.

Figure 4 shows the residual activities of the free and immobilized enzymes after 60 min of exposure to different temperatures in the absence of the substrate. It can be seen that immobilization made the enzyme more stable. After 30 min at 55°C, the free enzyme from *G. lucidum* retained 27,24% of its initial activity, whereas the immobilized enzyme retained 67,41%.Changes in the stability parameters can be determined by several factors such as the number and nature of the bonds formed between the enzyme and the carrier, the degree of confinement of the enzyme in the support and by the conditions under which immobilization occurs. The fact that immobilized enzymes often display greater thermal stability than the corresponding free enzymes may also occur in

consequence of a diminished conformational flexibility and a reduced exposure to denaturing factors because the support itself can act as a physical protector (Marconi, 1989).

3.6. Kinetic parameters of free and immobilized laccases and reuse in the oxidation of the substrate ABTS

Substrate saturation curves of the reaction rates were measured with the free and immobilized enzymes. Fig. 5 shows the Michaelis-Menten plot (Fig 4A-B) and the Eadie-Hofstee plot (Fig. 4C-D) for the data obtained with the free and immobilized laccases. The Eadie-Hofstee representation was used because it is the most adequate to detect deviations of the experimental data from the Michaelis-Menten equation. Free and immobilized laccases from *G. lucidum* and *P. pulmonarius* obeyed the Michaelis-Menten equation. The immobilization of the laccases on MANAE-agarose caused increases in both kinetic parameters, K_M and V_{max}. The increases in the K_M values were more pronounced in relative terms than those in the V_{max} values, what resulted in decreased V_{max}/K_M ratios. The latter modification represents a decreased catalytic efficiency at low substrate concentrations (Table 2). These observations about the modifications of the enzymatic properties may occur due to the following factors: conformational effects (conformational modification of the enzyme molecule due to alteration in the active site tertiary structure; stereochemical effects); microenvironmental effects (electrostatic interactions between support and substrate, the conditions of the microenvironment close to the enzyme are different from those of the reaction solution, and diffusion or mass transfer effects) arise from the diffusion resistance of the substrate to the catalytic site of the enzyme and from the product to the solution.

The possibility of reuse of the enzymatic derivatives from the MANAE-agarose support was high: 85.69% and 75.29% residual activity of *G.lucidum* and *P.pulmonarius* laccase was still present after the tenth reuse with ABTS as the substrate (Fig. 5). For the enzymatic application in the industrial sector to be viable, it is necessary to obtain a stable biocatalyst, which has its properties preserved during the process and that, if possible, offers the possibility of being reused. The development of enzymatic immobilization techniques in solid supports allows the preservation of the kinetic properties and structural

stability of the enzyme, reducing its inactivation by chemical, physical or biological factors, as well as allowing its reuse, making the process economically viable (Castro et al., 2010).The results of immobilization of the laccase in Manae-agarose with possibility of reuse are quite positive, since agarose based gels are known for the ease with which they can be activated in several ways as well as for the ease in handling them. They can be evaluated by spectrophotometric methods and can even be used in reactors because they are compressible and resistant to stirring devices (Guisán, 1988).

3.7. Evaluation of free and immobilized laccase efficiency in the degradation of bisphenol A and reuse of the MANAE-agarose derivative

The capability of free and immobilized laccases to degrade BPA is shown in Fig. 6. Immobilized laccase was more efficient than free laccase in the biodegradation of BPA: after 1 h, the immobilized laccase degraded 96,6% of BPA at the initial concentration of 100 mg/L, whereas the free laccase removed 90% (Fig. 6). As the reusability is an important parameter to be considered in a large scale application, the reuse of immobilized laccase was examined in consecutive cycles of BPA degradation. As can be seen in Fig. 6, the immobilized enzymes retained more than 80% of their initial capability to degrade BPA after 15 cycles of reuse.

Immobilized laccases have been largely used to degrade endocrine disrupting chemicals, including BPA. The enzyme from *T. versicolor* is preferentially used (Barrios-Estrada et al., 2018). Up to now no study has been done using immobilized *P. pulmonarius and G. lucidum* laccases for the continuous degradation of BPA. The present study, to our knowledge, is the first attempt at using these immobilized enzymes as tools for degrading BPA. Table 3 compares the results obtained in the present study with those previously reported for the *T. versicolor* enzyme. It can be concluded that both laccases investigated in the present study are comparable to the *T. versicolor* enzyme in terms of efficiency and reusability.

4. Conclusion

The data obtained in this study showed that the immobilization of the laccase of *G. lucidum* and *P. pulmonarius* in MANAE-agarose support was efficient,

whereas the percentage of immobilization in the glyoxyl-agarose and agarose-epoxide supports were low. Compared to free laccases, thermal stabilities of the immobilized laccases were improved. Immobilization changed the kinetic constants of the enzymes, reduced the V_{max}/K_M ratio, but both laccases maintained their Michaelian behavior. The immobilized laccases were more efficient in the degradation of BPA than the free laccases. In addition to this, the immobilized enzymes retained more than 80% of their initial capability to degrade BPA after 15 cycles of reuse.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Proc. 3079/2015-8) for funding this study. Author T. Brugnari, E.N. Freitas, thanks Coordenação de Aperfeiçoamento do Pessoal do Ensino Superior (CAPES) for the financial support provided for their graduate studies in Universidade Estadual de Maringá. G. A. Bubna was a post-doc research grant recipient of CAPES. A. Bracht, M.L.T.M. Polizeli and R.M. Peralta are research grant recipients of CNPq.

References

Aghaie-Khouzani, M., Forootanfar, H., Moshfegh, M., Khoshayand, M.R., Faramarzi, M.A., 2012. Decolorization of some synthetic dyes using optimized culture broth of laccase producing ascomycete *Paraconiothyrium variabile*. Biochem. Eng. J. 60, 9-15.

Ba, S., Arsenault, A., Hassani, T., Peter Jones, J., Cabana, H., 2013. Laccase immobilization and insolubilization: from fundamentals to application for the elimination of emerging contaminants in wastewater treatment. Crit. Rev. Biotechnol., 33(8), 1809-1825.

Baldrian, P., 2006. Fungal laccases: ocurrence and properties. FEMS Microbiol. Ver. 30, 215-242.

Barrios-Estrada, C., Rostro-Alanis, M.J., Muñoz-Gutiérrez, B.D., Iqbal, H.M.N., Kannan, S., Parra-Saldívar, R., 2018. Emergent contaminants: endocrine

disruptors and their laccase-assisted degradation: a review. Sci. Total Environ. 612, 1516-1531.

Bolivar, J.M., Mateo, C., Grazu, V., Carrascosa, A.V., Pessela, B.C., Guisan, J.M., 2010. Heterofunctional supports for the one-step purification, immobilization and stabilization of large multimeric enzymes: Amino-glyoxyl versus amino-epoxy supports. Process Biochem. 45, 1692-1698.

Brijwani, K., Rigdon, A., Vadlani, P.V., 2010. Fungal laccases: Production, function, and applications in food processing. Enzyme Res. Article ID 149748, 10 pages DOI: 10.4061/2010/149748, 2010.

Castro, H.F., Zanin, G.M., De Moraes, F.F., Sá-Pereira, P., 2010. Imobilização de enzimas e sua estabilização. Capítulo 6, In: Bon, EPS, Pereira Jr, N, Gottschalk LMF, Sá-Pereira, P, Roseiro J C, Ferrara MA, editors. Enzimas em biotecnologia: Produção, aplicação e mercado. Rio de Janeiro: Interciência.

Daâssi, D., Prieto, A., Zouari-Mechichi, H.,Martínez,M.J., Nasri, M.,Mechichi, T., 2016. Degradation of bisphenol A by different fungal laccases and identification of its degradation products. Int. Biodeterior. Biodegrad. 110, 181–188.

Dai, J., Wang, H., Chi, H., Wang, Y., Zhao, J., 2016. Immobilization of laccase from *Pleurotus ostreatus* on magnetic separable SiO_2 support and excellent activity towards azo dye decolorization, J. Environ. Chem. Eng, 4, 2585-2591.

Fernández-Fernández, M., Sanromán, M.A., Moldes, D., 2013. Recent developments and applications of immobilized laccase. Biotech. Adv., 31(8), 1808-1825.

Fernandez-Lafuente, R., Rodriguez, V., Guisán, J.M., 1998. The co-immobilization of D-aminoacid oxidase and catalase enable the quantitative transformation of D-amino acid (phenylamine) into an alpha-keto acids (phenylpyruvic acid). Enzyme Microb. Technol. 23, 28-33.

Freitas, E.M., Bubna, G.A., Brugnari, T., Kato, C.G., Nolli, M., .Rauen, T.G., Moreira, R.F.P.M., Peralta, R.A., Bracht, A., Souza, C.G.M., Peralta, R.M., 2017. Removal of bisphenol A by laccases from *Pleurotus ostreatus* and *Pleurotus pulmonarius* and evaluation of ecotoxicity of degradation products. Chem. Eng. J. 330, 1361-1369.

Guisán, J.M., 1988. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. Enzyme Microb.Technol. 10, 375-382.

Hanefeld, U., Gardossi, L., Magner, E. 2009. Understanding enzyme immobilization. Chem. Soc. Rev. 38, 453-68.

Hou, J., Dong, G., Ye, Y., Chen, V., 2014a. Enzymatic degradation of bisphenol-A with immobilized laccase on TiO_2 sol-gel coated PVDF membrane. J. Memb. Sci. 469, 19-30.

Hou, J., Dong, G., Luu, B., Sengpiel, R.G., Ye, Y., Wessling, M., 2014b. Hybrid membrane with TiO_2 based bio-catalytic nanoparticle suspension system for the degradation of bisphenol-A. Bioresour. Technol. 169, 475-483.

Inácio, F.D., Ferreira, R.O., Araujo, C.A.V., Peralta, R.M., Souza, C.G.M. 2015. Production of enzymes and biotransformation of orange waste by oyster mushroom, *Pleurotus pulmonarius* (Fr.) Quél. Adv. Microbiol.5, 1-8.

Kordon, K., Mikolasch, A., Schauer, F., 2010. Oxidative dehalogenation of chlorinatedhydroxybiphenyls by laccases of white-rot fungi. Int. Biodeter. Biodegr. 64, 203–209.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lin, J., Liu, Y., Chen, S., Le, X., Zhou, X., Zhao, Z., Ou, Y., Yang, J., 2016. Reversible immobilization of laccase onto metal-ion-chelated magnetic microspheres for bisphenol A removal. Int. J. Biol. Macromol. 84, 189-199

Marconi, W. 1989. Immobilized enzymes: their catalytic behaviour and their industrial and analytical applications. Reac.Polymers 11, 1-19.

Mate, D.M., Alcalde, M., 2017. Laccase: a multi-purpose biocatalyst at the forefront of biotechnology. Microb Biotechnol.10, 1457-1467.

Mateo, C., Palomo, J.M.G., Fernandez-Lorente, G., Guisán, J.M., Fernandez-Lafuente, R., 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb.Technol. 40, 1451-1463.

Mota, T.R., Kato, C.G.K., Peralta, R.A., Bracht, A., Morais, G.R., Baesso, M.L., Souza, C.G.M., Peralta, R.M., 2015. Decolourization of congo red by *Ganoderma lucidum* laccase: evaluation of degradation products and toxicity. Water Air Soil. Pollut. 226, 351-362.

Mueller, J.K., Heger, S., 2013. Endocrine disrupting chemicals affect the gonadotropin releasing hormone neuronal network, Reprod. Toxicol. 44, 73–84.

Souza, C.G.M., Tychanowicz, G.K., Souza, D.F., Peralta, R.M., 2004. Production of laccase isoforms by *Pleurotus pulmonarius* in response to presence of phenolic and aromatic compounds. J. Basic Microbiol. 2,129-136.

Zdarta, J., Antecka, K., Frankowiski, R., Zgola-Grzeskowiak, A., Ehrlich, H., Jesionowski, T., 2017. The effect of perational parameters on the biodegradation of bisphenols by *Trametes versicolor* laccase immobilized on *Hippospongia communis* sponging scaffolds. Sci. Total Environ. 615, 784-795.

Zilly, A., Coelho-Moreira, J.S., Bracht, A., Souza, C.G.M., Carvajal, A.E., Koehnlein, E.A., Peralta, R.M., 2011. Influence of NaCl and Na_2SO_4 on the kinetics and dye decolorization ability of crude laccase from *Ganoderma lucidum.* Int. Biodeter. Biodegr. 65, 340-344.

Table 1. Percentage of laccase immobilization in different substrates.

	MANAE-agarose		agarose-epoxide		glyoxyl-agarose	
	IM (%)	AR	IM (%)	AR	IM (%)	AR
G. lucidum	100	1.35	67.9	0.22	15.0	0.12
P.pulmonarius	100	0.92	67.7	0.27	1.25	0.05

IM(%)= percentage immobilization; AR= activity recovery

Table 2**.** Kinetic constants of free and immobilized laccases from *P. pulmonarius* and *G. lucidum.*

	K_M (µmol/mL)	V_{max} (µmol/min)	V_{max}/K_M
P. pulmonarius laccase			
Free enzyme	33.96± 2.32	24.262±5.52	714
Immobilized enzyme	221.60±19.30	30.567±12.37	138
G. lucidum laccase			
Free enzyme	32.31±2.30	27.617±6.44	855
Immobilized enzyme	263.20±25.43	49.969±23.59	190

Table 3. Comparison of removal of BPA by immobilized laccases.

Immobilized laccase	Removal of BPA	Reusability	Reference
Trametes versicolor laccase immobilized on *Hippospongia communis* sponging scaffolds	BPA (2 mg/L) was removed after 25 h	5 reuses	Zdarta et al., 2018
Trametes versicolor laccase immobilized on electrospun fibrous membranes by carbon nanotubes modification	BPA (50 mg/L) was removed after 5 h	Nd	Dai et al., 2016
Trametes versicolor laccase immobilized onto metal-ion-chelated magnetic microspheres	BPA (20 mg/L) was removed after 12 h	5 reuses	Lin et al., 2016
Trametes versicolor laccase immobilized laccase on TiO_2 sol–gel coated PVDF membrane	BPA (34 mg/L) was removed after 5 h	4 reuses	Hou et al., 2014a
Trametes versicolor laccase immobilized on hybrid membrane with TiO_2 based bio-catalytic nanoparticle suspension	BPA (34 mg/L) was removed after 24 h	15 reuses	Hou et al., 2014b
Pleurotus pulmonarius laccase immobilized on MANAE-agarose	BPA (100 mg/L) was removed after 1 h	15 reuses	This work
Ganoderma lucidum laccase immobilized on MANAE-agarose	BPA (100 mg/L) was removed after 1 h	15 reuses	This work

Nd= not determined

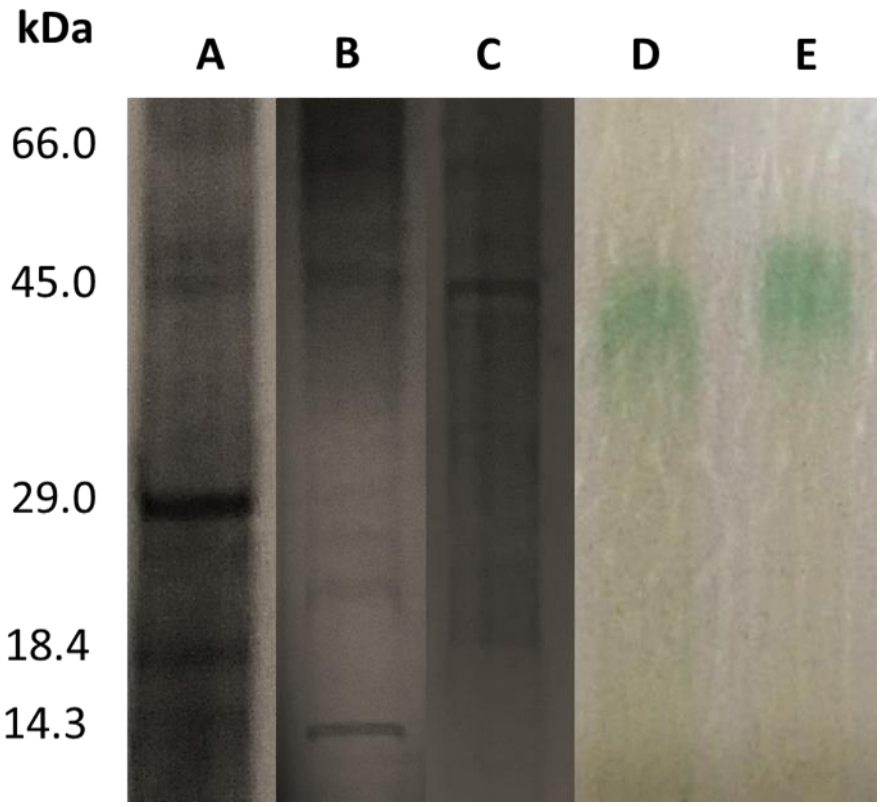

Figure 1. Electrophoresis gel of crude enzymatic extract of laccase. (A) Molecular patterns stained with silver staining; (B) crude enzymatic extract of *G.lucidum* stained with silver staining;(C) crude enzymatic extract of *P.pulmonarius* stained with silver staining; (D) Zymogram of *G.lucidum laccase*; (E) Zymogram of *P.pulmonarius*.

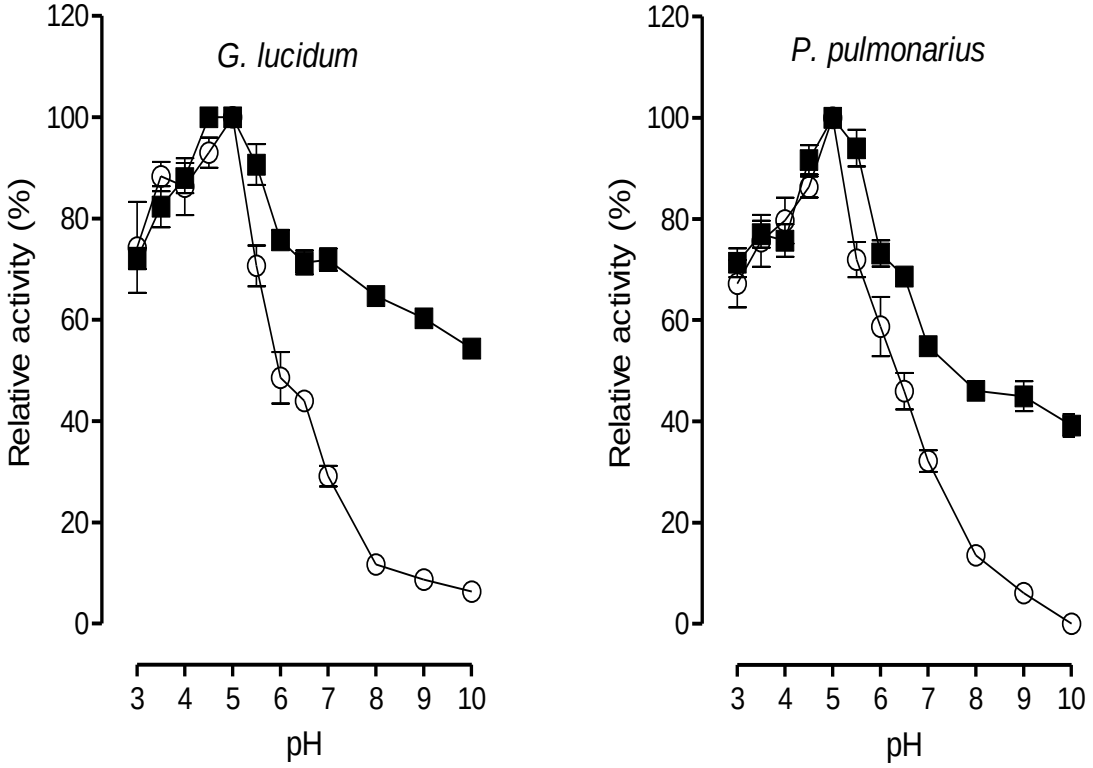

Figure 2. Effect of pH on the activities of free (○) and immobilized (■) laccases on MANAE-agarose support.

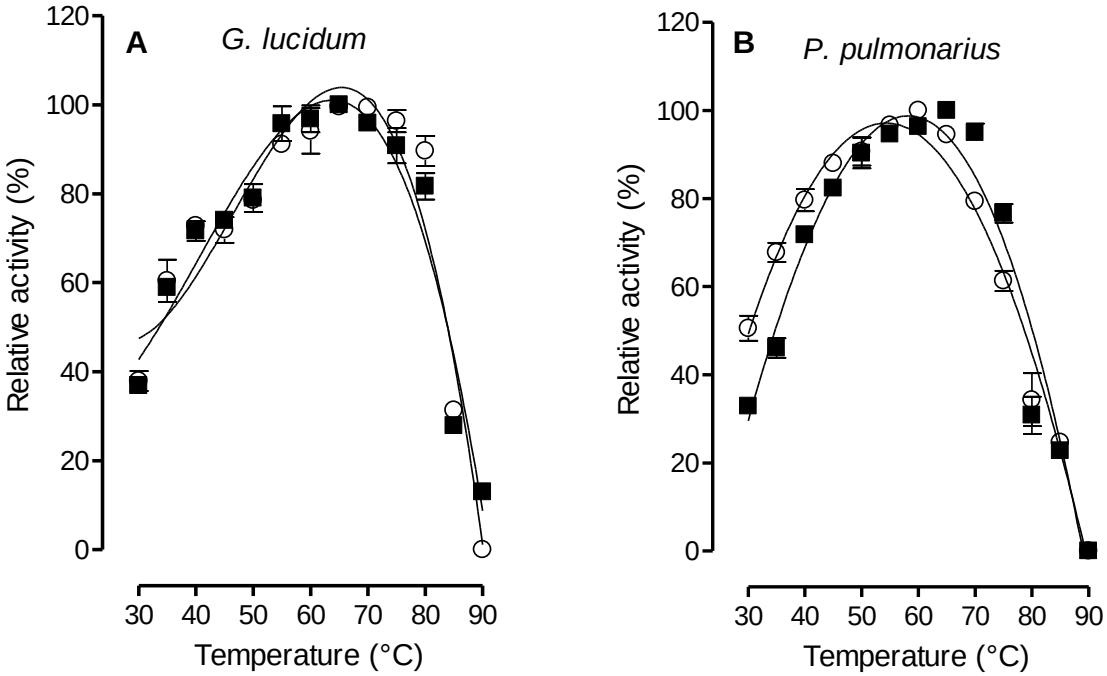

Figure 3. Effect of temperature on the activities of free (○) and immobilized (■) laccases on MANAE-agarose support.

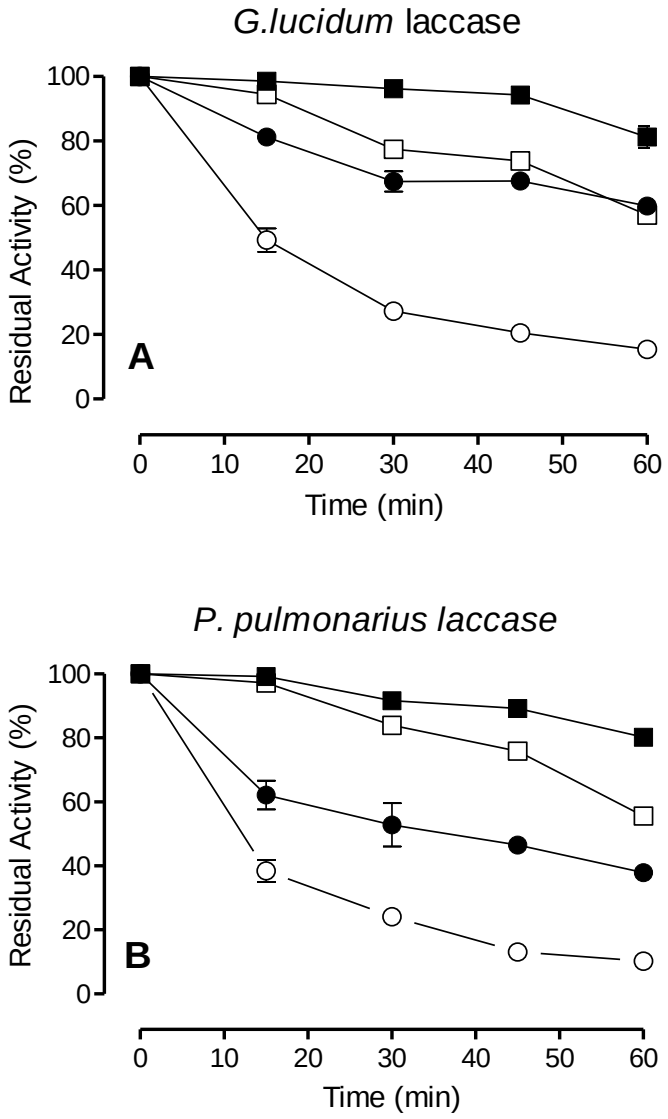

Figure 4. Stability of laccases, free and immobilized on MANAE-agarose support. Free laccase at 40°C (□); immobilized laccase at 40°C (■); free laccase at 55°C (○); immobilized laccase at 55°C (●). Data are expressed as mean ± SD (n=3).

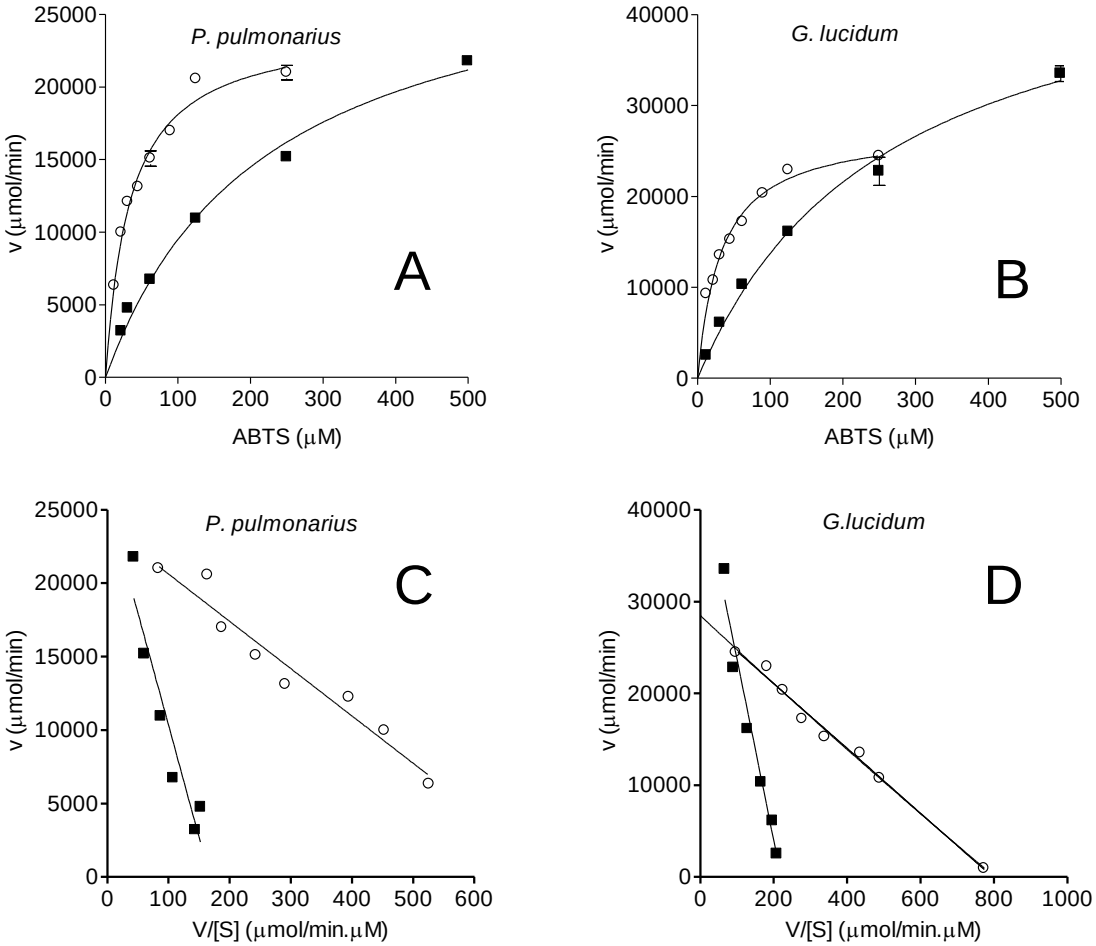

Figure 5. Initial reaction rates at different concentrations of ABTS with free (from 12 to 250 μM) and immobilized (from 12 to 500 μM) laccases. Free laccase (○); immobilized laccase (■). In A and B, the solid lines represent the fitted Michaelis–Menten equation. In C and D the corresponding Eadie-Hofstee representations are shown. Data are expressed as mean ± SD (n=3).

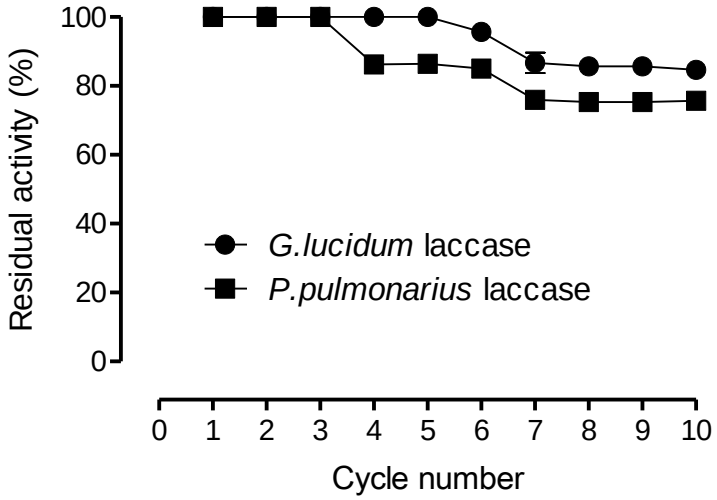

Figure 5. Reusability potential of the immobilized laccases using ABTS as substrate. Data are expressed as mean ± SD (n=3).

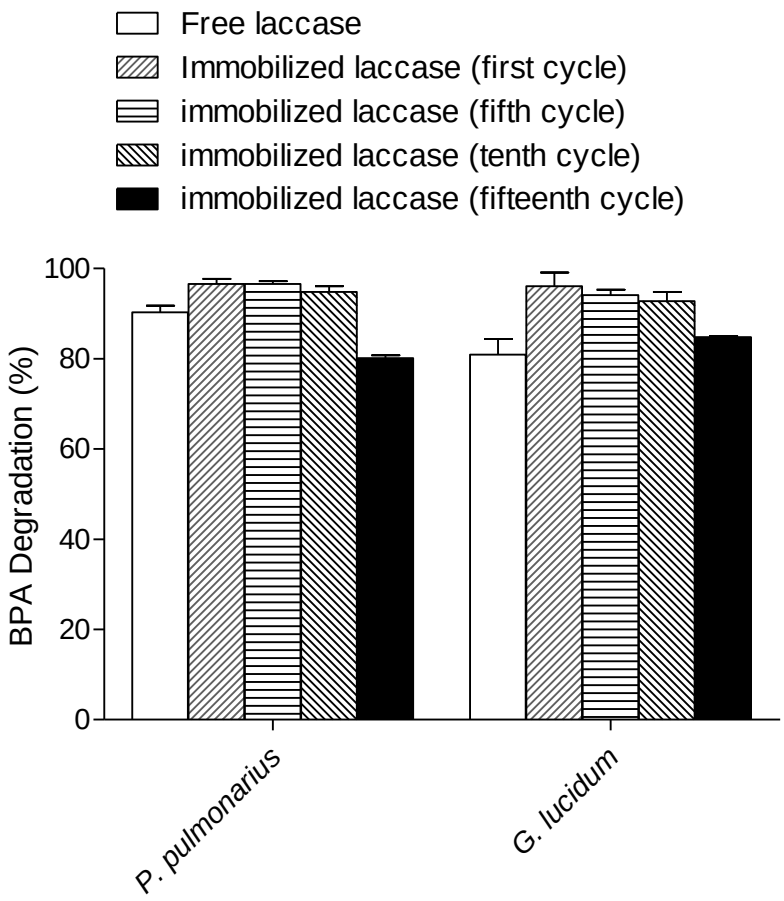

Figure 6. Reusability potential of immobilized laccases in the BPA degradation. Data are presented as mean ± SD (n = 3).