JULIANA ARRIEL TORRES

OBTENÇÃO E PERFORMANCE DE BIOCATALISADORES:

IMOBILIZAÇÃO E EFEITO DE ADITIVOS QUÍMICOS

LAVRAS - MG
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Profa. Dra. Angelita Duarte Corrêa
Orientadora
Dra. Maria Cristina Silva
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OBTENÇÃO E PERFORMANCE DE BIOCATALISADORES: IMOBILIZAÇÃO E EFEITO DE ADITIVOS QUÍMICOS

OBTAINING AND PERFORMANCE OF BIOCATALYSTS: IMMOBILIZATION AND THE EFFECT OF CHEMICAL ADDITIVES

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agroquímica, área de concentração Química/Bioquímica, para a obtenção do título de Doutor.

Aprovada em 27 de março de 2017.
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2017

A Deus, por tantas graças alcançadas durante toda minha caminhada, por fazer meus sonhos realidade e me guiar em cada momento sem desanimar.

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RESUMO

As peroxidases são enzimas com várias aplicações, principalmente, na degradação de compostos orgânicos. Pesquisas têm sido desenvolvidas com estas enzimas obtidas de várias fontes vegetais, sobretudo de resíduos agroindustriais. Entretanto, estes biocatalisadores estão sujeitos à inibição, como por exemplo pelos produtos finais da degradação de poluentes fenólicos. Para contornar esta limitação se pode incorporar aditivos químicos no meio reacional, além de imobilizar estas enzimas em diferentes suportes. Neste trabalho, aplicou-se peroxidases obtidas do nabo e de cascas de soja, um resíduo agroindustrial, na degradação de compostos fenólicos padrões na presença dos aditivos polietilenoglicol (PEG) e triton X-100. Avaliou-se também a degradação de compostos fenólicos totais de águas residuárias do processamento do café (ARC) utilizando a peroxidase de soja (PS). Além disso, buscou-se outra alternativa para melhorar a estabilidade enzimática, bem como facilitar a remoção do biocatalisador e possibilitar a reutilização do mesmo, imobilizando este biocatalisador em carvão ativado (CA), que foi sintetizado empregando o resíduo sólido recolhido da extração da PS. A imobilização da PS em CA (PS-CA) foi avaliada em diferentes condições de tempo, carga de enzima, pH e temperatura. Sintetizou-se um compósito magnético carvão ativado/magnetita para facilitar a remoção do biocatalizador imobilizado, com a aplicação de um campo magnético. Técnicas de caracterização foram realizadas nos diferentes materiais. As peroxidases de nabo e soja foram eficientes na degradação dos compostos fenólicos, entretanto, apenas para o fenol e o ácido cafeico (pH 3,0), o efeito protetor dos aditivos foi observado. PS apresentou 31,15% de oxidação da ARC, entretanto, na presença de PEG e triton X-100 nenhuma influência na oxidação de compostos fenólicos foi observada. O CA apresentou elevada área superficial específica de 1.603 $m^2\ g^{-1}$, que o torna ideal para imobilização de enzimas, além de ter sido obtido com aproveitamento total da biomassa, com geração mínima de resíduos. A imobilização PS-CA alcançou 100% na proporção suporte:enzima de 1,0:0,05 (m/m), pH 3,0, temperatura 40 °C por um período de 1 hora. PS-CA foi reutilizada durante 10 ciclos, apresentando um declínio no primeiro ciclo e mantendo-se constante até o último. Essa queda pode estar associada a inibição da enzima pelos produtos de reação, uma vez que a enzima não foi lixiviada. Apesar da vantagem de se utilizar o CA como suporte para imobilização de enzimas, sua recuperação do meio reacional é um processo tedioso. Para contornar este inconveniente sintetizou-se um compósito magnético. A imobilização da PS-CA magnético foi de 100% na proporção suporte:enzima de 1,0:0,05 (m/m), pH 3,0, temperatura 50 °C por um período de 7 horas. Esse biocatalisador imobilizado magnetizado foi reutilizado por 11 ciclos, com uma queda de aproximadamente 30% em relação ao primeiro ciclo e mantendo-se constante até o último. Conclui-se que utilizar aditivos na oxidação de compostos fenólicos da ARC não traz benefícios, o que representa uma resposta positiva do ponto de vista econômico. O CA obtido representa um suporte promissor para imobilização de PS, com aproveitamento máximo da biomassa, e o compósito magnético sintetizado facilita a recuperação e reutilização da PS imobilizada.

Palavras-chave: Peroxidase. Compostos fenólicos. Polietilenoglicol. Triton X-100. Água residuária. Carvão ativado. Estabilidade enzimática. Magnetita.

ABSTRACT

Peroxidases are enzymes with various applications, mainly in the degradation of organic compounds. Researches have been developed with these enzymes obtained from various vegetable sources, mainly agroindustrial waste. However, these biocatalysts are subject to inhibition, as for example by the final products of phenolic pollutants degradation. To overcome this limitation, chemical additives can be incorporated into the reaction medium, besides immobilizing these enzymes in different supports. In this work, were applied peroxidases obtained from turnip and soybeans, an agroindustrial residue, in the degradation of standard phenolic compounds in the presence of the additives polyethylene glycol (PEG) and triton X-100. It was evaluated also the degradation of total phenolic compounds from coffee processing wastewater (CPW) using soybean peroxidase (SP). In addition, another alternative was sought to improve the enzymatic stability, as well as to facilitate the removal of the biocatalyst and to enable the reuse of this, immobilizing this biocatalyst in activated carbon (AC), which was synthesized using the solid residue collected from the SP extraction. The immobilization of SP in AC (SP-AC) was evaluated at different time conditions, enzyme loading, pH and temperature. An activated carbon/magnetite composite was synthesized to facilitate the removal of the immobilized biocatalyst with the application of a magnetic field. Characterization techniques were performed in different materials. The turnip and soybean peroxidases were efficient in the degradation of the phenolic compounds, however, only for phenol and caffeic acid (pH 3.0), the protective effect of the additives was observed. PS presented 31.15% of CPW oxidation, however, in the presence of PEG and triton X-100 no influence on the oxidation of phenolic compounds was observed. The AC presented a high specific surface area of 1,603 m^2 g^{-1}, which makes it ideal for immobilization of enzymes, besides having been obtained with full use of biomass, with minimal waste generation. SP-AC immobilization reached 100% in the support:enzyme of 1.0:0.05 (m/m), pH 3.0, temperature 40 ° C for a period of 1 hour. SP-AC was reused for 10 cycles, presenting a decline in the first cycle and remaining constant until the last cycle. This drop may be associated with inhibition of the enzyme by the reaction products, once the enzyme has not been leached. Despite the advantage of using AC as a support for enzyme immobilization, its recovery from the reaction medium is a tedious process. To overcome this inconvenient a magnetic composite was synthesized. The immobilization of the magnetic SP-AC was 100% in the support:enzyme relationship of 1.0:0.05 (m/m), pH 3.0, temperature 50 ° C for a period of 7 hours. This magnetized immobilized biocatalyst was reused for 11 cycles, with a decrease of approximately 30% over the first cycle and remaining constant until the last. It is concluded that using additives in the oxidation of phenolic compounds of the CPW does not bring benefits, which represents a positive response from the economic point of view. The AC obtained represents a promising support for SP immobilization, with maximum biomass utilization, and the synthesized magnetic composite facilitates the recovery and reuse of the immobilized SP.

Keywords: Peroxidase. Phenolic compounds. Polyethylene glycol. Triton X-100. Wastewater. Activated carbon. Enzymatic stability. Magnetite.

SUMÁRIO

APRESENTAÇÃO

Este trabalho de tese está dividido em duas partes:

Na primeira parte constam a Introdução, Referencial teórico e as Considerações finais.

Na segunda parte constam os resultados deste trabalho que estão apresentados sob a forma de artigos. Cada artigo está estruturado de acordo com as normas das revistas científicas escolhidas para submissão ou publicação dos mesmos.

PRIMEIRA PARTE

1 INTRODUÇÃO

A aplicação de catalisadores é notável nos mais variados campos de atuação da indústria química, com destaque para a produção de combustíveis e biocombustíveis, petroquímica, fertilizantes, tintas, solventes, lubrificantes, fibras e polímeros. Na área ambiental, a mesma envolve a degradação de poluentes orgânicos e consequente tratamento de águas residuárias, bem como na produção de fármacos e na indústria alimentícia.

Ainda dentro do contexto da catálise, ressalta-se a utilização de enzimas como uma alternativa efetiva na degradação de diversos contaminantes, como por exemplo, os compostos fenólicos, os quais têm origem em diferentes segmentos industriais, estando presentes em diversos tipos de efluentes, tais como refinarias de petróleo, indústrias de corantes, papel e celulose, processamento e beneficiamento do café, entre outros. Quando as enzimas ou células são utilizadas como catalisadores tem-se a biocatálise.

Diversas enzimas apresentam várias aplicações nas mais diferentes áreas, ressaltando-se na biorremediação de efluentes contaminados e, entre elas, estão as peroxidases. Várias fontes vegetais contêm uma quantidade expressiva de peroxidases, como o nabo e a soja. O nabo é um vegetal que pode ser cultivado em diversos países, e já a soja possui peroxidase nas várias partes do grão, principalmente nas cascas, que são resíduos gerados em grande abundância na industrialização de alimentos. Dessa forma, estes vegetais representam uma fonte de enzima atraente do ponto de vista ambiental e econômico.

As peroxidases catalisam a oxidação de compostos orgânicos, tais como aminas aromáticas e compostos fenólicos, enquanto o peróxido de hidrogênio é reduzido, produzindo uma molécula de água. Durante o ciclo catalítico, produtos radicalares são formados os quais tendem a iniciar uma reação em cadeia até a formação de polímeros insolúveis, que podem ser facilmente removidos do meio reacional por meio de processos simples. A eficiência de biocatalisadores está diretamente relacionada com sua estabilidade, a qual pode ser afetada pela formação destes polímeros. Alguns estudos apontam os possíveis mecanismos envolvidos na inibição das enzimas por estes produtos finais, além de discutirem alternativas para manter a estabilidade e a eficiência catalítica dos biocatalisadores.

A incorporação de alguns aditivos químicos no meio reacional de degradação, tais como o polietilenoglicol (PEG) e o surfactante triton X-100, pode minimizar o fenômeno de inibição

das enzimas, por protegerem as mesmas dos produtos finais. Diferentes possibilidades são levantadas para explicar o mecanismo de proteção, uma vez que em alguns casos estes compostos são altamente eficazes.

Apesar da grande variedade de aplicações de enzimas em diferentes campos da pesquisa, sua utilização é ainda limitada na biocatálise homogênea, uma vez que na sua forma livre este biocatalisador está sujeito à inúmeras formas de inibição. Além da incorporação de aditivos químicos no meio reacional, existem outras alternativas que visam aumentar a estabilidade das enzimas, como por exemplo, o confinamento destes biocatalisadores em uma determinada matriz, em um processo denominado imobilização.

Diferentes técnicas são utilizadas na imobilização de enzimas, e a mais simples delas é a adsorção. Durante este processo, a enzima pode se ligar ao suporte por meio de ligações de hidrogênio, interações eletrostáticas ou por interações de Van der Waals. A natureza e a intensidade dessas interações estão relacionadas com o tipo de enzima e suporte empregados. A imobilização além de aumentar a estabilidade da enzima, permite o reuso da mesma e a sua separação dos produtos da reação. Diversos tipos de suportes são empregados na imobilização de enzimas, tais como zeólitas, polímeros naturais como quitosana, e os carvões ativados, os quais possuem estruturas porosas e uma elevada área superficial específica, além de grupos funcionais superficiais que podem auxiliar no processo de imobilização.

O carvão ativado pode ser produzido utilizando diferentes precursores carbonáceos, desde que apresentem um elevado teor de carbono e baixo teor de cinzas. Pesquisas recentes têm focado no aproveitamento de resíduos para produção destes adsorventes.

A extração da enzima peroxidase utilizando cascas de soja, um resíduo agroindustrial, gera outro resíduo sólido, e o aproveitamento deste para produção de carvão ativado é uma alternativa que agregará valor, contornará problemas relacionados com a disposição inadequada de resíduos no meio ambiente, além de ser uma alternativa aos carvões comerciais utilizados.

Apesar das inúmeras vantagens da utilização do carvão ativado como suporte para imobilização de enzimas, recuperar o biocatalisador imobilizado do meio reacional é uma tarefa dispendiosa, podendo levar até a perda de material. Sendo assim, inúmeras pesquisas têm focado na incorporação de nanopartículas magnéticas na estrutura do carvão ativado, facilitando sua remoção do meio com a simples aplicação de um campo magnético externo, e possibilitando uma reutilização mais eficaz do biocatalisador imobilizado.

Tendo em vista o crescente avanço da biocatálise e a elevada eficiência dos biocatalisadores em diversas áreas, a busca pelo aumento da estabilidade das enzimas no meio

reacional, seja pela adição de agentes estabilizantes ou por meio de técnicas de imobilização é de suma importância.

Diante do exposto, busca-se por meio deste projeto extrair peroxidases vegetais (soja e nabo) com proposição de alternativas (incorporação de aditivos químicos e imobilização da enzima) para o aumento da estabilidade destes biocatalisadores; produzir carvão ativado utilizando o resíduo da extração da peroxidase de casca de soja e utilizá-lo como suporte para imobilizar a peroxidase de soja. Busca-se ainda produzir um compósito magnético, no intuito de facilitar a recuperação e reutilização da peroxidase imobilizada.

2 REFERENCIAL TEÓRICO

2.1 Biocatálise ambiental

A química é uma área da ciência essencial ao desenvolvimento de inúmeros produtos fundamentais à humanidade, mas que enfrenta desafios relacionados ao desenvolvimento de tecnologias “verdes”, cada vez mais discutidas na atualidade. A necessidade de processos e alternativas que diminuam a geração de resíduos e minimizem os danos causados ao meio ambiente torna indiscutível a urgência em se desenvolver tecnologias limpas que satisfaçam as necessidades atuais sem comprometer as gerações futuras. Este novo caminho a ser delineado pela química é denominado como química verde ou química sustentável (PRADO, 2003, p. 738).

Neste sentido, a química verde nada mais é do que a busca pelo desenvolvimento e aplicações de produtos e processos químicos que reduzam ou eliminem a geração de substâncias perigosas à saúde humana e ao meio ambiente (ALCALDE et al., 2006). Alguns princípios básicos regem esta área da ciência, e entre eles, o uso da catálise para melhoria de processos industriais, maximização das reações e redução da formação de subprodutos indesejáveis durante o processo reacional, entre outros (PRADO, 2003, p. 738).

Uma área em amplo desenvolvimento nas últimas décadas, conhecida como biocatálise, desempenha um papel crítico e extremamente importante na concepção de processos sustentáveis em química sintética e na gestão de resíduos (JAIN et al., 2016; LIMA-RAMOS; NETO; WOODLEY, 2014). Esse processo envolve a utilização de enzimas para catalisar transformações químicas e para fabricar produtos para as indústrias químicas, farmacêuticas e alimentícias, sendo considerada uma alternativa aos processos convencionais de catálise industrial (HARTMANN; JUNG, 2010).

No contexto da biocatálise, se tem a utilização de enzimas para propostas de remediação ambiental, prática que tem aumentado de forma expressiva devido às propriedades peculiares dessa classe das proteínas (OLIVEIRA et al., 2016; RAO et al., 2014). A ação catalítica das enzimas é extremamente versátil, eficiente e seletiva comparada aos catalisadores químicos, ao passo que atuam em condições brandas de reação (temperatura e pressão), em uma ampla faixa de concentração de contaminantes, além de se tratar de catalisadores biodegradáveis e não tóxicos (JAIN et al., 2016). Estes biocatalisadores são capazes de atuarem em reações

específicas muitas vezes em uma taxa de reação elevada e não acessível pela catálise tradicional (RAO et al., 2014).

2.2 Peroxidases

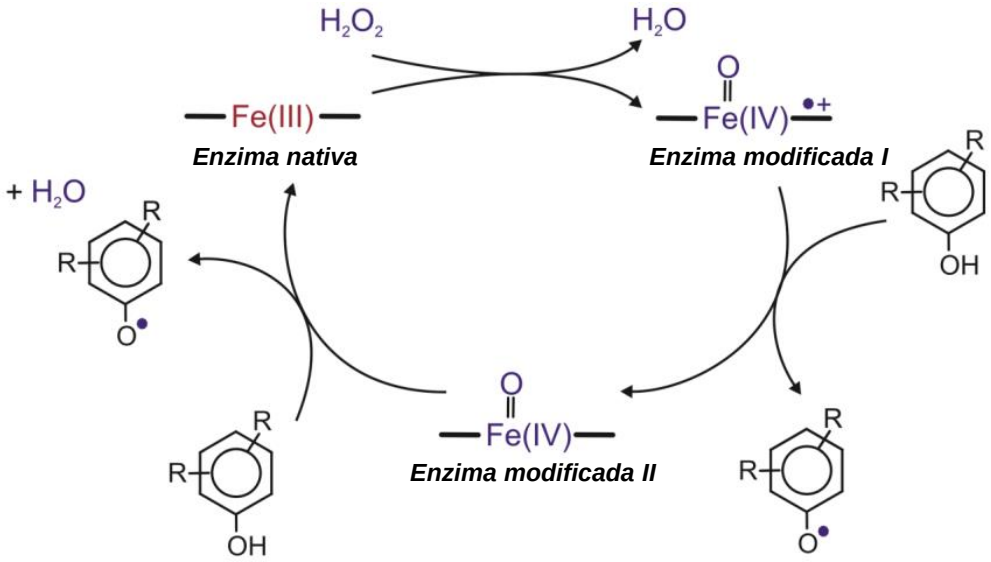
Diversas enzimas são utilizadas na biocatálise e, entre elas, as peroxidases (E.C.1.11.1.7) têm recebido atenção especial pelo seu reconhecido papel em aplicações ambientais (CHAGAS et al., 2015; SILVA et al., 2015; TORRES et al., 2016). Essas enzimas catalisam a oxidação de uma ampla variedade de substratos orgânicos e inorgânicos na presença de peróxido de hidrogênio como um cosubstrato. Podem ser obtidas de bactérias, fungos ou mais facilmente de organismos superiores, como as plantas, onde estão presentes em diferentes isoformas e disponíveis em todas as partes da célula (ASHRAF; HUSAIN, 2009; ZHENG; JIANG, 2014; TANDJAOUI et al., 2015).

As peroxidases possuem um amplo espectro de aplicações em diferentes áreas da ciência, como na bioquímica, biotecnologia, em áreas industriais e ambientais, no estabelecimento de diagnósticos clínicos, na construção de biossensores, entre outras (ASHRAF; HUSAIN, 2009, 2010; MACIEL; GOUVÊA; PASTORE, 2007). Nos últimos anos, têm sido empregadas na biotransformação de vários tipos de fenóis, aminas aromáticas, corantes, bisfenóis e bifenilos presentes em efluentes industriais (ASHRAF; HUSAIN, 2009; DIAO et al., 2011; TORRES et al., 2015).

O ciclo catalítico da peroxidase de raiz forte (horseradish peroxidase), a peroxidase mais estudada até o momento, é o melhor descrito entre essas proteínas, sendo considerado um sistema modelo de reação das peroxidases (GUZIK et al., 2014). No primeiro estágio do processo ocorre a reação entre o peróxido de hidrogênio e o Fe (III) da enzima na sua forma nativa com a liberação de uma molécula de água para produzir a forma oxidada da enzima (enzima modificada I), uma forma intermediária reativa compreendendo um centro oxiferril Fe (IV) e um cátion radical porfirínico (Figura 1). No segundo estágio da reação, a enzima modificada I oxida uma molécula de substrato, gerando um produto radicalar e a enzima modificada II, uma espécie oxiferril Fe (IV), um equivalente de oxidação superior ao estado nativo. Tanto a forma I quanto a forma II da enzima são potenciais oxidantes. Finalmente, a enzima modificada II é reduzida por uma segunda molécula de substrato, fazendo com que a enzima retorne a sua forma inicial (DERAT; SHAIK, 2006; HINER et al., 2001; SILVA et al., 2015; VEITCH, 2004, p. 249). Os radicais livres formados se acoplam formando dímeros, os

quais se solúveis, tornam-se substratos para um novo ciclo formando oligômeros superiores. O processo continua até a formação de polímeros de solubilidade limitada que acabam precipitando da solução (AL-ANSARI et al., 2010).

Figura 1 - Ciclo catalítico das peroxidases.



Fonte: GUZIK et al. (2014).

A fonte comercial mais utilizada de peroxidase é a horseradish peroxidase. Inúmeras fontes desta enzima tais como nabo, rabanete branco, soja, entre outras, têm sido amplamente investigadas na biorremediação de diferentes efluentes industriais com elevada eficiência (GALÁRRAGA et al., 2013; SILVA et al., 2013; STEEVENSZ et al., 2014). Entretanto, é necessária a busca por fontes mais baratas e acessíveis desta enzima, além da utilização de extratos brutos vegetais, que não necessitem de processos onerosos de purificação, os quais podem ser aplicados com a mesma eficiência de enzimas purificadas.

As cascas de grãos de soja são identificadas como uma fonte rica de peroxidase, e são obtidas no processo de extração do óleo de soja, uma vez que é necessário remover o filme de revestimento do grão. Atualmente, uma determinada quantidade dessas cascas é utilizada como fonte de fibras na alimentação de gados, ao passo que uma enorme quantidade é classificada como resíduo (NETO et al., 2013; SILVA et al., 2013). Tendo em vista a concentração elevada

de peroxidase nas cascas de grãos de soja e a excessiva quantidade gerada deste subproduto na industrialização de alimentos (cerca de 200 milhões de toneladas anualmente), o seu emprego como fonte enzimática se torna extremamente atraente do ponto de vista econômico, representando um biocatalisador promissor para utilizações industriais comparado com enzimas comerciais.

Além de cascas de soja, a raiz do nabo é outra fonte vegetal alternativa que possui uma elevada atividade enzimática da peroxidase, podendo ser aplicada sem processos onerosos de purificação. As raízes de nabo são facilmente cultivadas em vários países sendo excelentes fontes de peroxidase, e devido às suas propriedades cinéticas e bioquímicas, apresentam uma alternativa potencial à enzima comercial horseradish peroxidase (AHMEDI et al., 2015). Esta fonte de enzima já foi extensamente estudada em outros trabalhos publicados por nosso grupo de pesquisa tanto na descoloração de corantes têxteis (SILVA et al., 2012a, 2012b, 2012c), quanto na oxidação de compostos fenólicos em águas residuárias do processamento do café (TORRES et al., 2016), podendo ser aplicada como um processo aliado aos tratamentos convencionais.

2.3 Compostos fenólicos em águas residuárias

O aumento da contaminação mundial do sistema hidrográfico com compostos químicos é um dos principais problemas ambientais. A presença de contaminantes orgânicos emergentes em estações de tratamentos de águas residuárias, em águas superficiais e subterrâneas, tornou-se uma questão de constante preocupação nas últimas décadas (BHATNAGAR et al., 2014; GASSER et al., 2014).

Entre os poluentes orgânicos presentes em águas residuárias, a atenção é reportada aos compostos fenólicos, frequentemente consumidos e produzidos por uma ampla variedade de indústrias e consequentemente presentes nos efluentes gerados por estes setores, como nas refinarias de petróleo, fundição de metais, conversão de carvão, indústrias de corantes, plásticos, papel, nas de processamento úmido do café, entre outras (STEEVENSZ et al., 2014; TORRES et al., 2016). Estas substâncias são altamente recalcitrantes, apresentando um elevado grau de toxicidade à saúde humana, a vida aquática e aos microrganismos decompositores da matéria orgânica nos tratamentos biológicos. A exposição aos fenóis pode induzir a mutagenicidade, carcinogenicidade, imunossupressão, desregulações endócrinas, infertilidade, problemas na visão, entre outros (ABUSSAUD et al., 2016; DUAN et al.; 2014).

De acordo com a Resolução nº 430 do CONAMA, de 13/05/2011, os efluentes de quaisquer fontes poluidoras somente poderão ser lançados, direta ou indiretamente, em corpos de água se apresentarem concentrações máximas de fenóis totais de 0,5 mg L^{-1}. Esta resolução dispõe sobre a classificação dos corpos de água e diretrizes ambientais para seu enquadramento, bem como estabelece as condições e padrões de lançamento de efluentes. A realidade dos efluentes industriais é extremamente diferente e expressivamente fora dos padrões de lançamento estabelecidos, ressaltando a necessidade de tecnologias eficientes para o tratamento de águas residuárias contaminadas por estes compostos recalcitrantes.

Um exemplo de efluente contendo elevado teor de compostos fenólicos totais é a água residuária do processamento úmido do fruto do café. O café pode ser processado por via úmida ou via seca. Enquanto no processamento via seca os grãos são secos na íntegra (com casca), na via úmida, utiliza-se uma grande quantidade de água em todas as etapas do processamento. Apesar deste último atribuir uma melhor qualidade ao produto final, o efluente gerado contém uma ampla variedade de compostos orgânicos, tais como cafeína, açúcares e compostos fenólicos, sendo impraticável seu descarte em corpos hídricos receptores sem tratamento prévio (TORRES et al., 2016).

Considerando que o Brasil é o maior produtor e exportador de café, e o segundo maior consumidor do produto, uma considerável quantidade de efluente é gerado a cada dia. Desta forma, estudos que avaliem os vários aspectos relacionados a este tipo de efluente, bem como seu impacto ao meio ambiente e à saúde humana, se tornam indispensáveis para o desenvolvimento de alternativas e processos economicamente viáveis e tecnologicamente sustentáveis para a remediação destas águas residuárias (TORRES et al., 2016).

2.4 Tratamento enzimático de águas residuárias contendo poluentes fenólicos

O tratamento enzimático de águas residuárias contaminadas por substâncias tóxicas, como os compostos fenólicos, é uma alternativa eficiente na remoção destes compostos, e entre as enzimas utilizadas, tem-se a peroxidase. Este biocatalisador atua na oxidação de fenóis com a formação de radicais livres na presença de peróxido de hidrogênio, conforme o ciclo citado anteriormente (Figura 1). Esses radicais formados se difundem do sítio ativo das enzimas para a solução, onde se acoplam formando dímeros, os quais por sua vez, se solúveis tornam-se substratos para um novo ciclo enzimático formando oligômeros superiores. Este processo continua até que os polímeros gerados atinjam uma solubilidade limitada e precipitam da

solução. Estes produtos podem ser facilmente removidos por processos simples, como filtração ou centrifugação (AL-ANSARI et al., 2010).

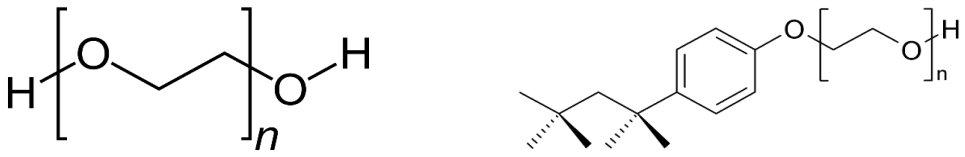
A peroxidase de soja é uma das peroxidases mais empregadas no tratamento de efluentes contaminados em função das suas inúmeras vantagens, como econômica, estabilidade conformacional, baixa suscetibilidade à inibição irreversível por peróxido de hidrogênio, entre outras (AL-ANSARI et al., 2010). Não obstante as vantagens da utilização da peroxidase na biorremediação de águas residuárias, este processo apresenta desvantagens: (1) os radicais livres gerados durante o ciclo catalítico podem reagir com a enzima inibindo-a e (2) a enzima pode adsorver os produtos poliméricos formados levando a co-precipitação e obstruindo o sítio ativo. O fenômeno predominante em determinada situação dependerá das condições reacionais como, concentração da enzima, do substrato, entre outros (KIM; CHAE; CHU, 2007; KLIBANOV et al., 1983; NAKAMOTO; MACHIDA, 1992; TORRES et al., 2016; WRIGHT; NICELL, 1999).

2.5 Estabilidade enzimática no meio reacional

2.5.1 Efeito protetor de aditivos químicos na oxidação de compostos fenólicos catalisada por peroxidase

A inibição das enzimas pelos processos descritos anteriormente é muitas vezes atenuada pela adição de polímeros solúveis no meio reacional, conhecidos como “polímeros de sacrifício”, a exemplo do polietilenoglicol (PEG), proporcionando uma maior eficiência no tratamento enzimático. Na presença deste polímero a peroxidase é retida na fase aquosa, evitando sua co-precipitação com os produtos poliméricos, devido a maior afinidade destes produtos com os aditivos (MAO et al., 2013). A adição de surfactantes como o triton X-100 também tem sido pesquisado, uma vez que estes aditivos aumentam ou estabilizam a atividade enzimática por diminuírem as interações entre a enzima e os produtos da polimerização oxidativa. Além disso, as interações entre a enzima e o surfactante podem induzir uma mudança na conformação ou no sítio ativo da enzima, alterando desta forma a estabilidade enzimática (STEEVENSZ et al., 2014; ZHANG et al., 2012). A fórmula química de ambos os aditivos está apresentada na Figura 2.

Figura 2 – Fórmula química do polietilenoglicol (a) e triton X-100 (b).

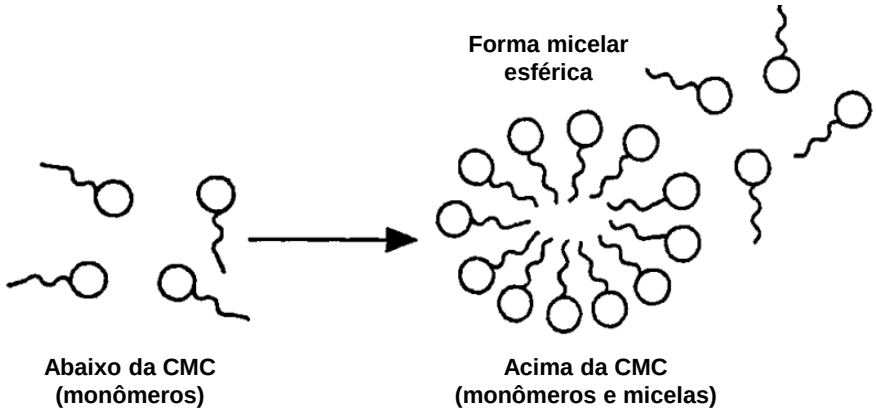


(a) **(b)**

Fonte: SIGMA-ALDRICH (2017).

Os surfactantes são moléculas anfifílicas que contém uma unidade polar hidrofílica (cabeça) e uma cadeia hidrofóbica (cauda). São solúveis em água, mas em concentrações elevadas as moléculas se associam para formar micelas (Figura 3). A concentração em que este fenômeno ocorre é conhecida por concentração micelar crítica (CMC), que é sensível a mudanças no sistema de solvente. O estado dos surfactantes em solução depende de vários fatores, como a natureza do surfactante, concentração, temperatura da solução, entre outros (GOLDFEDER; FISHMAN, 2014; HAMED, 2014, p. 303).

Figura 3 – Formação do agregado micelar.



Legenda: CMC = concentração micelar crítica.
Fonte: MANIASSO (2001, p. 87).

Algumas explicações sugerem o efeito dos surfactantes na conservação da atividade enzimática. Assim como o PEG, acredita-se que esses aditivos se associam com os produtos poliméricos formados durante a reação impedindo a adsorção dos mesmos às enzimas. Este

fenômeno pode ser explicado pelo particionamento dos produtos formados nas micelas do surfactante ou, alternativamente, pelo particionamento dos monômeros destes surfactantes nos agregados do produto, alterando a sua característica de superfície de modo que a enzima não adsorva mais (AL-ANSARI et al., 2010). Portanto, o efeito promovido pelo surfactante dependerá da sua forma de organização.

Várias pesquisas buscam uma maior estabilidade das enzimas no meio reacional, principalmente quando o objetivo é aplicá-las em áreas de remediação ambiental, uma vez que estarão sujeitas a um ambiente inóspito em que inúmeros contaminantes poderão estar presentes, e a complexidade da poluição pode implicar em efeitos diversos sobre a atividade enzimática. Além da incorporação de aditivos químicos no meio reacional, tais como o PEG e surfactantes, a imobilização de enzimas é uma alternativa que além de aumentar a estabilidade do biocatalisador, facilita sua remoção do meio reacional e permite seu reuso.

2.5.2 Imobilização de enzimas

Em aplicações ambientais e/ou industriais, o ideal é que os biocatalisadores sejam heterogêneos, razoavelmente estáveis sob condições que possam estar na maioria das vezes muito longe do seu ambiente fisiológico e capazes de manter uma boa atividade e seletividade quando atuarem com substratos que são, em alguns casos, diferentes dos seus substratos fisiológicos (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011). Essas características são desejáveis para a produção de biocatalisadores mais eficientes visando avanços substanciais em diferentes processos catalíticos.

A imobilização de enzimas em suportes sólidos é uma estratégia potencial para o aumento da estabilidade do biocatalisador, uma vez que prolonga sua vida útil, proporciona uma melhora na especificidade, além de permitir seu reuso e uma fácil separação dos produtos finais, minimizando ou eliminando a contaminação do biocatalisador. Isso significa que a imobilização não é uma etapa adicional na biocatálise, mas sim um passo necessário (BILAL et al., 2017; BOLIVAR; EISL; NIDETZKU, 2016; RUEDA et al., 2016). Sendo assim, estratégias para fixação de biomoléculas em algum suporte mantendo a sua atividade, conformação e funcionalidade é uma evolução no campo da pesquisa (BARBOSA et al., 2013; DATTA et al., 2013; GALÁRRAGA et al., 2013; GUNDA et al., 2014; SHELDON; PELT, 2013).

O termo “enzimas imobilizadas” refere-se a enzimas confinadas ou localizadas em outra fase (matriz ou suporte) podendo ser reutilizadas (DATTA et al., 2013; MOHAMAD et al., 2015). A aplicação de enzimas imobilizadas tem sido muito relatada, particularmente no campo das tecnologias ambientais, envolvendo o tratamento de águas residuárias, purificação de água, conversão de biomassa, recuperação de recursos, além de aplicações relacionadas a outras áreas, tais como na construção de biossensores, na administração de fármacos, diagnóstico clínico, biocélulas de biocombustíveis, entre outros (AMMANN et al., 2014; MAGNER, 2013, p. 6213; OGORZALEK et al., 2015). Portanto, a imobilização desempenha um papel significativo na relação custo-benefício, no aumento da eficiência do catalisador e na simplificação dos processos operacionais (JAIN et al., 2016).

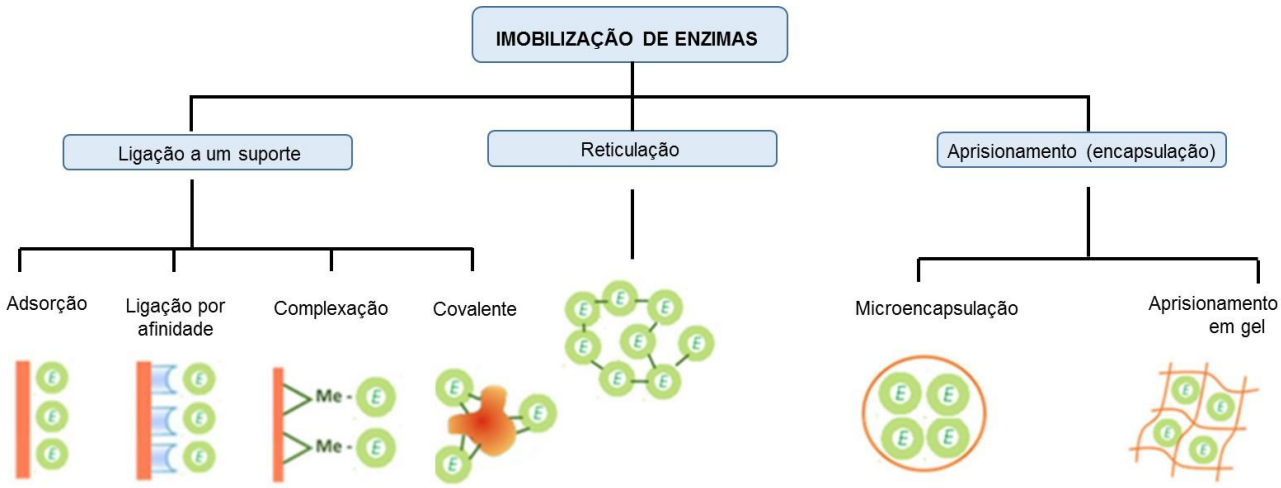
Os diferentes métodos de imobilização exploram o fato de que as proteínas possuem vários aminoácidos com características distintas, nos quais seus grupos funcionais das cadeias laterais podem estar diretamente envolvidos nas ligações e interações com o suporte (MOHAMAD et al., 2015). Estes diferentes procedimentos produzem algumas alterações na estrutura da enzima e na sua mobilidade em geral, o que pode conduzir em alterações nas propriedades intrínsecas das mesmas, como atividade, especificidade ou estabilidade. Estes efeitos podem ser imprevisíveis dependendo da área da enzima envolvida na imobilização, do tipo de imobilização, da intensidade da ligação (nos casos de imobilização química), entre outros (BARBOSA et al., 2013, 2015; CARLSSON et al., 2014). Apesar das inúmeras aplicações de enzimas imobilizadas, a compreensão de como este biocatalisador interage com a superfície dos suportes a nível molecular ainda é um grande desafio (OGORZALEK et al., 2015).

Ao contrário da estabilidade enzimática que é frequentemente favorecida após o processo de imobilização, a enzima imobilizada é geralmente menos ativa comparada com a enzima livre, o que pode ser atribuído às distorções estruturais na enzima resultantes da sua ligação a uma superfície sólida e/ou devido às reações enzimáticas estarem ocorrendo em um ambiente heterogêneo (BOLIVAR; EISL; NIDETZKU, 2016).

Inúmeras técnicas são empregadas para imobilizar enzimas em suportes sólidos, principalmente baseadas em mecanismos químicos e físicos. Os métodos físicos envolvem o aprisionamento de moléculas de enzima dentro de uma matriz porosa, enquanto os químicos incluem ligação da enzima a uma matriz por ligações covalentes, ligação da enzima com o auxílio de reagentes multifuncionais ou bifuncionais, entre outros (DAOUD et al., 2010;

DATTA et al., 2013). Sendo assim, existem basicamente três categorias para imobilização de enzimas: ligação a um suporte, reticulação e aprisionamento (Figura 4).

Figura 4 - Diferentes métodos de imobilização de enzimas.



Fonte: Adaptado de VAGHARI et al. (2016).

Talvez o procedimento mais simples para imobilizar a enzima seja por adsorção. Esse tipo de imobilização depende da estrutura da enzima e do suporte, e da interação entre ambos. Uma vez estabelecidas as condições de imobilização (força iônica, pH e temperatura), a imobilização é possível, geralmente em poucos minutos.

A adsorção é o procedimento de imobilização mais antigo, fácil e de baixo custo, governado pela interação das partes funcionais da cadeia da enzima com a superfície do suporte por ligações de hidrogênio, interações eletrostáticas e forças de Van der Waals. Uma das vantagens desta técnica é que normalmente a funcionalização da superfície do suporte não é necessária, não havendo a necessidade de outros reagentes, o que reduz extremamente o custo de todo processo. Além disso, a ligação entre a enzima e a matriz é fraca comparada com a imobilização química, o que torna este processo interessante, uma vez que a estrutura nativa da enzima não é alterada. Isto impede que o sítio ativo do biocatalisador sofra alguma perturbação permitindo que a enzima mantenha sua atividade catalítica. Outro benefício deste tipo de imobilização é a facilidade de regeneração do suporte pela remoção da enzima inativa e a possibilidade de recarregá-lo com uma nova alíquota do biocatalisador ativo (ABUSSAUD et

al., 2016; ALKAN et al., 2009; HARTMANN; KOSTROV, 2013; JESIONOWSKI et al., 2014; MOHAMAD et al., 2015; NISHA et al., 2012).

Apesar das inúmeras vantagens deste método de imobilização, a adsorção pode apresentar uma limitação relacionada com a possibilidade de lixiviação da enzima, principalmente durante o processo de lavagem e operação (EŞ; VIEIRA; AMARAL, 2015). Uma alternativa para evitar ou minimizar este fenômeno é variar as condições de imobilização, o que pode favorecer a interação enzima-suporte. Ainda assim, esta técnica é regularmente utilizada em processos de larga escala, especialmente quando a enzima é mais acessível (BRADY; JORDAAN, 2009; HARTMANN; KOSTROV, 2013).

Entre os vários aspectos importantes no processo de imobilização de enzimas, a seleção do suporte é um parâmetro crucial para a obtenção de um biocatalisador imobilizado eficiente. O suporte deve acomodar a enzima e protegê-la contra a desnaturação garantindo assim a sua estabilidade. A eficiência de uma enzima imobilizada depende diretamente do suporte e da maneira como a enzima se liga a ele. O ideal é que a matriz empregada tenha uma elevada afinidade com a enzima, uma estrutura química adequada que proporcione uma máxima atividade enzimática e um bom contato com o biocatalisador, seja termodinamicamente estável, resistente à contaminação e ao ataque microbiano, tenha grupos funcionais reativos, além de estar disponível a baixo custo (DAOUD et al., 2010; JAIN et al., 2016; KANDASAMY et al., 2010; MOHAMAD et al., 2015; SANTOS et al., 2015).

Embora não haja um suporte universal ideal para a imobilização de todas as enzimas, inúmeras pesquisas têm sido feitas empregando diferentes suportes levando-se em consideração as características enzimáticas e suas aplicações. Entre estes suportes estão matrizes a base de sílica, resinas acrílicas, polímeros sintéticos, óxidos metálicos, colágeno, quitosana, agarose, vidro poroso tratado, membranas ativas, entre outras. Apesar das vantagens de cada suporte, algumas limitações relacionadas às tecnologias necessárias para fixação da enzima no suporte acabam elevando o custo de produção destes biocatalisadores (EŞ; VIEIRA; AMARAL, 2015; MOHAMAD et al., 2015).

Materiais porosos apresentam características favoráveis para imobilização de enzimas. Uma área de superfície interna relativamente alta de um suporte poroso pode fornecer um refúgio seguro para as enzimas, onde elas são capazes de tolerar valores de pH mais extremos, temperaturas elevadas e altas concentrações de sais. Entretanto, o diâmetro do poro deve ser suficientemente grande para acomodar o biocatalisador. Caso contrário, a adsorção ocorrerá apenas na superfície externa, anulando a vantagem da presença de poros (CARLSSON et al.,

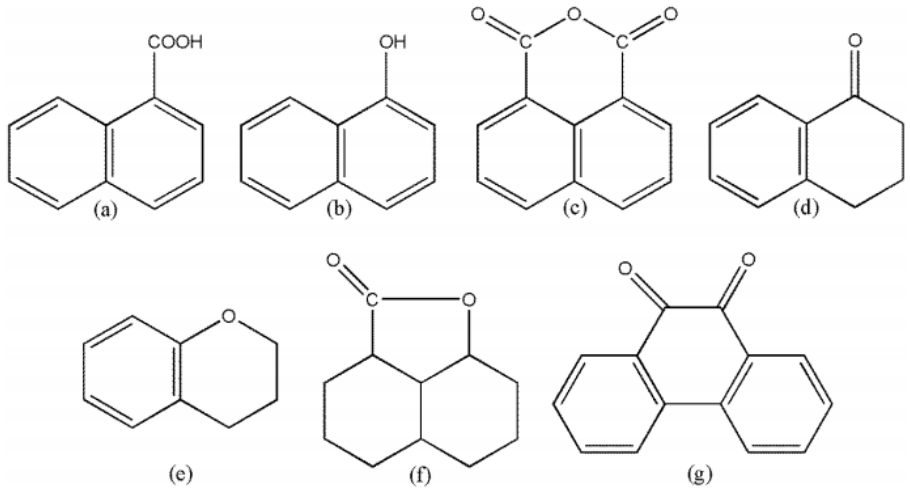
2014; MAGNER, 2013, p. 6213). Entre estes materiais tem-se o carvão ativado, um sólido amorfo, hidrofóbico que além dessas características possui uma forte capacidade e eficiência de adsorção, boas propriedades mecânicas e não são tóxicos (ABUSSAUD et al., 2016; DAOUD et al., 2010; JAIN et al., 2016; MOHAMAD et al., 2015; RAMANI et al., 2010, 2012; ZHENG; JIANG, 2014). A imobilização de enzimas neste tipo de suporte é rápida, fácil de manusear, de baixo custo e estável contra a desnaturação (SARANYA et al., 2015). Uma vez que a maioria dos processos químicos faz uso de um catalisador, o carvão ativado com elevada área superficial e uma ampla gama de grupos funcionais superficiais pode ser facilmente utilizado como um suporte para outros novos tipos de catalisadores (MENDES et al., 2015).

2.6 Carvão ativado e precursores agroindustriais

Carvão ativado é um termo comum utilizado para descrever materiais baseados em carbono que possuem estrutura de poros interna bem desenvolvida. As características típicas de um carvão ativado tais como elevada área superficial, grande porosidade, bem como um amplo espectro de grupos funcionais presentes em sua superfície faz deste material extremamente versátil com inúmeras possibilidades de aplicações em diferentes áreas, tais como na medicina, como suporte de catalisadores, materiais para eletrodo, filtros de ar, entre outras (ABUSSAUD et al., 2016; BHATNAGAR et al., 2013; MANEERUNG et al., 2016; MENDES et al., 2015).

As características químicas de carvões ativados são geralmente determinadas por certo grau de heterogeneidade química de sua superfície, que está diretamente relacionada com a presença de heteroátomos, ou seja, átomos que estão presentes na estrutura de carbono diferentes destes, como o oxigênio, nitrogênio, enxofre e fósforo. O tipo e a quantidade destes elementos são derivados da natureza do material precursor ou introduzidos durante o processo de ativação, do tratamento térmico ou tratamento pós-químico e geralmente estão sob a forma de grupos funcionais e/ou átomos quimicamente ligados na estrutura. Os grupos funcionais incluem carboxilas, carbonilas, fenóis, lactonas, quinonas, entre outros (Figura 5), e as propriedades de adsorção podem ser significativamente influenciadas por estes grupos (BHATNAGAR et al., 2013; SHAFEEYAN et al., 2010).

Figura 5 – Possíveis estruturas dos grupos funcionais contendo oxigênio presentes nas superfícies dos carvões ativados: grupos carboxílicos (a); grupos fenólicos (b); anidridos carboxílicos (c); grupos carbonila (d); grupos éter (e); lactonas (f); quinonas (g).



Fonte: ROVANI (2015).

Tendo em vista a vasta gama de aplicações de carvões ativados espera-se que a procura mundial aumente drasticamente (aproximadamente 8,1% por ano). China e Estados Unidos serão os dois principais mercados consumidores que impulsionarão fortemente a demanda por este material, o que levará consequentemente, a um aumento do custo do carvão ativado. O custo deste material acaba inviabilizando seu uso como adsorvente, principalmente em aplicações relacionadas ao controle da poluição (BRITO et al., 2017; DEMIRBAS, 2009, p. 1; MANEERUNG et al., 2016).

O carvão ativado pode ser produzido utilizando uma variedade de materiais ricos em carbono, como madeira, casca de coco, caroço de tâmara, sabugo de milho, casca de arroz, casca de soja, resíduos do café, entre outros. O custo do precursor e do material adsorvente é um dos fatores mais importantes e cruciais para se determinar a viabilidade econômica do processo, e alguns parâmetros como disponibilidade, forma (natural, resíduos agroindustriais ou produtos sintetizados), o processamento requerido, as condições de tratamento, são os responsáveis por este custo (BHATNAGAR et al., 2013; DEMIRBAS, 2009, p. 1; DURSUN et al., 2013; GONÇALVES et al., 2013).

O Brasil, por apresentar uma economia basicamente agrária, gera cerca de 597 milhões de toneladas de resíduos agroindustriais anualmente, cujo processamento seria extremamente vantajoso do ponto de vista econômico e ambiental. Nos dias atuais, uma grande quantidade de resíduos lignocelulósicos são descartados, simplesmente queimados ou depositados em aterros, constituindo um grave problema ambiental, relacionados à contaminação da água e do solo, além da poluição atmosférica (ANGIN, 2014, p. 259; GONÇALVES et al., 2013; MENDES et al., 2015). Desta forma, agregar valor a estes resíduos gerados em abundância, além de reduzir os problemas ambientais relacionados com a sua disposição, é o objetivo de diversas tecnologias propostas atualmente, e entre elas, a produção de carvão ativado, um material que pode ser empregado em diversos ramos das indústrias química, alimentícia e farmacêutica.

Além dos resíduos agroindustriais citados, a casca de soja, um resíduo lignocelulósico gerado em grande abundância na industrialização de alimentos, é um candidato promissor para produção de carvão ativado. Entre os resíduos agroindustriais gerados anualmente no Brasil, estão cerca de 200 milhões de toneladas de cascas de soja, as quais geralmente são queimadas para fins energéticos ou parcialmente coletadas para uso como ração animal (MENDES et al., 2015; MIAO et al., 2013). Verifica-se ainda que resíduos agroindustriais apresentam elevado teor de carbono e um baixo conteúdo de cinzas, os que os tornam excelentes precursores para síntese de carvão ativado (ZHOU; ZHANG; CHENG, 2015). Neste contexto, no presente projeto, as cascas de grão de soja serão utilizadas para obtenção da enzima peroxidase, e o resíduo resultante será empregado como precursor carbonáceo na preparação de carvão ativado.

2.6.1 Preparação de carvão ativado

Basicamente existem dois métodos para a produção de carvão ativado, a ativação química e a física. A ativação química envolve a impregnação da matéria prima com agentes ativantes, que podem ser o ácido fosfórico, hidróxido de potássio, hidróxido de sódio, cloreto de zinco, entre outros. Estes impregnantes são utilizados como agentes desidratantes e oxidantes que promovem a decomposição do material carbonáceo, induzindo a carbonização e aromatização do carbono, inibem a formação de alcatrão, aumentando desta forma o rendimento de carbono (LI et al., 2011; MIAO et al., 2013). O ácido fosfórico e o cloreto de zinco são os agentes de ativação mais comumente empregados para a obtenção de carvão mesoporoso (JAIN et al., 2014). Após a impregnação com agentes ativantes uma estrutura de poros é criada por

uma ativação térmica e, portanto, neste tipo de ativação a carbonização e a ativação são realizadas em uma única etapa (CHEN et al., 2011; LIU et al., 2010).

A ativação física por sua vez consiste de duas etapas, a carbonização de um precursor carbonáceo sob atmosfera inerte e, em seguida, a ativação do produto resultante em temperatura mais elevada com agentes ativantes como, CO_2, vapor d'água, ar ou a mistura desses. No entanto, a temperatura geral de carbonização é mais elevada, e um pouco de carbono é perdido. Além disso, o processo de ativação por CO_2 exige uma temperatura mais elevada do que o vapor d'água, pois o CO_2 possui menor reatividade devido ao seu maior tamanho molecular, o que impede a sua difusão através da rede porosa (LI et al., 2011).

A ativação química geralmente apresenta um maior rendimento, menor tempo de ativação, uma estrutura de poros mais desenvolvida, além de utilizar uma temperatura menor em todo o processo comparada com a ativação física (BRITO et al., 2017; CHEN et al., 2011; LIU et al., 2010). Apesar das inúmeras vantagens deste método, algumas desvantagens estão associadas ao mesmo, entre elas, a necessidade de uma etapa de lavagem do material obtido, um passo laborioso em função do número de lavagens necessárias para remover completamente o agente ativante, além de resíduo que é gerado durante esta etapa de lavagem (FU et al., 2013; LIM et al., 2010). Um diagrama geral dos dois métodos de produção de carvão ativado está representado na Figura 6.

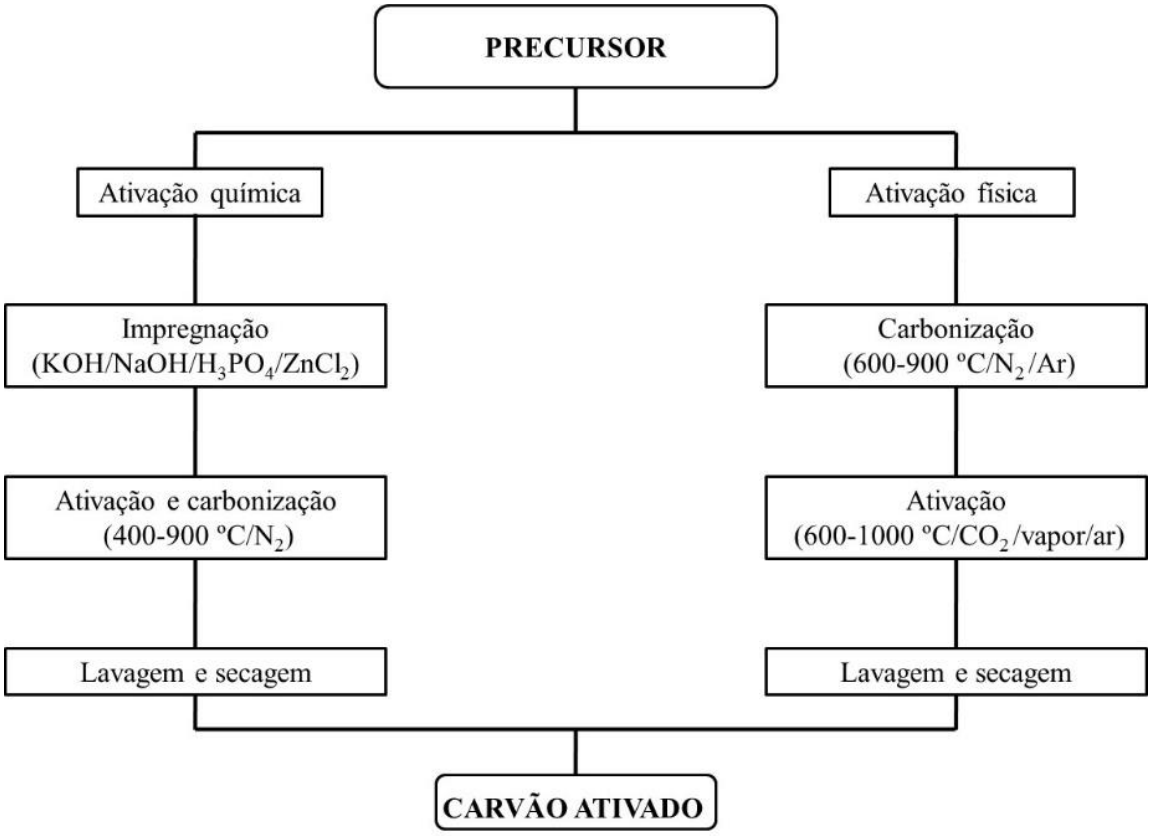
2.7 Compósitos de carvão ativado/óxido de ferro como suportes magnéticos

Apesar das inúmeras vantagens da utilização do carvão ativado como suporte para a imobilização de enzimas, sua aplicação é prejudicada devido à dificuldade de recuperação deste material do meio reacional, principalmente na forma de pó, além da baixa capacidade de reutilização. O método de filtração comumente utilizado causa bloqueio dos filtros e perda do carvão ativado (LI, et al., 2017; MAO et al., 2016; PARK et al., 2015). Além da filtração, outras técnicas convencionais tais como centrifugação, sedimentação e leitos fixos também são limitadas em função do custo operacional e de manutenção, bem como devido as consideráveis quedas de pressão que podem destruir o equipamento (MULIWA et al., 2016, p. 250).

A introdução de nanopartículas magnéticas (Fe_3O_4 e γ-Fe_2O_3) à materiais adsorventes tem atraído a atenção de diversos pesquisadores e de certos setores da indústria (LI et al., 2017). Esse tipo de material pode ser utilizado como adsorvente de contaminantes aquosos ou gasosos que, após adsorção, pode ser recuperado pelo uso de um campo magnético externo, sem a

necessidade de etapas tediosas de separação, permitindo também menos consumo de energia e perda do catalisador. Durante o processo de preparação de compósitos, suportes que contenham grupos hidroxilas abundantes em sua estrutura favorecem uma forte interação com os íons de ferro, devido principalmente a efeitos intermoleculares, tais como interações eletrostáticas e forças de Van der Waals (LI et al., 2017). Desta forma, os carvões ativados são considerados bons suportes para vários óxidos metálicos, em função das suas notáveis propriedades, principalmente devido aos vários grupos funcionais superficiais (OH et al., 2015).

Figura 6 - Métodos de preparação de carvão ativado.



Fonte: Adaptado de CHEN et al. (2011).

São reconhecidos universalmente como óxidos de ferro aqueles compostos formados por FeO e FeOH. Estes compostos são muito abundantes na crosta terrestre e de baixo custo, além de serem facilmente sintetizados em laboratório. Na maioria deles, o ferro está no estado trivalente, porém, alguns contêm Fe (II) na sua estrutura: FeO, $Fe(OH)_2$ e Fe_3O_4 (CASTRO et al., 2009; CORNELL; SSHWERTMANN, 2003).

Entre os vários tipos de óxidos de ferro existentes, a magnetita (Fe_3O_4) exibe excelentes propriedades físico-químicas, devido a presença de Fe (II) e Fe (III) em sua estrutura. A incorporação de magnetita na estrutura de carvão ativado tem sido foco de vários grupos de pesquisa, uma vez que se combinam as propriedades magnéticas destes óxidos com as adsortivas dos carvões ativados, com diversas aplicações, tais como materiais adsorventes (tanto para propósitos ambientais como em análise química), catalisadores em processos de oxidação avançada e como suporte para imobilização de enzimas (LIMA, 2013, p. 344; SU, 2017, p. 48).

Existem vários métodos de síntese da magnetita, tais como a decomposição térmica e/ou redução, síntese hidrotermal e a co-precipitação. Entre estes, a co-precipitação é um método simples mais utilizado, na qual soluções de bases são adicionadas às soluções aquosas de sais de Fe^{2+}/Fe^{3+} sob atmosfera inerte a temperatura ambiente ou a uma temperatura elevada. O tamanho, forma e composição das nanopartículas magnéticas depende do tipo de sal utilizado (por exemplo, cloretos, nitratos, sulfatos), a razão Fe^{2+}/Fe^{3+}, a temperatura da reação, valor do pH e da força iônica do meio, entre outros (MUNOZ et al., 2015; SU, 2017, p. 48).

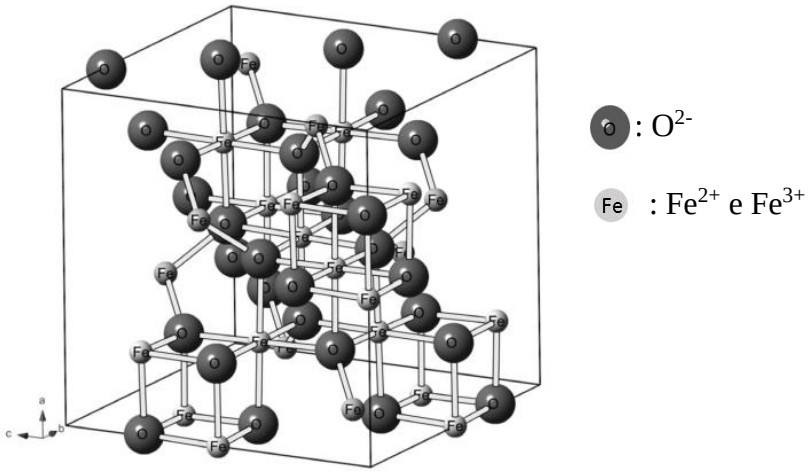
Tendo em vista a simplicidade do método de co-precipitação para síntese de nanopartículas, este também é o mais utilizado para combinar as propriedades do carvão ativado com partículas magnéticas na síntese de compósitos. Comparado com outros métodos existentes, tais como impregnação, tratamento térmico, impregnação de solução e método hidrotermal, este não requer produtos químicos e procedimentos especiais (KYZAS; DELIYANNI; LAZARIDIS, 2014; XU et al., 2014, p. 622).

A magnetita (Fe_3O_4) é um óxido de ferro misto com FeO e Fe_2O_3, um espinélio invertido em sua estrutura cristalina, com os íons O^{2-} coordenados tanto a íons Fe^{2+} e Fe^{3+}, nos interstícios octaédricos, quanto a íons Fe^{3+} em interstícios tetraédricos (Figura 7) (SCHWERTMANN; CORNELL, 2000). Como os íons Fe^{3+} são divididos igualmente entre as posições tetraédricas e octaédricas, não existe momento magnético resultante de presença destes íons. Entretanto, todos os íons Fe^{2+} residem nos interstícios octaédricos, sendo estes íons responsáveis pela magnetização de saturação ou, ainda, pelo comportamento magnético do material (OLIVEIRA; FABRIS; PEREIRA, 2013).

Li et al. (2017) sintetizaram nanoesferas de carvão mesoporoso magnético contendo ferro para remoção de cromo (VI) em solução aquosa. De acordo com estes autores, quando as nanoesferas de carvão mesoporoso são dispersadas na solução de Fe^{3+}, o material mesoporoso contendo vários grupos hidroxilas acabam atraindo facilmente o ferro devido a efeitos

intermoleculares. O material sintetizado apresentou considerável capacidade de adsorção de cromo (VI) de 87 mg g^{-1}, com excelentes propriedades magnéticas, as quais auxiliam na separação do adsorvente do meio reacional.

Figura 7 – Estrutura cristalina da magnetita.



Fonte: OLIVEIRA, FABRIS, PEREIRA (2013).

Já Zhou et al. (2015), sintetizaram carvão magnético utilizando resíduos de cascas de amendoim. O compósito sintetizado foi utilizado como catalisador de reações do tipo Fenton heterogêneo para remoção de azul de metileno com o auxílio de persulfato. Os autores encontraram uma eficiência de 90% de degradação e uma boa estabilidade operacional do catalisador, o qual manteve atividade após sete ciclos.

Liu et al. (2012) sintetizaram um compósito magnético mesoporoso baseado em carbono para imobilização da lacase. Este material apresentou uma elevada capacidade de adsorção de 491,7 mg g^{-1} e uma rápida taxa de remoção de fenol e p-clorofenol, 78% e 84% respectivamente, com uma contribuição de 20% de adsorção por parte do suporte mesoporoso. Além disso, os autores encontraram uma maior estabilidade frente ao pH e temperatura do biocatalisador imobilizado comparado à enzima livre.

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3 CONSIDERAÇÕES FINAIS

A biocatálise consiste de um processo catalítico no qual são utilizadas enzimas ou células como catalisadores. Tendo em vista que a biocatálise desempenha papel de suma importância na concepção de processos sustentáveis em química sintética e na gestão de resíduos, o presente trabalho teve por objetivo o desenvolvimento de biocatalisadores a partir de processos simples visando a mínima formação de resíduos, bem como a avalição da imobilização e da adição de aditivos químicos na estabilidade destes biocatalisadores. Em termos práticos, procedeu-se a aplicação do catalisador na degradação de compostos fenólicos da água residuária do processamento do café (ARC), cujos efeitos ambientais em decorrência da geração de resíduo líquido são cada vez mais crescentes.

As enzimas utilizadas nesse trabalho são enzimas oxidativas, a saber, peroxidases, as quais foram obtidas do nabo e de cascas de grãos de soja, sendo esse último resíduo agroindustrial gerado em abundância no beneficiamento da soja. Tendo em vista o maior desempenho catalítico da peroxidase de soja no decorrer do trabalho, procedeu-se aos estudos de imobilização da mesma. Ressalta-se ainda, o virtuoso ciclo de produção desse biocatalisador, uma vez que o próprio resíduo sólido gerado na extração da peroxidase de soja foi utilizado como precursor carbonáceo para a síntese do carvão ativado utilizado como suporte de imobilização da enzima.

As enzimas na sua forma livre apresentaram elevada capacidade de degradação de compostos fenólicos padrões e razoável remoção de fenóis da ARC. Diante desses resultados, ambas enzimas podem ser utilizadas na remediação de diferentes efluentes contendo substâncias tóxicas.

Em relação a adição de PEG e triton X 100, os quais impedem a inibição da enzima ao evitar o contato do catalisador com os produtos poliméricos formados durante a degradação, observou-se que estes exerceram efeito significativo apenas para soluções padrões de fenólicos, enquanto que para a ARC a adição desses químicos é dispensável. Este resultado é relevante, principalmente do ponto de vista econômico, uma vez que reagentes adicionais não são necessários durante o processo de degradação de compostos fenólicos utilizando biocatalisadores.

O resíduo sólido recolhido no processo de extração da peroxidase de soja foi eficientemente empregado como precursor carbonáceo para síntese de carvão ativado, e apresentou uma excelente área superficial específica de 1.603 m^2 g^{-1}. O reaproveitamento do

mesmo ressalta-se como uma alternativa economicamente viável com aproveitamento máximo da biomassa, agregando valor a este resíduo e contornando problemas relacionados com a disposição inadequada de resíduos no meio ambiente, além de ser uma alternativa aos carvões comerciais utilizados. O carvão ativado sintetizado foi utilizado como suporte para imobilização da peroxidase de soja. Com intuito de favorecer as interações enzima-suporte os parâmetros de imobilização foram variados permitindo alcançar 100% de imobilização da enzima nesse suporte. O biocatalisador utilizado pode ser reutilizado por vários ciclos.

Na biocatálise homogênea a reutilização do biocatalisador é impossível, uma vez que enzima, substrato e produtos estão na mesma fase. Desta forma, a imobilização é uma técnica promissora, pois permite a recuperação do biocatalisador imobilizado, além da possibilidade de reutilização do mesmo. O carvão ativado obtido neste trabalho foi um excelente suporte para imobilização da peroxidase e o processo de imobilização por adsorção mostrou-se altamente eficaz.

Apesar das inúmeras vantagens da utilização do carvão ativado como suporte para a imobilização de enzimas, sua aplicação é prejudicada devido à dificuldade de recuperação deste material do meio reacional, principalmente na forma de pó. Nesse contexto, a síntese de um compósito magnético foi uma alternativa eficaz, atribuindo propriedades magnéticas ao carvão ativado. Em sinergismo com a propriedade magnética, a característica adsortiva do carvão ativado contribui significativamente para o processo de imobilização de enzimas, facilitando a recuperação do biocatalisador imobilizado e possibilitando a aplicação do mesmo em diferentes campos da pesquisa. Neste adsorvente magnético a peroxidase de soja também obteve 100% de imobilização após a determinação dos parâmetros ótimos, e foi aplicada em 11 ciclos consecutivos.

Portanto, as peroxidases são biocatalisadores extremamente eficientes podendo ser facilmente imobilizadas nos materiais sintetizados. A obtenção dos suportes utilizados constitui uma rota limpa de produção com aproveitamento máximo da biomassa e geração mínima de resíduos, e os biocatalisadores imobilizados são passíveis de serem aplicados em diversas reações de cunho ambiental, em diagnósticos clínicos, produção de biossensores e em diversas outras aplicações biotecnológicas.

Em trabalhos futuros pretende-se efetuar um estudo comparativo da performance catalítica das enzimas na forma livre e imobilizada, bem como a aplicação dos catalisadores suportados em carvão ativado puro ou magnético na degradação de compostos fenólicos.

SEGUNDA PARTE - ARTIGOS

No primeiro artigo, intitulado **Evaluation of the protective effect of chemical additives in the oxidation of phenolic compounds catalysed by peroxidase**, avaliou-se o potencial da peroxidase de nabo e de soja na oxidação de compostos fenólicos, entre eles o fenol, ácido clorogênico e o ácido cafeico na presença de polietilenoglicol e triton X-100 em diferentes concentrações. O efeito protetor destes aditivos químicos também foi avaliado na oxidação da água residuária do processamento do café por peroxidase de soja.

No segundo artigo, intitulado **Novel eco-friendly biocatalyst: soybean peroxidase immobilized onto activated carbon obtained from agricultural waste**, investigou-se a imobilização da peroxidase de soja em carvão ativado sintetizado utilizando o resíduo proveniente da extração da enzima, com aproveitamento máximo da biomassa e geração mínima de resíduos. Os materiais obtidos foram caracterizados por diferentes técnicas e o biocatalisador imobilizado reutilizado por 10 ciclos.

No terceiro artigo, **Development of a reusable and sustainable biocatalyst by immobilization of soybean peroxidase onto magnetic adsorbent**, sintetizou-se um compósito adsorvente magnético. A peroxidase de soja foi imobilizada neste suporte facilitando sua recuperação do meio reacional com a simples aplicação de um campo magnético externo dispensando processos tediosos de separação. Os materiais obtidos foram caracterizados e a peroxidase de soja imobilizada foi reutilizada por 11 ciclos com uma excelente performance.

Além dos três artigos referidos acima, este trabalho de tese também gerou uma patente intitulada "**Processo de obtenção de carvão enzimático magnético a partir de resíduo agroindustrial para propósitos ambientais**", de titularidade da UFLA, depositada junto ao INPI em 19/10/2016, sob o n° BR 10 2016 024396 3.

ARTIGO 1 - Evaluation of the protective effect of chemical additives in the oxidation of phenolic compounds catalysed by peroxidase

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ABSTRACT

The use of oxidoredutive enzymes in removing organic pollutants has been the subject of much research. The oxidation of phenolic compounds in the presence of chemical additives has been the focus of this study. In this investigation, the influence of the additives polyethylene glycol and Triton X-100 was evaluated in the phenol oxidation, caffeic acid, chlorogenic acid and total phenolic compounds present in coffee processing wastewater (CPW) at different pH values, performed by turnip peroxidase and peroxidase extracted from soybean seed hulls. The influence of these additives was observed only in the oxidation of phenol and caffeic acid. In the oxidation of other studied phenolic compounds, the percentage of oxidation remained unchanged in the presence of these chemical additives. In the oxidation of CPW in the presence of additives, no change in the oxidation of phenolic compounds was observed. Although several studies show the importance of evaluating the influence of additives on the behaviour of enzymes, this study found a positive response from the economic point of view for the treatment of real wastewater, since the addition of these substances showed no influence on the oxidation of phenolic compounds, which makes the process less costly.

Keywords: Biocatalysis; peroxidases; phenolic compounds; polyethylene glycol; triton X-100; wastewater.

1 Introduction

The CONAMA Resolution Nº 430 from 05/13/2011 states that any effluents from polluting sources can only be released directly or indirectly into water bodies if their maximum phenol concentration is 0.5 mg L^{-1}.[1] This concentration is much lower than those found in wastewater of many industries such as petroleum refining, coal conversion, wood preservation, metal casting, pulp and paper, dyes, adhesives, resins, plastics, textile manufacturing [2] and notably in coffee processing wastewater (CPW).[3] The increasing presence of phenolic compounds in wastewater coming from these industries represents an important environmental toxicity hazard.[4] However, the toxicity and environmental impact of these compounds and their derivatives can vary depending upon the number, type and position of substituent groups on the phenol ring.[5]

The most used method for removal of phenolic compounds from wastewater is biological treatment, [6] due to lower costs and possibility of complete mineralization.[7] Despite being an effective method, the inhibitory and toxic nature of phenolic compounds at high concentrations, prohibits further enhancement of organic load with desired performance. Industrial effluents generally contain phenolic compounds at such concentrations, which are inhibitory to the bioactivity of microbes in biological systems.[8]

Enzyme-based treatments represent an alternative method for removing phenols from wastewater and it has been shown that peroxidases are able to react with aqueous phenolic compounds and form non-soluble materials that could be easily removed from the aqueous solution by filtration, sedimentation or centrifugation.[2,9] Peroxidases from several sources have been explored on a large scale including bitter gourd, soybean, turnip and horseradish.

Although the peroxidase-mediated oxidative coupling process has an enormous potential for the remediation of aqueous solutions contaminated by phenolic compounds, its application is hampered by the low operational stability of peroxidase as a result of enzyme inactivation, which increases treatment costs. Klibanov and coworkers [10] suggested that this inactivation occurred during the enzymatic reaction, due to the interactions of phenoxyl radicals with the enzyme active site.

On the other hand, Nakamoto and Machida [11] have reported that enzyme inactivation is a result of the end-product polymer, which adsorbs the enzyme and coprecipitates it, hindering the access of the substrate to the enzyme active site. Another possible way for the

inactivation of the enzyme is due to excess hydrogen peroxide.[12-14] Which of these might be dominant in a given situation depends very much on the specific concentrations of the enzyme, peroxide and the reducing substrate (e.g. phenol).[2]

To enhance the efficacy and cost-effectiveness of the enzymatic approach, various additives have been tested for their ability to minimize enzyme inactivation. These studies have shown that the oxidation of phenols catalyzed by peroxidases can be enhanced by adding additives, such as surfactants like Triton X-100, sodium dodecyl sulfate (SDS), sodium dodecylbenzenesulfonate (SDBS), hexadecyltrimethylammonium bromide (CTAB) sodium cholate and Tween 80, [2,15-19] polyethylene glycol (PEG), [2,20-26] among others.

PEG is believed to act as a ‘‘sacrificial polymer’’: it diverts the free radicals generated during the catalytic process from covalently binding to the active site, causing inactivation; alternatively, it substitutes for the enzyme in adsorption onto the solid polymeric products formed during the reaction, because it has a higher partition affinity with the polymer products than peroxidases, [11,27] thereby preventing the enzyme from settling with the solids.

The effects of surfactants on enzymes have been studied previously. It has been reported that the presence of surfactants can reduce the interactions between enzymes and the oxidative polymerization products, as well as protect them from inactivation.[28] In addition, the interactions between enzyme and surfactant can involve the free surfactant molecules and/or the micelles, leading to conformational changes which could modify the catalytic rate constant and/or the enzyme-substrate binding constant, thereby affecting the enzyme activity and stability.[15,29,30]

While many studies with selected aromatic compounds in buffered solutions have shown promise, few enzymatic treatment research with real industrial phenolic wastewater matrices have been investigated. Effluent streams often have fluctuating pH, temperatures and pollutant concentrations, and may contain heavy metals, inorganic salts and a plethora of organic compounds in various ratios.[31] In addition, in natural environments, mixtures or composed combinations of many organic and inorganic contaminants, rather than a single pollutant, are present, and the complexity of the pollution may entail possible negative or positive synergistic effects on the enzyme efficiency.[32] An example is the coffee processing wastewater, which is rich in total suspended and dissolved solids, containing a wide variety of organic compounds, such as caffeine, sugars and phenolic compounds, unsuitable for direct disposal in soil or waterways.[33]

Within this context, the objective of this study was to evaluate the behavior of non-ionic surfactant Triton X-100 and the polymer polyethylene glycol in the suppression of the adsorption of turnip peroxidase in the polymeric products formed by the oxidation of various phenolic compounds. In addition, the oxidation of phenolic compounds from a wastewater sample was evaluated in the presence and absence of these additives by peroxidase extracted from soybean hulls, in order to understand the behavior of these additives in a real sample.

2 Materials and methods

2.1 Chemicals and reagents

Catalase (EC 1.11.1.6 ≥10.000 units per mg protein) was purchased from Sigma-Aldrich. Polyethylene glycol (average molecular mass of 4000 Da), Triton X-100, guaiacol and H_2O_2 were purchased from Vetec. The phenolic compounds used were: phenol, caffeic acid and chlorogenic acid, all Sigma-Aldrich (St. Louis, MO, USA).

2.2 Collection of coffee processing wastewater

Coffee processing wastewater (CPW) was obtained on a farm located in Bom Sucesso, Minas Gerais, Brazil. This water was collected in a storage tank on the farm, where all the processing effluent is mixed. After collecting the samples, the effluent was stored in amber bottles at 4 ºC.

2.3 Obtention of the enzymatic extract

The enzyme was extracted from turnip roots purchased from the local market. The roots (with peel) were washed in water and cut into small uniform pieces. Turnip roots (30 g) were homogenized in a blender with 100 mL of 0.05 mol L^{-1} phosphate buffer, pH 6.5, containing 0.2 mol L^{-1} NaCl for 30 s. The homogenate was filtered in organza cloth and centrifuged at 10000 x g for 15 min, at 4 ºC.[34] The obtained solution was subjected to precipitation by adding cold acetone until reaching 65% (v/v). After a rest from 12 to 14 h, at -18 ºC, the homogenate was centrifuged at 11000 x g for 15 min, at 4 ºC. The precipitate obtained after the

removal of acetone by a treatment in refrigerator during 72 h was redissolved in phosphate buffer, pH 7.0 and then used for the studies on phenolic compound oxidation.[35]

To obtain the soybean seed hull extracts, the seeds were immersed in distilled water for 24 h and hull removal was performed manually. The procedure for the extraction of peroxidase was made similarly to the turnip roots, differing only in the amount the plant tissue used, which was 15 g of seed hull.

2.4 Enzymatic activity

The enzymatic activity was determined according to Khan and Robinson [1994, with modifications] by using the following reaction medium: 1.5 mL of 1% guaiacol (97%, v/v), 0.4 mL of 12.25 mmol L^{-1} H_2O_2 (PA), 0.1 mL enzyme (kept in ice bath) and 1.2 mL of 0.1 mol L^{-1} phosphate buffer pH 7.0 for turnip peroxidase (TPE) and 1.2 mL of 0.1 mol L^{-1} citrate phosphate buffer pH 6.0 for the peroxidase extracted from soybean seed hulls (SBP). The reaction was carried out for 3 min at 30 ºC in a Spectrovision spectrophotometer coupled to a thermostatic bath (λ = 470 nm).

One unit of peroxidase activity (U) represents the formation of tetraguaiacol (µmol) per minute of reaction in the assay conditions and it was calculated by using data relative to the linear portion of the curve.[36]

2.5 Effect of PEG and Triton X-100 dose

Phenol, chlorogenic acid and caffeic acid, all at 1 mmol L^{-1} (1.5 mL), were treated with turnip peroxidase (16.83 U mL^{-1}) in the presence of 5 mmol L^{-1} H_2O_2 (0.4 mL) and varying concentrations (0.025; 0.05; 0.10; 0.50 and 1.0 mg mL^{-1}) of PEG and Triton X-100 in a 0.1 mol L^{-1} phosphate buffer, pH 7.0 at 30 ºC for 30 min. The reaction mixture was stirred continuously.

The same oxidation assay was conducted for chlorogenic acid and caffeic acid at 1 mmol L^{-1} in citrate phosphate buffer pH 3.0 at 30 ºC for 30 min. The reactions were stopped by adding 0.1 mL of catalase solution (1.2 mg of the commercial enzyme in 1 mL of 0.1 mol L^{-1} phosphate buffer, pH 7.0).[23, with modifications]

The insoluble product was removed by centrifugation at 3000 x g, for 10 min at 25 ºC. The experiments were performed triplicate and the deviations are represented in the form of bar in all graphs presented.

2.6 Determination of phenolic compounds

The residual concentration of phenolic compounds was measured by the colorimetric method of Folin and Denis, using each phenolic compound as a standard for the calculation of oxidation.[37] For the preparation of the Folin-Denis reagent 50 g of $Na_2WO_4 \cdot 2H_2O$ was added to 10 g of phosphomolybdic acid. Then 25 mL of concentrated phosphoric acid (85%) was added to the above mixture, and the volume was completed to approximately 375 ml water maintaining the mixture under reflux for two hours. The resulting solution was then diluted to 500 mL. Sodium carbonate solution was prepared by dissolving Na_2CO_3 anhydrous at 70-80 ºC (20 g) in 100 mL of water. A standard curve was constructed for each phenolic compound (100 mg/1000 mL), and the caffeic acid was diluted in 1% ethanol/water. Each solution was prepared on the day it is used.

Folin-Denis reagent (100 µl) was added at a series of dilutions of the solutions of phenolic compounds prepared (40, 80, 120, 160 and 200 µl) and the volume completed up to 1.7 mL of distilled water. The solution was shaken vigorously and then added 200 µl saturated sodium carbonate solution. The final volume was again shaken and allowed to stand for thirty minutes. After this period of time, the absorbance was measured at 760 nm by UV/VIS Spectrophotometer (Spectrovision). A blank was also analyzed in each instance. After the absorbance readings a curve was plotted for each phenolic compound analyzed. Duplicate determinations were carried out for each sample.

2.7 Effect of PEG and Triton X-100 dose on the oxidation of CPW

Wastewater samples were previously centrifuged at 10000 x g for 10 min to remove interfering impurities. The supernatant was analyzed in order to determine the initial concentration of phenolic compounds according to item 2.6.

CPW (1.5 mL) was treated by SBP (25.24 U mL^{-1}) in the presence of 7 mmol L^{-1} H_2O_2 (0.4 mL) at concentrations of 0.025; 0.05; 0.10; 0.50 and 1.0 mg mL^{-1} [5,20] of PEG and Triton X-100 in a 0.1 mol L^{-1} tris-HCl buffer, pH 8.0 at 30 ºC for 30 min. The reaction mixture was stirred continuously during 30 min. The enzymatic reaction was stopped by adding 0.1 mL catalase solution (1.2 mg of the commercial enzyme in 1.0 mL of 0.1 mol L^{-1} phosphate buffer, pH 7.0). The residual concentration of phenolic compounds was carried out according to item 2.5. The controls were carried out in the absence of hydrogen peroxide.

3 Results and discussion

3.1 Effect of PEG and Triton X-100 dose

At first, the influence of the addition of PEG and Titron X-100 was evaluated on the oxidation of caffeic acid (concentration in the reaction medium - 1 mmol L^{-1}) in phosphate buffer pH 7.0 (0.1 mol L^{-1}) for turnip peroxidase (16.34 U mL^{-1}). According to the results shown in Figure 1, there was no increase in the percentage oxidation of caffeic acid with the addition of these additives, since oxidation in the absence of these additives was 55.14%.

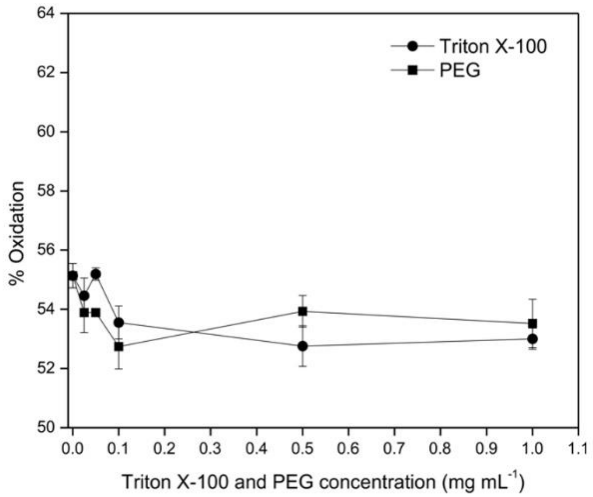

Figure 1. Effect of the concentration of Triton X-100 and PEG on the oxidation of caffeic acid in 0.1 mol L^{-1} phosphate buffer, pH 7.0 by turnip peroxidase at 16.34 U mL^{-1} and 30 ºC for 30 min.

According to Nakamoto and Machida,[11] the main reason for enzyme inactivation in the oxidation of phenolic compounds is their adsorption in the polymeric products formed. It is inferred that peroxidase molecules first adhere to the polymerized phenol, while retaining their activity, and then they are apparently inactivated, probably because the polymerized phenol encloses them to hinder the access of the substrates to the enzyme active site. Several studies have described a significant improvement in oxidation when the process is performed in the presence of additives, such as PEG and Triton X-100, which are considered to competitively suppress the adsorption of peroxidase molecules.

However, in the oxidation of caffeic acid at pH 7.0, the formation of polymeric products was not observed during the reaction, which possibly explains the non-influence of additives on the oxidation of this phenolic compound, once the formation of these polymers is directly related to enzyme inactivation, decreasing its catalytic activity. Therefore, to confirm the effect of these additives on the oxidation of phenolic compounds, a study was conducted with phenol since, in previous studies (data not shown) evaluating the oxidation of phenol by turnip peroxidase, the formation of the polymer product was clearly observed.

The percentage of oxidation obtained after the addition of different concentrations of PEG and Triton X-100 in the oxidation of phenol is shown in Figure 2. When the enzyme (TPE) was added into the phosphate buffer containing phenol (concentration in the reaction medium of 1mmol L^{-1}), H_2O_2 (concentration in the reaction medium of 5 mmol L^{-1}) and PEG or Triton X-100, the mixture turned gradually black and a precipitate began to form. The obtained polymer is a kind of black powdery material.

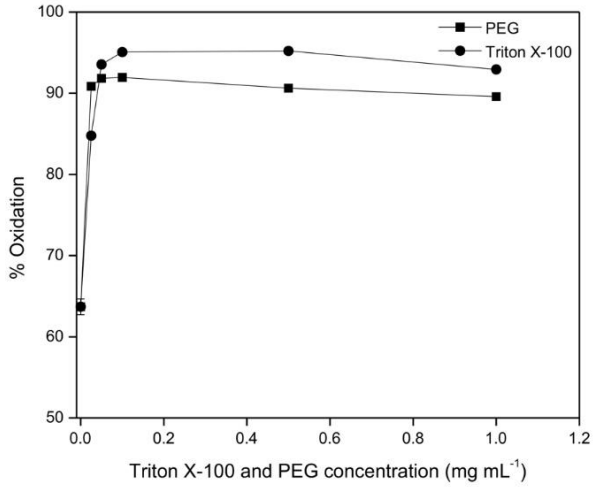

Figure 2. Effect of the concentration of Triton X-100 and PEG on the oxidation of phenol by turnip peroxidase at 16.83 U mL^{-1}, pH 7.0, and 30 ºC for 30 min.

The percentage of oxidation in the absence of additives was 63.69%, while in the presence, there was an increase of about 46.82%. As shown in Figure 2, the phenol oxidation efficiency increased rapidly with a concentration of PEG up to 0.1 mg mL^{-1}; at higher concentrations, the percentage oxidation suffers a slight decline. PEG protection efficiency varies with its concentration and with its average molecular mass (as long as average molecular mass is not lower than about 1.500 g mol^{-1}). As PEG concentration increases, the efficiency

also increases until a threshold is reached, the minimum effective concentration, beyond which the effect of the additive shows no further improvement. PEG protection efficiency also depends on the enzyme and the aromatic substrate being investigated.[2]

The increase in the efficiency of phenol oxidation by turnip peroxidase was practically the same with the addition of PEG additive (91.94%) and the surfactant Triton X-100 (95.10%). Surfactants are amphiphilic molecules that posses both hydrophilic and hydrophobic parts. In aqueous solutions, surfactants can exist as monomers (below the critical micelle concentration - CMC) or as micelles (above the CMC); thus, the effects caused by surfactants on the behavior of enzymes could depend on their organization forms.[38] According to studies conducted by Zhang and coworkers, [2012b] Triton X-100 was added to the reaction solution at various concentrations below or above its CMC (about 310 μmol L^{-1} ~ 0.2 mg mL^{-1}).

Various possibilities were considered for the ability of surfactants to suppress enzyme deactivation. These surfactants, like PEG, could prevent enzyme adsorption to the polymeric products by associating themselves with the products. Two scenarios are envisaged for surfactants preventing the products from adsorbing the enzyme and precipitating it: either by partitioning of the products as formed into any surfactant micelles present or, alternatively, by surfactant monomers partitioning into product aggregates, changing their surface features so as not to adsorb the enzyme. As the effects were evident for Triton X-100 at concentrations below the CMC, it is inferred that partition of the products into surfactant micelles can be ruled out as a mechanism for the observed surfactant effects. In that case, it is suggested that the effect of Triton X-100 is consistent with its modifying the product particles, so that they do not adsorb the enzyme.[2]

As observed, after the addition of both PEG and Triton X-100, the phenol oxidation efficiency increased rapidly with a concentration up to 0.1 mg mL^{-1}; from this concentration, the percentage oxidation suffers a slight decline. The aggregation process of surfactant molecules was a stepwise process.[39] The hydrophobic tails of surfactant molecules collected together and formed aggregates. The large and incompact micelle aggregates turned into small and compact micelles with increasing surfactant concentrations. Therefore, surfactant molecules at higher concentrations adsorbed more phenol in order to partition it into the micelles which reduced the free phenol concentration, [40] and prevented its contact with peroxidase, leading to a decrease in oxidation.[16]

The results suggest that the effect of Triton X-100 on phenol oxidation related closely to its concentration. Surfactants in aqueous solutions can affect enzymatic reactions either

below or above the CMC.[15] The similarities between Triton X-100 and PEG noted above are not surprising in view of their structural similarity.[28]

Similar results were observed by Al- Ansari and coworkers, [2] who studied the effect of the addition of surfactants, including Triton X-100, on the conversion efficiency of 1 mmol L^{-1} phenol by soybean peroxidase at pH 7.0. The maximal or optimal surfactant effects observed in the 125–200 mg L^{-1} range, were within the CMC range for Triton X- 100.

As described above, some authors suggest possibilities that explain how PEG and surfactants extend the removal activity of phenols and anilines by oxidative enzymes. Enzymes such as peroxidases are associated with the solid particles of polymeric products through hydrophobic interactions. At a certain minimum effective concentration, PEG may associate with these particles, being predominantly [41] or completely [27] taken up, replacing the enzyme from binding to the surface of these products, and being subsequently removed by sedimentation with solid particles. The surfactants (Tritons and Tweens) interact with the polymeric product solids in the same way, through their tails.[2]

In previous studies of the effect of pH on the oxidation of caffeic acid by turnip peroxidase (data not shown), it was noted that only the pH values 2, 3 and 4 have the formation of a precipitate, unlike other analyzed, since only at these pH values, the metabolites formed during the oxidation reaction bind to form polymeric products. Therefore, once these pH values were observed the formation of polymer precipitate, the effect of adding PEG and Triton X-100 on the oxidation of caffeic acid (concentration in the reaction medium of 1 mmol L^{-1}) was evaluated in phosphate citrate buffer 0.1 mol L^{-1}, pH 3.0 (Figure 3). As noted in the oxidation of phenol after the addition of these additives, the percentage oxidation of caffeic acid in the presence of Triton X-100 also increased (around of 22%); however, practically no effect was found on oxidation after the addition of PEG.

It was observed again that when there is the formation of polymeric products during the oxidation reaction, the presence of chemical additives positively influences the percentage of oxidation of the studied phenolic compound, corroborating the study by Nakamoto and Machida, [11] who reported that these products inactivate the enzyme and, therefore, the addition of these additives suppresses inactivation, improving the percentage of oxidation.

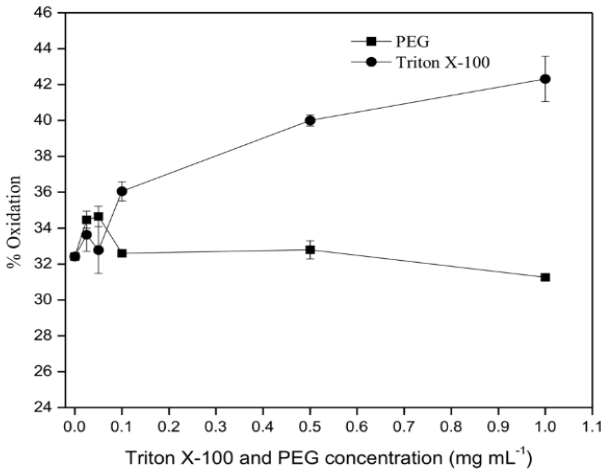

Figure 3. Effect of the concentration of Triton X-100 and PEG on the oxidation of caffeic acid by turnip peroxidase at 16.34 U mL^{-1}, pH 3.0 and 30 ºC for 30 min.

In order to evaluate this theory that the final polymeric product adsorbs enzyme molecules preventing the access of substrate to the active site of the enzyme during the oxidation reaction, a study on the oxidation of chlorogenic acid by turnip peroxidase was conducted. The choice of this phenolic compound was due to the fact that the formation of any precipitate in the reaction medium was not observed when the oxidation reaction was conducted in phosphate buffer at pH 7.0 and in citrate phosphate buffer at pH 3.0. Thus, the effect of adding PEG and Triton X-100 in the oxidation of chlorogenic acid (concentration in the reaction medium of 1 mmol L^{-1}) by turnip peroxidase (16.34 U mL^{-1}) was observed in phosphate citrate buffer at 0.1 mol L^{-1}, pH 3.0 and in phosphate buffer at 0.1 mol L^{-1}, pH 7.0 (Figures 4 A and B).

Rather, the oxidation of chlorogenic acid at pH 7.0, at a concentration of 0.1 mg mL^{-1} Triton X-100, decreased the percentage of oxidation, confirming the theory that surfactant molecules at higher concentrations adsorbed more phenol in order to partition it into micelles, which reduced the free phenol concentration, [40] preventing its contact with peroxidase, leading to a decrease in oxidation. The same behavior was observed in the oxidation of phenol by turnip peroxidase presented earlier. At pH 3.0, no effect on the oxidation of chlorogenic acid was observed in the presence of the additives PEG and Triton X-100, an expected result, due to the non-formation of polymeric precipitates during the reaction.

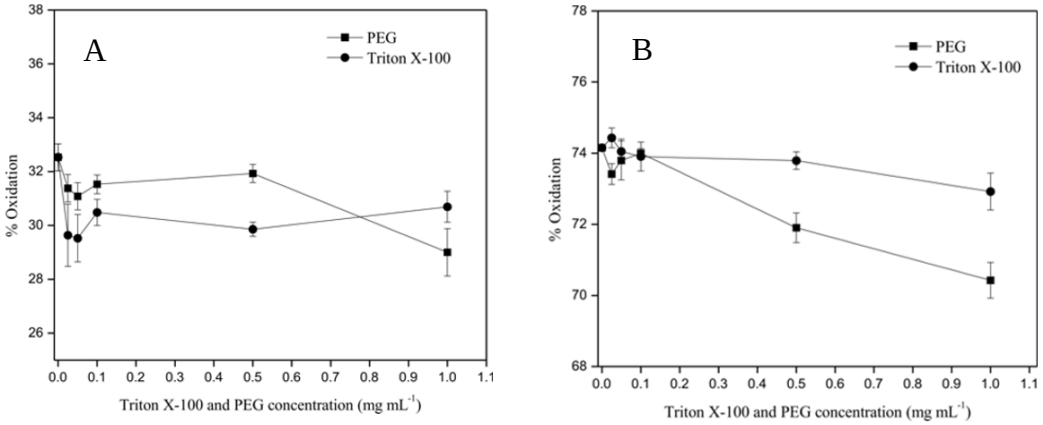

Figure 4. Effect of the concentration of Triton X-100 and PEG (A) on the oxidation of chlorogenic acid in phosphate citrate buffer at 0.1 mol L^{-1}, pH 3.0, (B) in phosphate buffer at 0.1 mol L^{-1}, pH 7.0, by turnip peroxidase at 16.34 U mL^{-1}.

Since studies evaluating the influence of the addition of chemical additives on the oxidation of phenolic compounds in real wastewater are scarce, a study with CPW was performed using SBP as a catalyst and standardized reaction conditions after studies previously conducted, in order to evaluate the influence of the addition of PEG and Triton X-100 on the oxidation of phenolic compounds present in wastewater.

3.2 Effect of PEG and Triton X-100 dose on the oxidation of wastewater from coffee fruit processing

In this study, SBP showed a promising performance as a biocatalyst in the oxidation of CPW. In order to be an economically feasible treatment alternative, the enzyme must be cheap enough and available in quantities to meet the consumer's needs. Hence, SBP was selected for this study, since soybeans are grown on a massive scale worldwide and SBP is located in the seed coat or hull.[42,43] Furthermore, the waste generated from one industry can provide the catalyst to detoxify the effluent of another industry.[31] Given the ability of SBP to oxidize phenolic compounds from wastewater, besides representing one low cost alternative, the influence of the addition of PEG and Triton X-100 on the oxidation of this effluent was evaluated, since this additive has the ability to minimize enzyme inactivation.

The CPW used in this study showed a concentration of 257.84 mg L^{-1} ± 4.09 total phenols, which exceeds the CONAMA Resolution 430/2011.[1] The high concentration of these pollutants justifies the need for the treatment of this effluent. Other effluents also were characterized by higher concentrations of phenols as wastewater from alkyd resin manufacture. [43] The only phenolic compound detected in this effluent was phenol, ranging from 640.00 to 2607.00 mg L^{-1}.

There was no influence toward the oxidation of phenolic compounds in CPW in the presence of PEG and Triton X-100 (Figure 5).

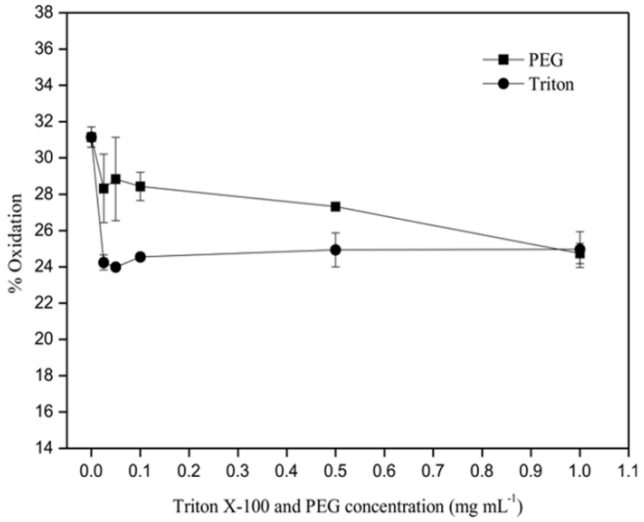

Figure 5. Effect of the concentration of Triton X-100 and PEG on the oxidation of coffee processing wastewater in 0.1 mol L^{-1} tris-HCl buffer, pH 8.0 at 30 ºC for 30 min, by soybean peroxidase at 25.24 U mL^{-1}.

The oxidation of phenolic compounds of CPW in the absence of these additives was 31.15 ± 0.55%. Employing the additive PEG and Triton X-100 at a concentration of 0.025 mg mL^{-1}, the oxidation was 28.33 ± 1.89% and 24.24 ± 0.19%, respectively, while at a concentration of 1 mg mL^{-1}, the oxidation was 24.74 ± 0.56% for PEG and 24.96 ± 0.97% for Triton X-100.

As previously discussed the addition de PEG and Triton inhibits the enzyme deactivation enzyme due to the adsorption to the polymeric products by associating themselves with reaction products. Thus, since it was not observed the formation of polymeric products in the oxidation of the CPW, it is suggested that the addition of these chemical additives have no effect in this case.

The controls made without hydrogen peroxide did not cause any conversion, confirming that peroxidases are activated only in the presence of peroxide.

Similar results were also reported by Al-Ansari and coworkers, [2] who studied the effect of the addition of PEG (3350 g mol^{-1}) to increase the efficiency of phenolic compounds removal from coal tar wastewater. At a PEG concentration of 0.6 mg mL^{-1}, no effect on phenol removal was observed.

4 Conclusions

The peroxidase extracted from turnip by a low-cost process, was effective in the oxidation of the studied phenolic compounds. Thus, the enzymatic oxidation may be an alternative and/or complementary technology to treat wastewater containing such toxic compound.

No change was observed in the oxidation of caffeic acid in the presence of the additives Triton X-100 and PEG at pH 7.0. However, at the same pH, the presence of these additives showed a significant change in the oxidation of phenol, about 46.82%. For the oxidation of caffeic acid at pH 3.0, only in the presence of Triton X-100 there was a 22% increase in oxidation, while practically no effect was observed in the presence of PEG. In the oxidation of chlorogenic acid, Triton X-100 caused a decrease in oxidation at pH 7.0. At pH 3.0, no effect on the oxidation was observed in the presence of additives. However, the addition of these additives in the CPW oxidation reaction medium with the enzyme SBP hardly changed.

It is worth noting that the use of additives showed no influence on the oxidation of phenolic compounds. Considering the treatment process of real wastewaters containing phenols, this is a positive response from the economic point of view.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ARTIGO 2 - Novel eco-friendly biocatalyst: soybean peroxidase immobilized onto activated carbon obtained from agricultural waste

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Abstract

The immobilization of enzymes is an excellent alternative to overcome the drawbacks of using these biocatalysts in free form. This process plays a significant role in cost-effective recovery, increased catalyst productivity and in simplifying process operations. After the soybean peroxidase (SP) extraction, a residue at high carbon and low ash content is generated. This residue was used as carbonaceous precursor for production of activated carbon (AC) with high surface area (1603 m^2 g^{-1}). The AC produced was used as support for SP immobilization. The immobilization of SP was evaluated in different time conditions, enzyme load, pH and temperature. The samples, before and after immobilization, were characterized by thermogravimetric analysis, elemental analysis composition, specific surface area, X-ray powder diffraction, scanning electron microscopy and Fourier transform infrared spectroscopy. In addition, repeated applications of immobilized biocatalyst were made in order to evaluate its

operational stability and capacity to recover the reaction medium, in which was observed that after a decline in activity from the first to the second cycle, it remained constant until the tenth application. In the context, the process of material obtainment constitutes a clean route for the development of more sustainable biocatalysts capable of applications in various areas.

Keywords: Biocatalyst; Waste recycling; Soybean Peroxidase; Activated carbon.

Introduction

Enzymes are biocatalysts widely used in several areas, including the synthetic, pharmaceutical chemistry, wastewater bioremediation, fabrication of high performance biosensors, among others.[1-5] They exhibit a high catalytic activity and selectivity, acting as an environmentally efficient catalyst without the need to work at high pressures, temperatures and harsh chemical environments. However, their use still is hampered due to some limitations related to their low stability front a pH, temperature, loss of activity due to the changes in environmental conditions and presence of the interferents, as well as the impossibility of reuse. The high cost associated with their production and purification, their fragile nature highly dependent on the reaction conditions and high loads required for commercial production are factors that hamper their free-form application.[6-8] Due to these factors, the immobilization of enzymes has been an industrially and commercially viable alternative, solving the disadvantages of their use in solution related to stability and recovery of the reaction medium.

The enzyme immobilization process may lead to an improved specificity and especially the catalyst stabilization, protecting the enzymes from denaturants agents and proteolysis.[9,10] Several authors have describe processes of immobilization of different enzymes in various carriers which can be achieved by physical methods, that involve the entrapment of enzyme molecules within a porous matrix, and chemical methods, which, in turn, include enzyme attachment to the matrix by covalent bonds, cross-linking between enzyme and matrix, or enzyme cross-linking by multi-functional reagents.[7,8,11-14] Among the various techniques used for the immobilization of enzymes, we can highlight adsorption, a simple, fast and economically viable process that can occur through physical interactions generated between the enzyme and the carrier. The physic-chemical parameters of the carrier that should be taken into an account are the surface area, pore structure and type of functional groups present on the surface. Adsorbed enzymes are shielded from aggregation, proteolysis and interaction with hydrophobic

interfaces. In this process, the bond between the enzyme and the carrier is relatively weak compared to the chemical immobilization. That is very interesting, since the native structure of the enzyme is not altered enabling it to maintain its activity.[7,11,15]

In general, the carriers used for the enzyme immobilization by adsorption can be divided into both organic and inorganic origin. Among the various organic carriers used, the use of activated carbon can be emphasized, which presents a large surface area, a high pore volume and higher hydrothermal resistance compared to other porous materials. In addition, the immobilization process in this matrix is nontoxic, fast, inexpensive, easy to handle and stable against denaturation by oxidants.[8,16]

The search for different precursors for the synthesis of activated carbon has been increasing and the use of agro-industrial waste makes the process even more viable economically, besides meeting current environmental needs. Consequently, various types of precursors of vegetable origin have been researched, such as wood, apricot and/or cherry pits, wheat straw, bean and rice husks, nut husks, corn husks, among others.[17] The soybean seed hulls are an abundant waste from soybean-processing industries and may offer an inexpensive and renewable additional source of activated carbons. Thus, conversion of this kind of waste in active carbon reduces the cost of waste disposal and provide an alternative to the existing commercial active carbon.[18]

Peroxidases (E.C.1.11.1.7) are enzymes that catalyze the reduction of peroxides, such as hydrogen peroxide and the oxidation of a variety of organic and inorganic compounds, such as, phenolic compounds, aromatic amines and dyes and, therefore, they have a great potential as bioremediation catalysts.[19]

Thus, the focus of this work was to synthesize an activated carbon from residue originating from enzymatic extraction of an agribusiness byproduct, optimize soybean peroxidase immobilization parameters in the activated carbon obtained and to evaluate the reuse of the immobilized biocatalyst in order to obtain a capable environmental friendly biocatalyst for applications in numerous technological areas.

Experimental

Obtention of soybean peroxidase (SP)

Initially the soybean seeds were immersed in distilled water for 24 h and then the husks were removed manually (Figure 1). The process of obtaining the biocatalyst was conducted according to Silva et al.[19] The peroxidase activity assay was based on the rate of guaiacol oxidation for the formation of tetraguaiacol (ε =26.6 mM^{-1} cm^{-1}). One unit of peroxidase activity (U) at pH 6.0 represents the formation of tetraguaiacol (μmol) per minute of reaction.[20]

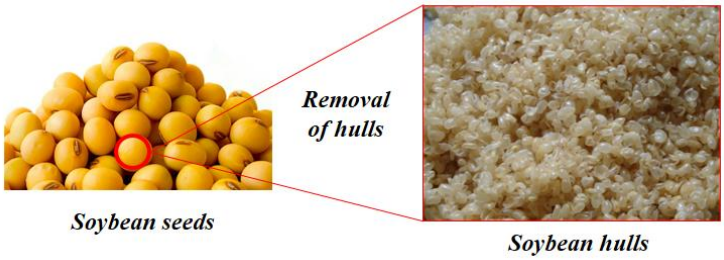

Fig. 1 Removal of soybean hulls.

Activated carbon preparation

The waste originating from the process of obtaining the soybean peroxidase was used as a carbonaceous precursor for synthesis of activated carbon. This waste was washed with distilled water successive times to remove excess phosphate buffer pH 6.5, dried at 60 °C for 24 h and then treated with a zinc chloride ($ZnCl_2$, 97% P. A. Sigma-Aldric) solution at a 1:1 mass proportion (salt: raw material) and pyrolyzed in a tubular oven, under a N_2 flow of 150 mL min^{-1}, at 550 °C, for 3 h. The material was then washed with hot distilled water for 1h to remove the excess activating agent and dried at 60 °C for 24 h.

Adsorption experiments (immobilization of soybean peroxidase)

For the immobilization process, 1 g of activated carbon was kept under constant stirring in a water bath with 20 ml of SP solution in buffer. The following parameters were evaluated: time (30 min to 300 min), enzyme load (0.05, 0.1, 0.25, 0.5 and 1 g), pH (3-6 citrate phosphate buffer, 7-9 tris-HCl buffer) and temperature (20 to 70 °C). To assist in understanding the enzyme immobilization process at different pH values, the pH corresponding to the point of zero charge (pH_{PCZ}) for the AC was determined. The zeta potential of the dilute suspensions of the materials was measured in a Zeta Potential Meter (Malvern – Zeta Sizer model nano-ZS) in

a pH range of 9.7 to 3.0, the pH value was adjusted by adding HCl (0.1 mol L^{-1}) or NaOH (0.1 mol L^{-1}). The immobilization procedure was stopped by separation of the immobilized material by centrifugation at 11,000 x g, for 10 min at 25 °C. After the procedure, the material obtained was washed under vacuum with citrate-phosphate buffer pH 6.0 to remove excess unbound enzyme, and then dried at 4 °C overnight, and finally stored in a freezer. The immobilized material was called SP-AC.

Immobilization of SP was investigated by measurements of enzymatic activity according to Khan and Robinson.[20] The immobilization yield was calculated from the difference between the initial and residual activity according to the following Equation 1:[21,22]

$$\boldsymbol{\varphi_{(\%)} = (A_0 - A_F)/A_0 \quad x\,100} \tag{1}$$

where: $\varphi_{(\%)}$ is the immobilization yield, A_0 is the enzyme activity of the supernatant before incubation, and A_F is the enzyme activity of the supernatant after the incubation period.

Characterization

Thermogravimetric analysis (TG)

The AC and SP-AC were characterized by thermogravimetric analysis (TG) to determining the ash content. TG analysis (10 mg of sample) were performed on a TG/DTA 6200 simultaneous TG/DTA thermogravimetric analyser (TG/DTA 6200, SII NanoTechnology Inc., Tokyo, Japan). Samples were heated under air (100 mL min^{-1}) from room temperature to 1000 °C at 10 °C min^{-1}. The weight loss (TG curve) and the derivative weight loss (DTG curve) were recorded as a function of temperature.

Elemental analysis composition (CHN), specific surface area, total pore volume and average pore diameter

The determination of carbon, hydrogen and nitrogen in the carbonaceous precursor and the activated carbon were performed in triplicate, using 10 mg of sample in a CHN elemental analysis equipment from Perkin Elmer 2400. The samples were weighed in tin capsules using a microbalance (Perkin Elmer AD-6 Auto Balance Controller) connected to CHN for direct

acquisition of the masses. All results are based on a value of a standard of known elemental composition.

The specific surface area, total pore volume and average pore diameter were determined by physical N_2 adsorption/desorption at 77 K using a Quantachrome Nova 1200 instrument. Prior to each analysis the materials were degassed at 250 °C under vacuum for 5 h. The total pore volume was calculated at a relative pressure at P/P0 ≈ 0.985.

X-ray powder diffraction (XRD)

XRD pattern of materials was analyzed by X-ray powder diffraction (XRD) using a diffractometer (XRD-7000, Shimadzu Scientific Instruments, Tokyo, Japan) with a Bragg Brentano camera geometry, Cu-K_α incident radiation (λ = 1.5418 A), 40 kV, 30 mA and acquisition rate of 2° min^{-1} between 10-60° (2θ) range.

Scanning electron microscopy (SEM)

The morphology was investigated by scanning electron microscopy (FEI QUANTA FEG 250 ESEM, FEI Company, Hillsboro, Oregon, USA). Samples were carbon coated using carbon thread on a sputter-coater (BALTEC Maschinenbau AG Med model 020, Pfaffikon, Switzerland) with a carbon evaporative attachment prior to Secondary Electron Imaging (SE).

Fourier transform infrared spectroscopy (FTIR)

Surface groups of the materials (SP, AC and SP-AC) were characterized by Fourier transform infrared spectroscopy (FTIR) on an interferometric spectrometer operating with a ceramic bright light source and high-sensitivity DLATGS detector (Shimadzu Prestige-21, Shimadzu Corporation, Tokyo, Japan). The Happ-Genzel function was used to achieve a good balance between ripple size and resolution.[23] CsI pellets were prepared by mixing 1 mg of filtered powders with 100 mg of cesium iodide. FTIR spectra were acquired in the 4000-300 cm^{-1} region with spectral resolution of 2 cm^{-1} and 128 scans. During all analyses, the IR system was continuously purged with N_2 gas to reduce the interference due to water vapor and carbon dioxide.

Operational stability of immobilized enzyme

The operational stability of the SP-AC was determined by repeated applications of the immobilized enzyme in the enzyme activity assay. After each cycle, the activated carbon containing the immobilized enzyme (20 mg) was removed by centrifugation at 11,000 x g for 2 min at 20 °C, washed with citrate phosphate buffer (0.1 mol L^{-1}) pH 6.0 to remove excess unbound enzyme before the next cycle. The washing residue of SP-AC was collected and the residual enzymatic activity was determined. Then, the SP-AC was dispersed again in a fresh reaction medium.

Results and discussion

Effect of time and enzyme load on the immobilization process

The effect of time on the enzyme immobilization process was evaluated in order to determine the equilibrium point, from which the yield of immobilization is maximum. According to Figure 2 (a) it can be observed that from 1 h the immobilization yield is not changed, keeping constant until 5 h (≈17.75%).

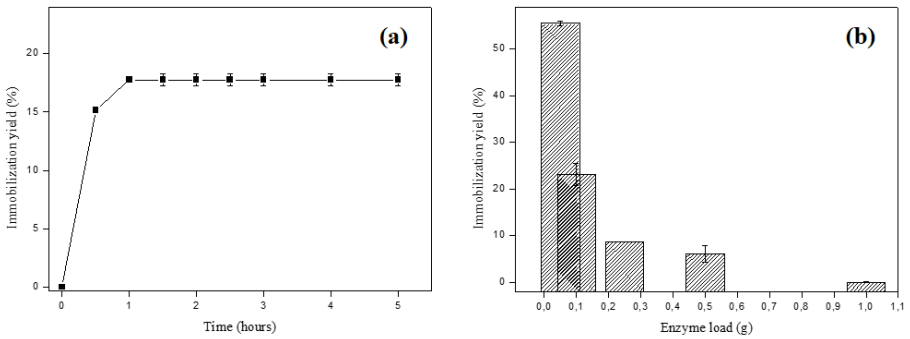

Fig. 2 (a) Effect of time on the SP immobilization process in AC. Reaction conditions: initial enzyme load – SP 2.31 U; 1 g of activated carbon, pH 6.0, 30 °C; (b) Effect of enzyme load on the SP immobilization process in AC. Reaction conditions: 1 g of activated carbon, pH 6.0, 30 °C for 1 h.

At the beginning of the process there is a high number of available adsorption sites and the mass transfer is favored. The extent that SP molecules are adsorbed to the bare sites becomes scarce and therefore less available, causing the enzymes to become clustered decreasing the immobilization yield. This may explain why, after a certain time, the immobilization process becomes constant.[8] Based on these data, the time of 1 h was maintained for the next immobilization experiments.

The second parameter evaluated was the variation of enzyme loading and the optimal enzyme load found was 0.05 g SP (in 20 ml phosphate citrate buffer pH 6.0), with immobilization yield of 55.52%, whereas using 1 g SP the yield was practically zero (Figure 2 (b)). The immobilization yield is greater at low enzyme loads, suggesting that lower yields may be associated with agglomeration of enzymes near the pore entrances and the active sites of the support used.[24] Liu and coworkers[16] found similar results which suggest that the resulting steric constraints could interfere in the dispersion of enzyme molecules on the activated carbon surface, which can be observed by the immobilization yield decrease.

Jain and coworkers[25] also evaluated the effect of horseradish peroxidase (HRP) loadings on surface-modified mesoporous active carbon (SMAC) for different initial values of the enzyme:substrate ratio. For all ratios studied, the immobilization decreases with increasing enzyme:support ratio. Analyzing the commercial activated carbon as support for immobilization of horseradish peroxidase in a higher ratio (1 mg of HRP:0.1 g SMAC) the percentage of HRP loading was 49.9%.

Effect of pH and temperature on the immobilization process

In order to evaluate the optimum pH for the immobilization of SP on AC, the pH of the enzyme solution ranged from 3 to 9. The higher immobilization yield (86.17%) was obtained at pH 3.0, which may be related to the surface charge on the adsorbent and the enzyme (Figure 3 (a)).

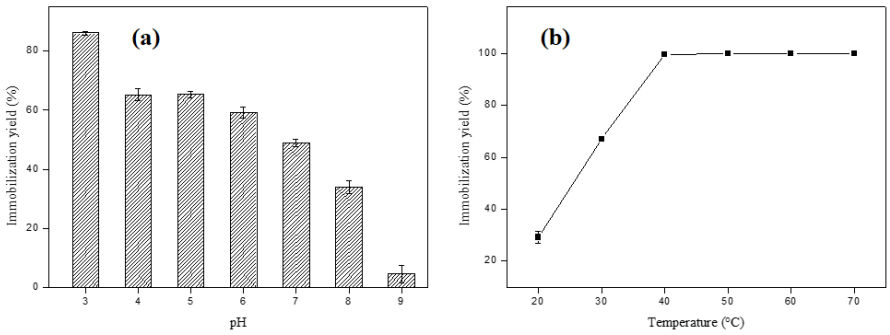

Fig. 3 (a) Effect of pH on the SP immobilization process in AC. Reaction conditions: 1 g of activated carbon, 0.05 g of SP, 30 °C for 1 h; (b) Effect of temperature on the SP immobilization process in AC. Reaction conditions: 1 g of activated carbon, 0.05 g of SP, pH 3.0, for 1 h.

The functional group and delocalized electrons present in the activated carbon is that which will characterize the acidic or basic character of its surface. Thus, the modification of pH of the liquid phase strongly influences the surface properties of activated carbon. The surface of the adsorbent is neutral (surface charge equal to zero) when the solution pH is equal to point of zero charge (PCZ). The surface is negatively charged at pH values greater than pH_{PCZ} ($pH > pH_{PCZ}$), and positively charged at pH values lower than pH_{PCZ} ($pH < pH_{PCZ}$).[41] The pH at the point of zero charge found for the AC obtained in this study was 7.36. Thus, at pH values in the 3 to 6 ($pH < pH_{PCZ}$) range of the AC surface is positively charged, while at pH values 8 and 9, the AC surface is negatively charged ($pH > pH_{PCZ}$).

Peroxidases are classified into three superfamilies: plant peroxidase, animal peroxidase and catalase peroxidase. The plant peroxidase superfamily is further subdivided into three classes: Class I, the intracellular peroxidases; Class II, which consists of extracellular fungal peroxidases and Class III, which comprises the plant class.[26] However, the three peroxidases classes show low identity in their primary amino acid sequences and have distinct functions and reaction mechanisms. It is important to highlight that plant peroxidases contain isoforms different, and because of this factor, it is generally difficult to determining the isoelectric point of a specific isoform of peroxidase, mainly because protein purification is not straightforward[27]. In this way, it is inferred that at pH values > pH_{PCZ} where the immobilization yield was less pronounced, the enzyme was negatively charged, which can have led to an electrostatic repulsion between the adsorbent surface and the enzyme. On the other hand, at pH values < pH_{PCZ} the observed effect was the opposite, with an increase in the immobilization percentage.

The next step in the evaluation of the optimal parameters consists in the evaluation of the effect of temperature on the immobilization of soybean peroxidase in activated carbon. This effect was evaluated at different temperatures and the pH maintained constant at 3.0.

The last parameter analyzed during the SP immobilization process in AC was temperature, and the results obtained are shown in Figure 3 (b). An increase was observed of the immobilization yield with increasing the temperature from 20 to 50 °C, reaching 100% at 40 °C. After this temperature, the immobilization percentage remained constant up to 70 °C. At high temperatures the diffusion rate of the enzyme molecules increases through the external boundary layer and in the internal pores of the adsorbent as a function of the solution viscosity decrease, besides acquiring sufficient kinetic energy to interact with the active sites of the surface.[28,29]

From the results obtained in this manuscript, the best conditions for soybean peroxidase immobilization in activated carbon were obtained in a period of 1 h, with an enzyme: support ratio of 0.05:1 g, pH 3.0 (citrate-phosphate buffer) at temperature of 40 °C.

Characterization

CHN analysis and specific surface area, average pore diameter and total pore volume of materials

The elemental compositions of the carbonaceous precursor and AC are presented in Table 1. The carbon content found in the precursor was 38.84%, close to that found by Gonçalves and coworkers,[30] which makes the residue used an excellent precursor for activated carbon synthesis, since the carbon content is one of the fundamental parameters for the development of a material with a high surface area and porosity. In addition, it is important to note that the decrease in the hydrogen content in AC compared to the precursor may be associated with the release of volatile compounds during the pyrolysis process.

Table 1 Summary of the catalyst nomenclature, its composition and specific surface area (BET), total pore volume, average pore diameter

Materials	Carbon (%)	Hydrogen (%)	Nitrogen (%)	BET (m^2 g^{-1})	Total pore volume (cm^3 g^{-1})[a]	Average pore diameter (Å)[b]
Carbonaceous precursor	38.84	5.66	1.27	----	----	----
Activated carbon	67.65	3.45	1.88	1603	0.847	15.6

[a]N_2 adsorption method
[b]BJH desorption

The AC production from waste generated during the enzyme extraction process presents a high specific surface area 1603 m^2 g^{-1} and total pore volume 0.847 cm^3 g^{-1} with the average pore diameter obtained being 15.96 Å. In the adsorption process there is little control over the orientation of the enzyme in the pores of the support. However, some amino acid residues may interact more favorably with the pore walls by acquiring a certain orientation. In addition, the pore size can also force a certain orientation. The enzyme immobilization efficiency and its retention is directly dependent on enzyme size and pore diameter of the carrier.[31]

It is worth mentioning that the cost of supports for enzyme immobilization is one of the essential factors to know the economic feasibility of their use, which may be related to several factors, including the cost of the precursor used (natural agro-industrial byproducts, or synthesized products) and it availability.[32] In addition, the obtaining of the catalyst described in this work complies with the principles of green chemistry,[33] since the waste generated during the enzyme extraction process is repurposed, which makes it a process with minimum residue generation.

Scanning electron microscopy (SEM)

SEM images of precursor (waste from the enzyme extraction process) and activated carbon before and after SP enzyme immobilization are shown in Figure 4 (a)-(d). The micrographs of the precursor show skeletons with clearly identifiable fibrous forms from the soybean hulls (Figure 4 (a)). However, after the activation process with $ZnCl_2$ there are cavities between the fibrous structures which play a positive role in increasing the specific surface area

(Figure 4 (b)). These cavities, after activation, may be the result of the release of volatile compounds during the pyrolysis process.[34]

The Figures 4 (c) and 4 (d) show the micrographs of the activated carbon after SP immobilization. The presence of small structures on the SP-AC suggested that SP remains clustered after the immobilization procedure.

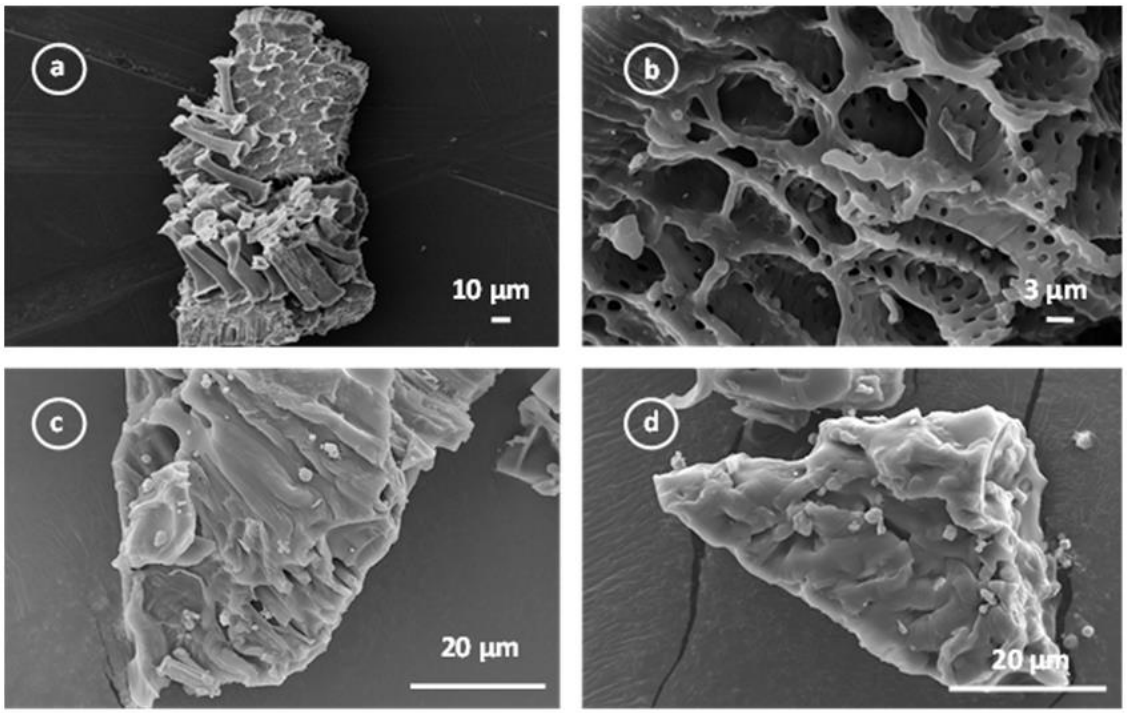

Fig. 4 SEM images of precursor (a); activated carbon after activation with $ZnCl_2$ (b); activated carbon after SP immobilization (c)-(d) at different magnifications.

X-ray powder diffraction (XRD) and FTIR spectra

The XRD patterns, recorded for the AC and SP-AC. Figure 5 (a), reveal the presence of an amorphous halo centered at $2\Theta = 23°$ degree which is a common feature of non-crystalline structures typically present in activated carbon.[35,36] In addition, the XRD result confirms the absence of possible residual crystalline contaminants from the chemical activation process. The XRD pattern of SP-AC reveals the maintenance of non-crystalline structure characteristic of activated carbon, and also displays small peaks at 2Θ values around 31°, 34° and 36° that, by matching to JCPDS files (JCPDS 35-0735), are identified as the reflections of residual sodium hydrogen phosphate hydrate and other salts derived from the buffer solution. The presence of

impurities was expected since the SP used in this work is a by-product without any previous purification stage.

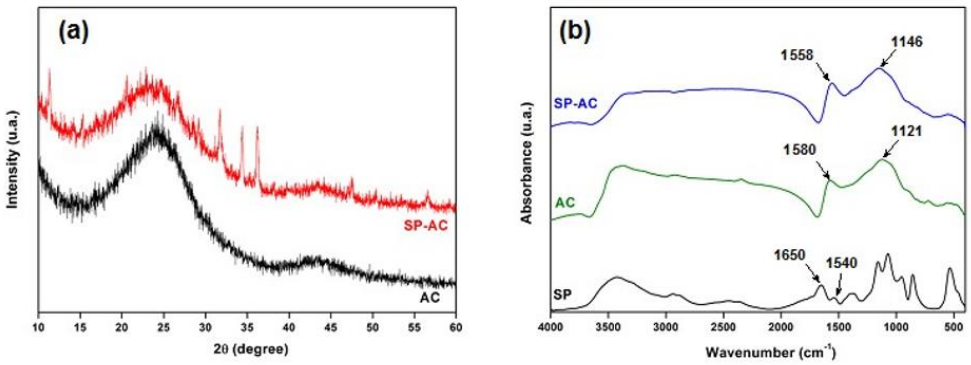

Fig. 5 (a) DRX diffractograms and (b) FTIR spectra corresponding to pure AC, SP-AC and SP samples. The SP pure analyses were added to the comparison.

The activated carbon and immobilized soybean peroxidase on activated carbon were characterized by FTIR spectroscopy. FTIR spectrum of pure SP enzyme displays absorbance bands between 3000 and 3500 cm^{-1} that are attributed to the N-H and O-H stretching, respectively, whereas the C-H asymmetric stretching mode is observed at 2930-2900 cm^{-1} (Figure 5 (b)). In addition, IR absorbance displays bands at 1640-1650 and 1540-1550 cm^{-1}, which are attributed to the amide I and amide II absorbance bands, respectively.[37,38] These regions consist mainly of the C=O stretching vibration of the backbone peptide bonds in proteins.[39] The region between 1200 and 1400 cm^{-1} involves mainly C-H bending motions,[40] whereas the 1260-1000 cm^{-1} and 700-400 cm^{-1} absorptions are attributed to phosphate groups (impurities in the activated carbon matrix) and C-C stretching modes, respectively. AC infrared spectrum exhibits a wide absorption at 3700-3200 cm^{-1} attributed to the presence of strong hydrogen bonds, i.e., O-H stretching or water adsorption. The position of this band at lower wave numbers indicate the presence of strong hydrogen bonds.[38,42] Furthermore, the FTIR spectrum of AC displays absorptions at 1680-1480 cm^{-1} corresponding to the overlapping of stretching modes in aromatic skeletal and C=C groups. The complex bands in the range from 1400 to 900 cm^{-1} are related to superposition of the following vibrational modes: C-H symmetric and asymmetric bending, C-O asymmetric stretching of aromatic ethers, esters and phenols, and C-O in carboxylic acids, alcohols, phenols and esters or the P=O bond in phosphate esters. Similar absorptions bands were observed for the SP-AC sample, however, with some

subtle spectral changes owing to the SP immobilization. The FTIR spectrum of SP-AC is characterized by the maximum displacement of the absorption band from 1580 cm^{-1} in AC to 1558 cm^{-1} in SP-AC. This red shift is associated with changes in the functional groups present on the activated carbon owing to SP immobilization. In fact, this spectral frequency has been attributed to overlapping of two absorptions ascribed to the (C=O) stretching mode of the carbonyl group and associated with a combination of N–H and C–N stretching vibrations.[37-39]

Thermal analysis

Thermal analysis showed both samples presented one single event related to the backbone carbon oxidation, as confirmed by exothermic peaks present in the DTA curves (Figure 6 (a)-(b)). TGA curve for AC shows a weight loss of 92% between 490-590 °C, whereas for SP-AC the similar residual mass was reached between 463-578 °C. In fact, DTG maximums were at 545 and 520 °C for AC and SP-AC, respectively. The combustion profile of activated carbon depends on several factors such as specific surface area, porosity and particle size, among others. It is important mentioned that, AC and SP-AC have the same carbonaceous matrix. Thus, this thermal shift is associated with the SP on the activated carbon surface. On the other hand, the residual mass for SP-AC was slightly greater in comparison with AC, 12 % and 8 %, respectively. Such difference in mass loss confirms the presence of SP and its thermostable impurities in the activated carbon matrix.

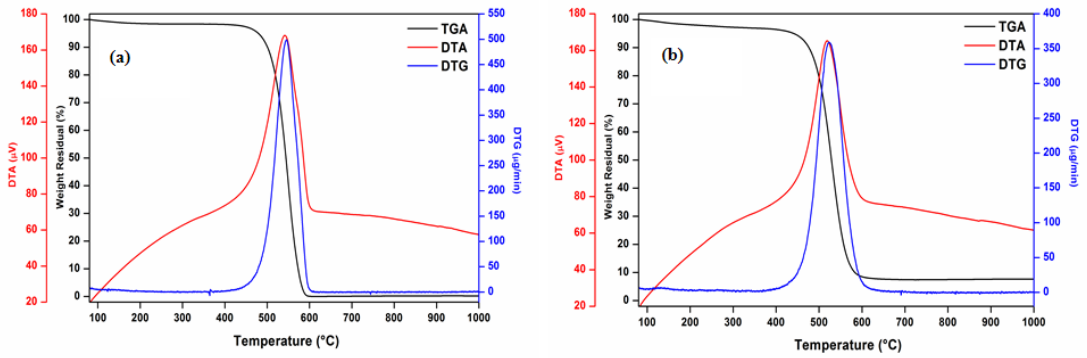

Fig. 6 TGA/DTG/DTA curves for AC as-prepared (a) and SP-AC samples (b).

Operational stability of immobilized enzyme

The viability of immobilized enzymes for industrial applications is dependent on whether the enzyme can be regenerated and consequently whether the biocatalyst can be reused.[43] This makes the enzyme immobilization process highly attractive, due to the current demands of the biotechnology industry for the development of productive catalysts and new techniques that increase their shelf life.[15]

The SP-AC was used in 10 cycles and the performance of the immobilized biocatalyst was analyzed as to determination the peroxidase activity at the end of each cycle. The results obtained are shown in Figure 7. It was observed that the activity of the SP-AC undergoes a decline from the first to the second cycle, becoming constant until the last.

To evaluate the amount of enzyme loss during the each cycle, the washing residue of SP-AC was collected and the residual enzymatic activity was determined. From the first cycle, no catalyst loss was detected, implying a strong bonding of SP on the support. Several authors suggest that the decrease in enzymatic activity after successive cycles is related to some enzyme leaching from the support.[44,45] In this work, enzyme leaching after successive cycles was no detected, implying a strong bonding of SP on the activated carbon.

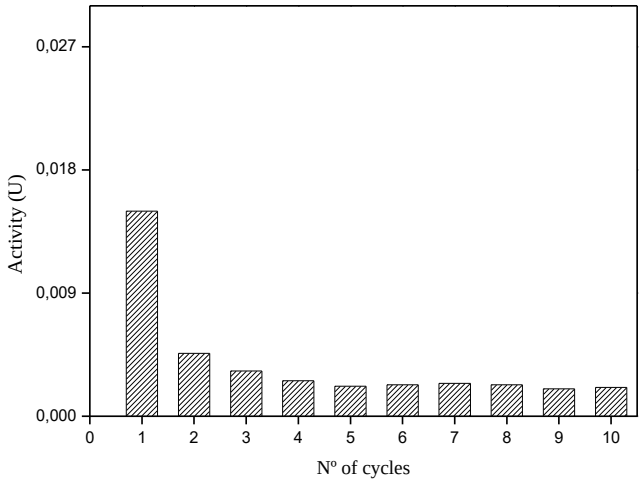

Fig. 7 Operational stability of immobilized soybean peroxidase in activated carbon in the activity assays. Reaction conditions: 20 mg of soybean peroxidase immobilized in activated carbon, citrate-phosphate buffer pH 6.0 (0.1 mol L^{-1}), guaiacol (1% v/v) and hydrogen peroxide (0.3% v/v).

The decrease performance of the catalyst after each cycle may be attributed to the accumulation of the end-product polymer (formed during the oxidative polymerization of phenolic compounds by peroxidases) on the catalyst surface hindering the access of the substrate to the enzyme active site.[4,46]

Considering that the enzymes are not stable during storage, presenting an activity decrease over time, in addition to the impossibility of reuse in its free form, soybean peroxidase immobilized in activated carbon obtained from enzyme extraction waste was capable of maintaining the peroxidase activity over repeated uses, which is attractive from an economic point of view.

Conclusion

The immobilization of enzymes has become a topic of great interest in different technological areas. In this study, a support for soybean peroxidase immobilization was synthesized from the waste itself generated during the enzyme extraction process, characterizing a production cycle with a total utilization of biomass and minimal waste generation, which complies with green chemistry principles. The synthesized activated carbon exhibits a high surface area (1603 m^2 g^{-1}), an important feature during the immobilization process. An immobilization yield of 100% was achieved after optimizing the reaction parameters (enzyme: support ratio of 0.05:1 g, pH 3.0 (citrate-phosphate buffer) at temperature of 40 °C for a period of 1h) and after 10 reuse cycles the immobilized soybean peroxidase still showed activity, a considerable aspect compared to biocatalysts in their free form, which cannot be reused. From the results obtained we concluded that soybean peroxidase immobilized on activated carbon is amenable to differentiated applications, such as in the biocatalysis, bioremediation, in clinical diagnostics, biosensor construction and other biotechnological industrial processes.

Acknowledgments

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ARTIGO 3 – Development of a reusable and sustainable biocatalyst by immobilization of soybean peroxidase onto magnetic adsorbent

Artigo a ser submetido à revista Chemosphere

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Abstract

In this work we synthesized an activated carbon/magnetite composite by a co-precipitation method. The activated carbon was synthesized from the solid waste obtained in the extraction process of the peroxidase enzyme. The magnetic composite was used as support for immobilization of soybean peroxidase (SP). After the determination of the optimal immobilization parameters, a yield of 100% was achieved under the following conditions: proportion support:enzyme 1.0:0.05 g, equilibration time of 7 h, pH 3.0 (citrate buffer phosphate 0.1 mol L^{-1}) and temperature of 50 °C. The determination of pH of point of zero charge (pH_{PCZ}) also was done to assist in the understanding of the immobilization process at different pH values. Several techniques of characterization were used for the different materials before and after the immobilization, such as thermogravimetric analysis, elemental analysis composition (CHN), X-ray powder diffraction and Fourier transform infrared spectroscopy (FTIR). The biocatalyst presented excellent operational stability and was reused for 11 consecutive cycles, with a 30% decline in activity from the first cycle, remaining constant until the last. The magnetic properties inserted in the activated carbon contributed to removal of the

biocatalyst from the reaction medium without interfering in the adsorptive characteristics of activated carbon. Thus, the activated carbon/magnetite composite can be applied in different research fields with high performance.

Keywords: Composite; Biocatalysis; Magnetite; Immobilization**;** Agroindustrial waste.

1. Introduction

Enzymes are used in numerous biotechnological fields including biocatalysis, biosensors, biofuel cells, biomedicine and in emerging areas such as nanobiophotonics and nanobioelectronics (Secundo, 2013). These "green catalysts" are capable of performing specific reactions at elevated rate not reachable by traditional chemical or physical catalysis (Silva et al., 2016a).

Among several types of enzymes, peroxidases (E.C.1.11.1.7) have been receiving considerable attention owing to their ability to oxidize a wide spectrum of organic compounds, and are mainly used in the biotransformation of various phenolic compounds, aromatic amines, biphenyls and dyes present in different industrial wastewater (Bilal et al., 2017; Mohamed et al., 2013). In addition to applications related to environmental remediation of different organic compounds, peroxidases are widely used in organic synthesis, polymer synthesis, biosensor design, clinic studies, among others (Gao et al., 2016; Somturk et al., 2015).

Peroxidases are enzymes of the class of oxirredutases that use hydrogen peroxide as an electron acceptor to catalyze the oxidation of various types of compounds (Quintanilla-Guerrero et. al., 2008). Due to the extensive demand for peroxidase, numerous researches has been seeking alternative sources of reduced cost in replacement the commercial enzyme horseradish peroxidase. Among them, stands out soybean hulls, a lignocellulosic residue produced worldwide. About 200 million tons of soybean hulls are produced annually throughout the world, being generally burned for energy purposes or partially collected for use as animal feed (Miao et al., 2013).

In spite of numerous advantages of enzymatic treatments, these catalysts have some limitations, mainly in their free form, since they are susceptible to inhibition, in addition to reduced stability. Since both enzyme and product are in dissolved state, the separation becomes more difficult, consequently more costly (Eş et al., 2015).

In order to overcome these disadvantages and make biocatalysis more favorable, several researches have focused on the immobilization process (Bolivar et al., 2016; Jain et al., 2016; Prokopijevic et al., 2014; Saranya et al., 2015; Silva et al., 2016b; Torres et al., 2017). The term ‘immobilized enzymes’ refers to ‘enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously’ (Mohamad et al., 2015). Immobilizations of enzymes play an important role in cost-effective recovery, increased catalyst productivity and minimize or eliminate contamination by potential interferents (Jain et al., 2016; Mohamad et al., 2015).

Immobilization of enzymes can be achieved by different methods, including the entrapment, cross-linking, covalent linkage and adsorption (Zlateski et al., 2014). This latter process, in turn, is the simplest and most widely used method of immobilization, in which enzymes are adsorbed physically or attached onto the carrier material (Brito et al., 2017; Mohamad et al., 2015). Adsorption immobilization is maintained by weak non-specific forces such as van der Waals, ionic interactions and hydrogen bonds, depending on the natural properties of the substrate surface and the adsorbate (Eş et al., 2015). The weak nature of these interactions is interesting since they do not alter the native structure of the enzyme, avoiding any perturbation in its active site, maintaining the catalytic activity of the enzyme immobilized (Jesionowski et al., 2014).

Various types of supports have been employed in immobilization process, such as natural polymers like alginate, chitosan, collagen, cellulose, and synthetic polymers such as zeolites, ceramics, silica, glass and activated carbon, among others (Datta et al., 2013; Mehta et al., 2016). Among these can be highlighted the immobilization of enzymes in activated carbon since this support presents high specific surface area, well-developed porosity, good mechanical properties, inertness and non-toxicity, in addition to the presence of different surface functional groups, which together with other properties are directly related to their adsorption efficiency (Abussaud et al., 2016; Jain et al., 2016; Zhang et al., 2015).

These supports can be synthesized from numerous sources and efforts have been made to find low cost precursors that can be used with equal or superior efficiency to commercial activated carbons. Within this context, highlighted the agroindustrial wastes due to their desirable characteristics, such as availability at a low cost, considerable mechanical strength, among others. Examples of some by-products include rice straw, coconut shells, bagasse and sludge, among others. Torres et al. (2017) synthesized activated carbon of soybean hulls, a residue generated in large quantities in food industrialization, and used this material as a support

for the soybean peroxidase immobilization. After optimization of the immobilization parameters, a yield of 100% was achieved.

In spite of the numerous advantages of using activated carbon as a support for immobilization of enzymes, this matrix suffers some limitations, with regard the separation of the immobilized biocatalyst from the reaction medium. The introduction of magnetic nanoparticles (Fe_3O_4 and γ-Fe_2O_3, among others) to adsorbent materials has attracted the attention of several researchers and the industrial community (Abussaud et al., 2016; Li et al., 2017; Oh et al., 2015; Zarei and Sinapour, 2015). This technology allows effective separation and recovery of the activated carbon by a simple magnetic process (Zhang et al., 2015). The synthesis of composite results in the synergism between the magnetic property of the magnetite and the adsorptive of the activated carbon, contributing significantly to the immobilization process of enzymes.

Thus, the aim of this work was the development of a "magnetic green biocatalyst" synthesized via immobilization of soybean peroxidase (SP) on magnetic composite. This composite, in turn, was obtained by synthesis of the activated carbon from the solid residue obtained in the process of extraction of the enzyme peroxidase from the soybean hull, an abundant agroindustrial residue, and magnetized by the co-precipitation method. The immobilization parameters were evaluated, as well as the reutilisation capacity of the immobilized enzyme. The result of this work is a production of the magnetic biocatalyst with minimal waste generation and maximum biomass utilization.

2. Materials and methods

2.1 Enzyme obtention

The source of peroxidase enzyme used was soybean hull, a by-product generated in expressive amounts in the processing industries of soybean oil (Neto et al., 2013). The procedure for SP obtention was carried out according to Silva and collaborators with some modifications (Silva et al., 2016a). After the homogenization of the soybean hulls with pH 6.5 phosphate buffer, the homogenate was filtered in organza cloth generating a solid waste which is retained in this tissue. The enzyme extract was collected and subjected to precipitation with cold acetone until reaching 65% (v/v) and the waste was collected and stored for further synthesis of the activated carbon. After 12-14 h at -18 °C, the sedimentation was concluded and

the precipitate was separated by vacuum filtration. Finally, the precipitate containing the peroxidase was left at 30 °C for removal of waste acetone for approximately 72 h and stored.

The SP activity assay was based on the rate of guaiacol oxidation to the formation of tetraguaiacol (ε = 26.6 mM^{-1} cm^{-1}). One unit of peroxidase activity at pH 6.0 represents the formation of tetraguaiacol (μmol) per minute of reaction (Khan and Robinson, 1994).

2.2 Preparation of the activated carbon

The solid waste originated in the obtaining process of SP described above was used as a carbonaceous precursor for synthesis of activated carbon (AC). The synthesis of chemically activated carbon was carried out according to Torres and collaborators (2017).

2.3 Preparation of the magnetic adsorbent composite (MAC)

Activated carbon/magnetite composite was prepared from a suspension of 2.5 g AC in 140 mL of $FeSO_4.7H_2O$ (Synth; 72 mmol, 20 g in 140 mL of distilled water) aqueous solution at 90 °C and purged with N_2. Next, 11.23 g of KOH (Vetec; 200 mmol) and 1.62 g of KNO_3 (Vetec; 16 mmol) were added to 60 mL of distilled water and this solution was added dropwise over in the above suspension in order to precipitate the iron oxide onto the AC surface. Vigorous stirring was maintained for 1 h under N_2 atmosphere at 90 °C. The composite obtained was collected by magnetic separation and washed with distilled water until neutral pH, then oven dried at 60 °C overnight (Zhang et al., 2013 modified by Silva et al., 2016b).

2.4 Soybean peroxidase immobilization

The immobilization process was initially conducted by suspending 0.5 g of MAC in 10 mL of citrate phosphate buffer containing 0.025 g of SP. The first parameter evaluated was the variation of the enzyme mass (0.025; 0.05; 0.10; 0.25; 0.5 and 1.0 g) to a fixed amount of MAC (0.5 g). This suspension was kept under constant stirring for a period of 3 h at room temperature. Then, studies were made in order to achieve the highest percentage of immobilization of SP in MAC through the variation of different parameters, time (1; 2; 3; 4; 5; 6 and 7 h), pH (3.0; 4.0; 5.0; 6.0; 7.0; 8.0 and 9.0) and temperature (20; 30; 40; 50; 60 and 70 °C). The reactions were interrupted by the removal of the immobilized material from the reaction medium through the

application of a magnetic field. At the end, MAC was washed successively with distilled water to remove excess unbound enzyme, dried at 30 °C for 48 h and stored. To assist in understanding the enzyme immobilization process at different pH values, the pH corresponding to the point of zero charge (pH_{PCZ}) for the MAC was determined according to Torres and collaborators (2017).

Immobilization of SP was investigated by measurements of enzymatic activity according to Khan and Robinson (1994). The immobilization yield was calculated from the difference between the initial and residual activity according to equation 1 (Chagas et al., 2015; Silva et al., 2016b; Tavares et al., 2015; Torres et al., 2017):

$$\varphi_{(\%)} = (A_0 - A_F)/A_0 \quad x\,100 \qquad \text{Eq. (1)}$$

where: $\varphi_{(\%)}$ is the immobilization yield, A_0 is the enzyme activity of the supernatant before incubation, and A_F is the enzyme activity of the supernatant after the incubation period.

A schematic illustrating from the process of peroxidase enzyme extraction up to the immobilization of this biocatalyst in the obtained magnetic adsorbent composite is presented in Fig. 1.

2.5 Operational stability of immobilized enzyme

The operational stability of the SP immobilized in MAC (SP-MAC) was determined by repeated applications of immobilized enzyme in the assay of enzyme activity. After each cycle, the MAC containing the immobilized SP (80 mg) was removed by application of a magnetic field, and then dispersed again in a fresh reaction medium. At the end of each cycle, the MAC was washed with distilled water and the remaining SP activity was evaluated as described to item 2.1.

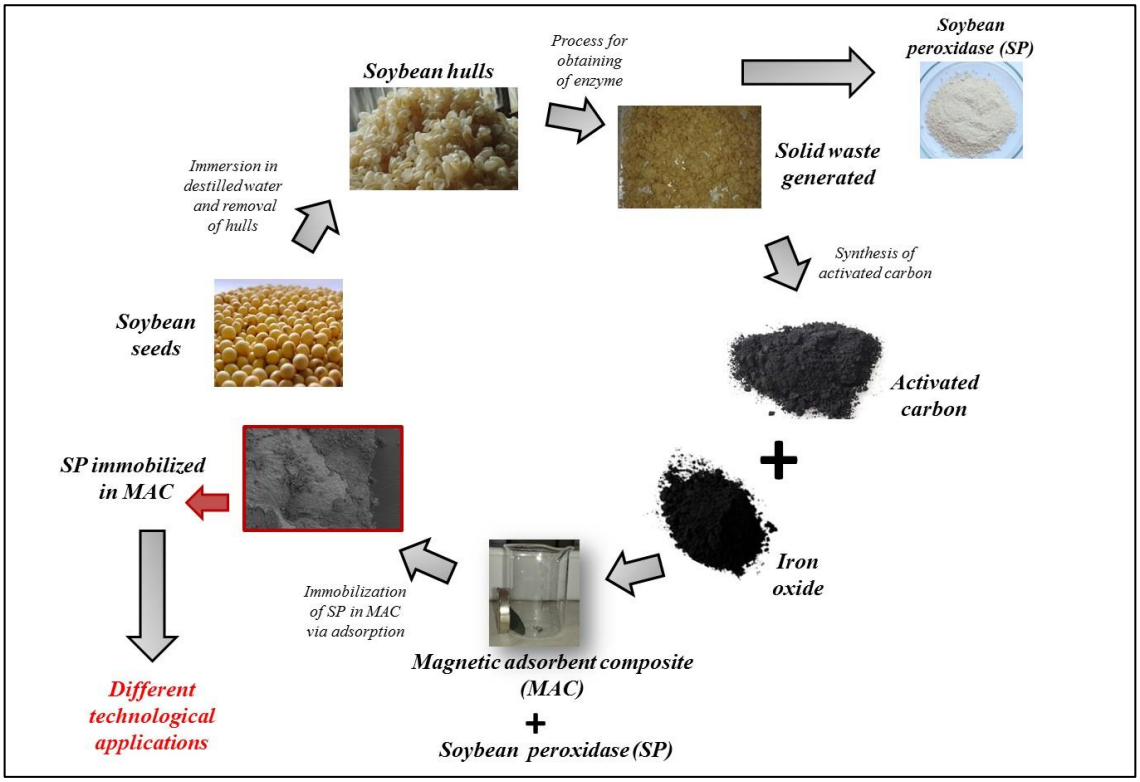

Fig. 1. Scheme of immobilized biocatalyst production with maximum biomass utilization.

2.6 Characterization

2.6.1 Thermogravimetric analysis (TG)

Thermogravimetric analysis (TG) of the materials (10 mg of sample) was performed in a Q50-TA Instrument. Analysis was performed under an air flow rate of 60 mL min^{-1} in the temperature range of 30 to 700 °C at a constant heating rate of 10 °C min^{-1}.

2.6.2 Elemental analysis composition (CHN) and specific surface area (BET)

The determination of carbon, hydrogen and nitrogen in the activated carbon and composite were performed in triplicate, using 10 mg of sample in a CHN elemental analysis equipment from Perkin Elmer 2400. The samples were weighed in tin capsules using a microbalance (Perkin Elmer AD-6 Auto Balance Controller) connected to CHN for direct

acquisition of the masses. All results are based on a value of a standard of known elemental composition.

The specific surface area of the activated carbon and magnetic adsorbent composite was measured with a Micromeritics ASAP-2020 instrument and calculated by the Brunauer-Emmett-Teller method. The materials were pre-treated (degasification) by heating at 70 °C under vacuum until reaching a degassing pressure of less than 20 mm Hg.

2.6.3 X-ray powder diffraction (XRD)

X-ray diffraction (XRD) patterns were recorded on a Shimadzu XRD6000 diffractometer using a nickel-filtered Cu-K_α radiation (λ = 1.5418 A). The diffractometer was operated at 30 kV and 30 mA in continuous scanning mode at a speed of 1.0° min^{-1} and a step width of 0.02° from 5° to 60° (2θ).

2.6.4 Scanning electron microscopy (SEM)

The morphology was investigated by scanning electron microscopy (FEI QUANTA FEG 250 ESEM, FEI Company, Hillsboro, Oregon, USA). Samples were carbon coated using carbon thread on a sputter-coater (BALTEC Maschinenbau AG Med model 020, Pfaffikon, Switzerland) with a carbon evaporative attachment prior to Secondary Electron (SE) and Backscattered Electron (BSE) imaging.

3 Results and discussion

3.1 Characterization

3.1.1 Thermogravimetric analysis (TG)

In order to evaluate the iron content and thermal stability, the materials were investigated in air atmosphere by TG (Fig. 2).

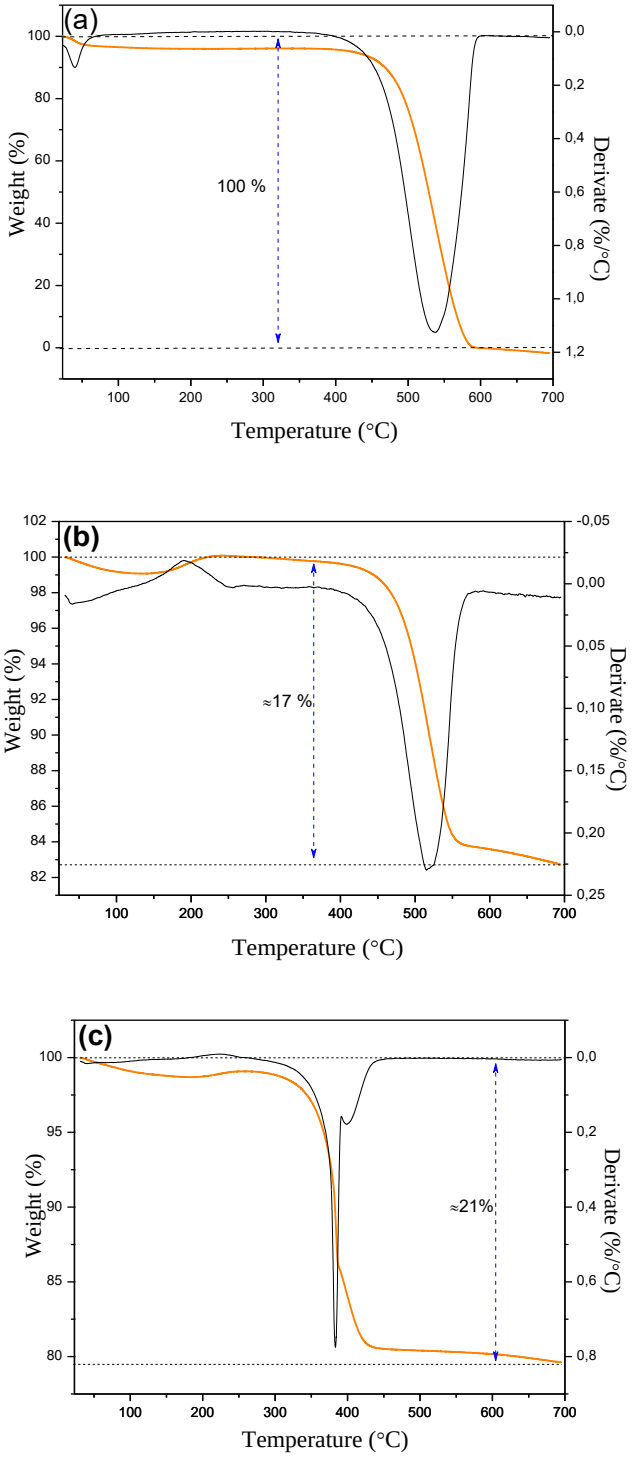

Fig. 2. Thermogravimetric analysis curves of activated carbon (a), magnetic adsorbent composite (b) and soybean peroxidase immobilized in magnetic adsorbent composite (c).

The slight mass loss of AC (Fig. 2 (a)) was attributed to the removal of adsorbed water molecules below 200 °C. In addition, the AC have not showed the presence of inorganic residues (ash), especially those related to the activating agent employed. The TG curve obtained for MAC and SP-MAC has typical characteristics of the presence of magnetite. The magnetite is readily oxidized to maghemite under air, and at temperatures above 300 °C this transformation continues until the formation of hematite (Cornell and Schwertmann, 1996). Thus, the increase in mass observed at approximately 200 °C was due to the oxidation of the magnetite to other forms.

The AC and MAC showed a significant weight loss in the range 420-580 °C, which can be attributed mainly to the decomposition of the carbon structure. The weight loss for the MAC was 17 wt% approximately, which may be related to the degradation of the activated carbon of the composite. On the other hand, weight loss of 21 wt% for SP-MAC was observed in a temperature range of approximately 275-425 °C, which may be associated with the presence of SP and its thermostable impurities on the MAC surface (Torres et al., 2017). In addition, the residual mass obtained for the MAC was 83%, whereas for the AC there was no residual mass, confirming the presence of magnetite in the synthesized composite. For the SP-MAC the percentage found was in 79%. Although the amount of activated carbon is much lower compared to the magnetite in the composite, this was sufficient to achieve an excellent immobilization of enzyme.

3.1.2 Elemental analysis composition (CHN) and specific surface area (BET)

The AC synthesized from the residue of SP extraction is essentially constituted by C, H and N as it can be seen in Table 1.

It is important to mention that the chemical and physics characteristics of activated carbon are directly related to the selected precursor and the conditions used during synthesis procedures (Bedin et al., 2016). An experimental study of Oliveira et al. (2009) showed that activated carbon from coffee husks, an agroindustrial residue, using different activating agents presented carbon content of 51.73% when using zinc chloride, corroborating with results obtained in this work (55.36%).

Table 1
Elemental analysis composition and specific surface area (BET).

Materials	Carbon (%)	Hydrogen (%)	Nitrogen (%)	BET (m^2 g^{-1})
Activated carbon	55.36	2.49	1.85	1581
Composite*	44.57	0.57	1.09	347

*Composite= activated carbon/magnetite (MAC).

The AC synthesized from the solid residue obtained after extraction of the SP presented a considerable specific surface area of 1581 m^2 g^{-1} (Table 1), which together with the surface functional groups, favour significantly the access of the adsorbate molecules to the surface of this adsorbent. After precipitation of the iron oxide on the support surface, there was a decrease in the specific surface area to 347 m^2 g^{-1}. This decrease may be related to the pores blocked of AC, as a result of the deposition of iron oxide particles and the low specific surface area of magnetite. These results are in agreement with numerous researches that evaluate the modification of the activated carbon from the incorporation of magnetic particles in its structure (Castro et al., 2009a,b; Gonçalves et al., 2013; Kyzas et al., 2014; Rey et al., 2016). Despite this effect, the magnetic adsorbent composite has a surface area close to that found in activated carbons synthesized by Brito et al., (2017), which ranges from 37 to 409 m^2 g^{-1}.

The determination of the textural characteristics of adsorbents is extremely important in adsorption processes, since the available surface area limits the amount of material that can be adsorbed by the matrix (Brito et al., 2017).

3.1.3 X-ray powder diffraction (XRD)

The XRD patterns of materials are given in Fig. 3. The XRD pattern of AC showed an amorphous halo centered at $2\theta = 23°$ degree which is a common feature of non-crystalline structures typically present in activated carbon (Li et al., 2017; Torres et al., 2017). In addition, the XRD results of MAC confirmed that the iron oxide was successfully synthesized, revealing the presence of the magnetite in the composite, due to the presence the Bragg diffraction at $2\theta = 18.3°$, 30.2^o, 35.6^o, 43.2^o, 53.5^o and 57^o referring, respectively, the diffraction planes (111), (220), (311), (400),(422) and (511). After the immobilization of SP, these peaks were maintained confirming the presence of the magnetite in the immobilized biocatalyst.

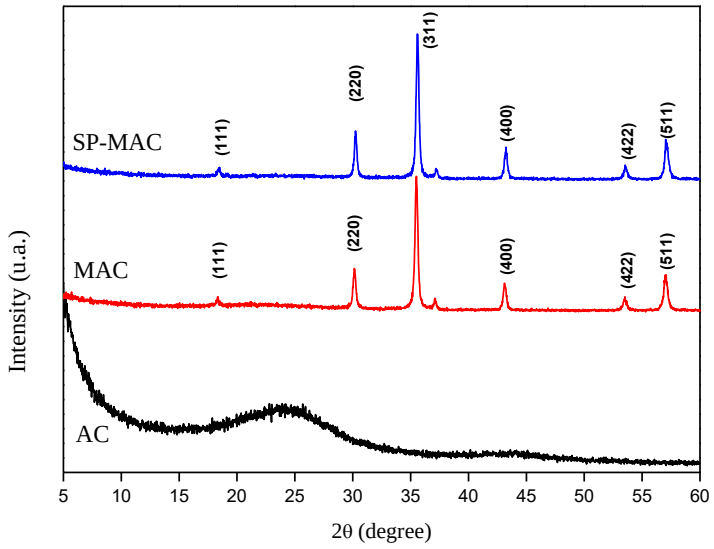

Fig. 3. X-ray powder diffraction patterns of activated carbon (AC), magnetic adsorbent composite (MAC) and soybean peroxidase immobilized in magnetic adsorbent composite (SP-MAC).

3.1.4 Scanning electron microscopy (SEM)

The SEM images of the activated carbon, the magnetic adsorbent composite and the composite after the immobilization of the soybean peroxidase are showed in Fig. 4.

The SEM images of the AC (Fig. 4 (a)) showed the presence of several cavities, indicating the development of porous structure characteristic of activated carbons after the chemical activation process. However, after addition of magnetite (Fig. 4(b) and (c)), the carbon matrix present several defects with a drastic morphological change, due to the presence of the magnetite to activated carbon. After the synthesis of the composite, the presence of several Fe_3O_4 nanocubes (approximately ~60 nm) covering the surface and pores of the AC with a good dispersion was observed. However, although the amount of magnetite was high, compared to the activated carbon, it was still possible to visualize a small region characteristic of the carbon matrix. After the immobilization of the SP onto MAC (Fig. 4 (d)), observed the formation of a different structure that covers the surface of this support, in the form of a shell, which may be related to the presence of the enzyme in MAC.

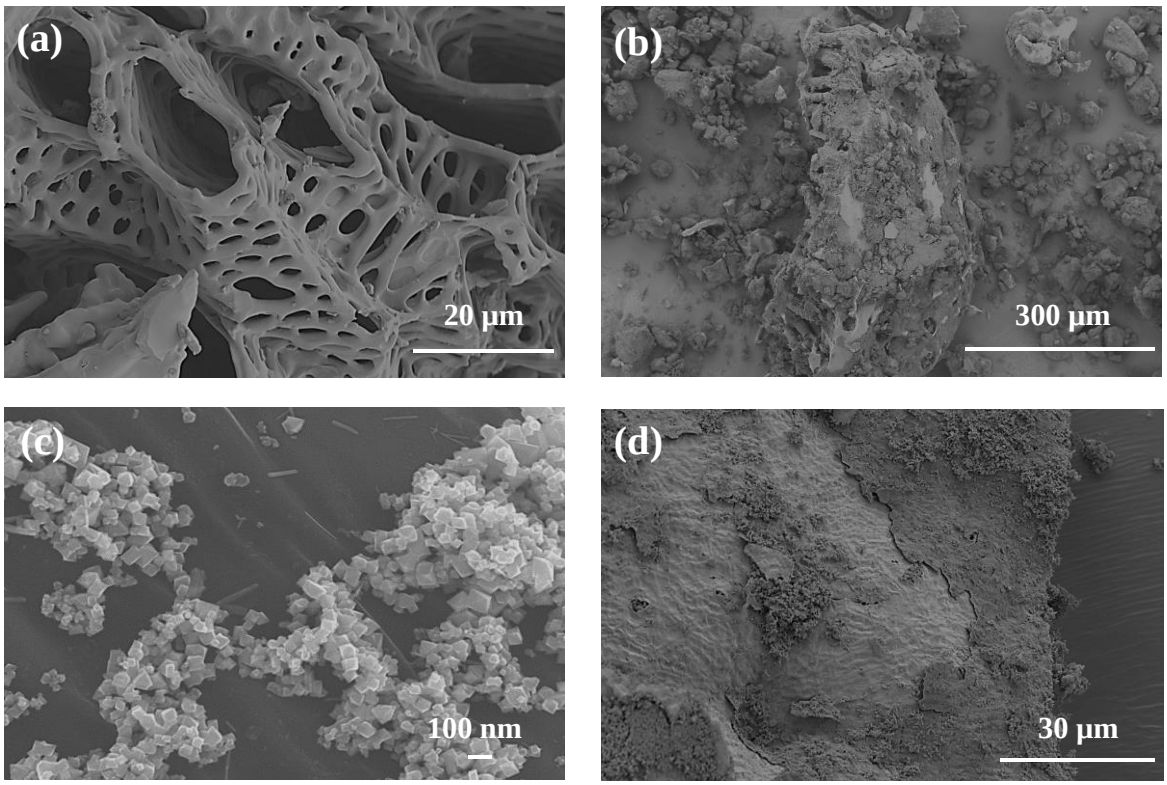

Fig. 4. Scanning electron microscopy images of activated carbon (a), magnetic adsorbent composite (b) and (c) at different magnifications, soybean peroxidase immobilized in magnetic adsorbent composite (d).

3.2 Soybean peroxidase immobilization

Although the immobilization process of enzymes by adsorption presents numerous advantages it is essential to optimize some reaction parameters in order to achieve a higher percentage of immobilization. Several parameters can be influence this process such as the proportion between carrier and enzyme, pore structure, particle size, surface area and mainly the type of functional groups present on the surface (Jain et al., 2016; Jesionowski et al., 2014). In addition, the activity of immobilized enzyme is influenced by some factors like effects of the support on the conformation of the enzyme and the diffusion limitations. The diffusion limitations occur when the protein-protein interactions lead to multilayers or clusters of enzyme molecules on the surface of the support, which generally occurs at elevated enzyme concentration, while in small amounts of enzyme, the low surface coverage leads to greater contact with the carrier used (Secundo, 2013).

In view of the above, the first parameter to be evaluated in the immobilization process of SP-MAC was the best relation between MAC and SP. According to Fig. 5, the highest immobilization (44.62%) was reached when the proportion of MAC to SP was 1.0:0.05 g.

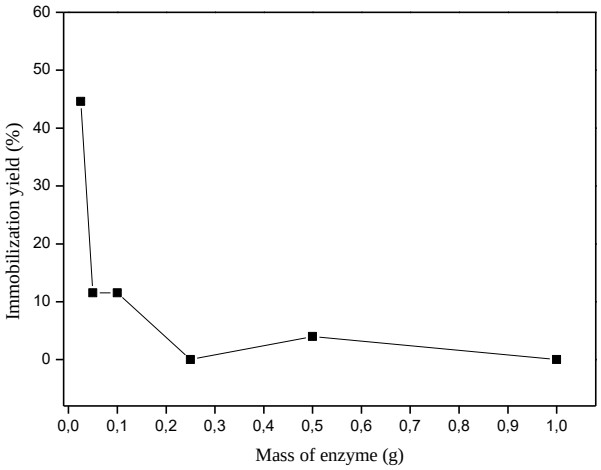

Fig. 5. Effect of enzyme load on the soybean peroxidase immobilization process in magnetic adsorbent composite (MAC). Reaction conditions: 0.5 g of MAC, pH 6.0, for 3 h at room temperature.

As the mass of enzyme increases, the tendency was to decrease the immobilization, since the excess of SP may result in an agglomeration of those enzymes inside the pore channels (Liu et al., 2012). Same result was found in other previous work, where we evaluated the immobilization of SP in pure AC. The higher yield of immobilization was also found by using low enzyme load (0.05 g), and the best proportion support: enzyme was 1.0: 0.05 g (Torres et al., 2017).

The second parameter to be evaluated in the immobilization process of soybean peroxidase in magnetic adsorbent composite to determine the equilibrium point, from which the yield of immobilization is maximum, was the time. The immobilization process through physical adsorption simply requires the contact of the enzyme solution with the support for a certain period of time for the physical adsorption to occur (Mohamad et al., 2015).

According to Fig. 6, it was observed that immobilization is a rapid process, and 26.15% of immobilization is reached in 1 h of reaction. This result is in agreement with numerous theories that consider that besides the versatility of the immobilization of enzymes through the adsorption, this method is characterized by the speed, simplicity and, mainly, the low cost, since the previous functionalization of the support is not required (Jesionowski et al., 2014; Silva et

al., 2016b; Zheng and Jiang, 2014). In 5 h immobilization there was a slight increase in yield (approximately 10%) reaching 37.45%, while in 7 h this yield was 49.56%. Thus, it was observed that the increase in immobilization yield was not very accentuated between the studied times, justifying the interruption of the reaction in 7 h.

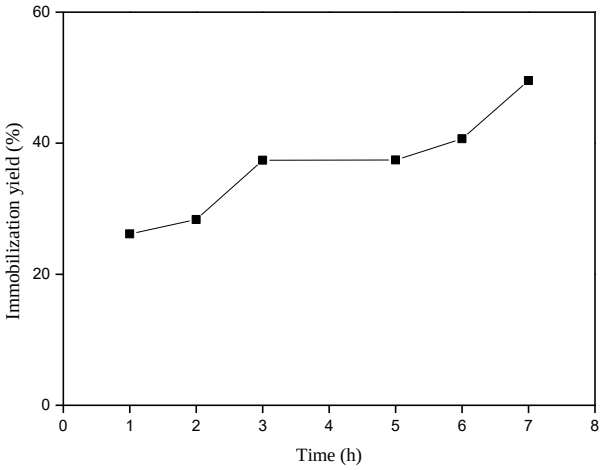

Fig. 6. Effect of time on the soybean peroxidase (SP) immobilization process in magnetic adsorbent composite (MAC). Reaction conditions: 0.5 g MAC; 0.025 g SP, pH 6.0, at room temperature.

Mohamad et. al., (2015) noted that this process is susceptible to pH and temperature variations. Thus, the third parameter evaluated was the variation of pH in the range from 3 to 9 during the immobilization SP. According to the Fig. 7 the higher immobilization yield of SP in MAC was obtained at pH 3.0 (100%).

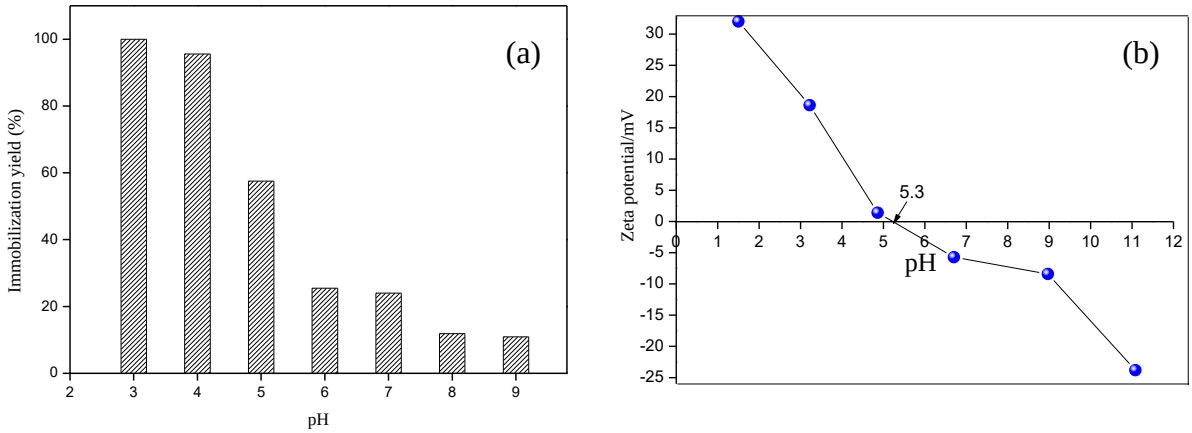

Fig. 7. Effect of pH on the soybean peroxidase (SP) immobilization process in magnetic adsorbent composite (MAC). Reaction conditions: 0.5 g of MAC, 0.025 g of SP, for 7 h at room temperature (a); Zeta potential of the MAC at different pH values (b).

The charged amino acids present in the structure of the enzyme can establish electrostatic interactions (attraction or repulsion) with the surface groups present in the support. Thus, the pH is parameter that can be optimized to modulate electrostatic interactions (Secundo, 2013).

The higher immobilization yield at pH 3.0 can be explained as a function of the MAC surface charge acquired in this pH value. The surface of the adsorbent is positively charged at pH values lower than pH_{PCZ} ($pH < pH_{PCZ}$), once the pH_{PCZ} found for this material was 5.3, which was observed a marked improvement in the immobilization yield. In contrast, at pH values higher than pH_{PCZ} the surface has negative charges ($pH > pH_{PCZ}$). Thus, this behavior in the immobilization of SP in MAC suggests that at pH values lower than pH_{PCZ} the side chain of the amino acids of the protein are negatively charged, which favors the electrostatic interaction between the surface of the support and the enzyme. On the other hand, at pH values above pH_{PCZ} the immobilization decreases.

The immobilization yields found at different pH values corroborate with the potentials obtained. At pH 3.0 the zeta potential was 18.63 mV and the immobilization yield was 100%, whereas at pH 5.0 the potential was 1.4 mV with a yield of 57.5%. The same decline in zeta potential and immobilization yield can be observed between pH 5.0 and 7.0 (potential zeta 1.4 and -5.72 mV, and immobilization yield 57.5% and 24.01%, respectively).

Torres et. al., (2017) evaluating the immobilization yield of SP in AC found similar behavior to that obtained in this work. The pH of the zero charge point for this adsorbent was 7.3, and a pronounced immobilization was achieved at pH values lower than pH_{PCZ}, suggesting that the enzyme surface was negatively charged in these pH, and at pH values above pH_{PCZ} there was a decrease in immobilization yield.

Although the immobilization yield at pH 3.0 have been 100%, the pH 4.0 was chosen to evaluate the next parameters, since at this pH the yield was also satisfactory (95.57%) and the difference found was minimal.

The adsorption process is governed basically by three steps: the external diffusion of the adsorbate through the boundary layer from the adsorbent surface, pore diffusion or intraparticle diffusion and the adsorption of the adsorbate on the surface active sites (Bedin et al., 2016). These phenomena are influenced directly by the temperature during the adsorption process, and may have positive and/or negative effects. Considering the importance of this parameter during the immobilization process by adsorption, was carried a study of the effect of temperature during the immobilization process of SP in MAC (Fig. 8).

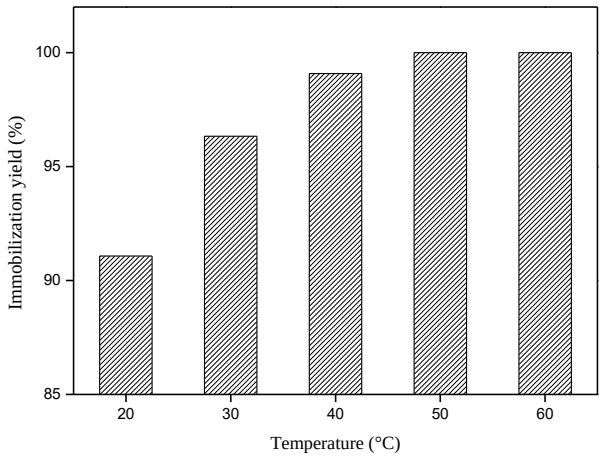

Fig. 8. Effect of temperature on the soybean peroxidase (SP) immobilization process in magnetic adsorbent composite (MAC). Reaction conditions: 0.05 g of MAC, 0.025 g of SP, pH 4.0, for 7 h.

According to the Fig.8, the immobilization suffer a slight increase with the temperature increase, since at 20 °C the immobilization yield reached was 91.07%, while at 50 °C this percentage was 100%, that was, an increase of only 8.93%. Thus, this difference was minimal, which is extremely advantageous, since the immobilization can be carried out at room temperature, reducing process costs. This phenomenon may be associated with the increase in the mechanism of diffusion (increase kinetic energy) of the enzyme molecules across the external boundary layer and in the internal pores of the adsorbent particle. In addition, at higher temperatures the enzyme molecules acquire enough energy to interact with the surface groups of the composite (Aboua et al., 2015; Ahmad and Alrozi, 2011; Brito et al., 2017; Kang et al., 2013).

Therefore, after a study evaluating of the best conditions for the higher immobilization of SP in MAC, the following conditions were established: relation support:enzyme 1.0:0.05 g, time of equilibrium of 7 h, pH 3.0 (citrate buffer phosphate 0.1 mol L^{-1}) and temperature of 50 °C.

Thus, the best parameters obtained are not necessarily the ideal conditions for immobilization, especially in industrial applications, where cost reduction is indispensable.

3.3 Operational stability of immobilized enzyme

One of the great advantages of the enzyme immobilization process is the possibility of reusing them, a powerful tool and extremely viable in differentiated industrial applications. Moreover, the magnetic properties of the composite facilitates their removal from the reaction medium by the application of a magnetic field, avoiding several steps in the separation process, frequently used in heterogeneous catalysis. Thus, in order to evaluate the reusability of SP immobilized in MAC, repeated applications of this biocatalyst were carried out in the reaction medium to determine its activity (Fig. 9).

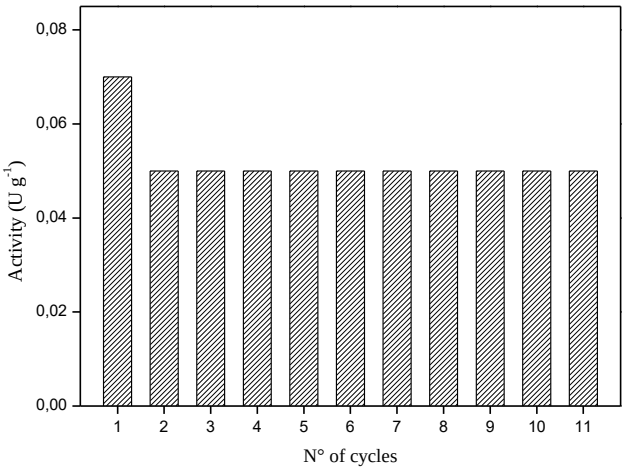

Fig. 9. Operational stability of immobilized soybean peroxidase (SP) in magnetic adsorbent composite (MAC) in the activity assays. Reaction conditions: 80 mg of SP immobilized in MAC, citrate-phosphate buffer pH 6.0 (0.1 mol L^{-1}), guaiacol (1% v/v) and hydrogen peroxide (0.3% v/v).

According to the results, SP-MAC showed an excellent operational stability, with a slight decline in its activity only from the first to the second cycle, approximately 30%. From then on, its activity remained constant until the eleventh application. This high reusability can be related to the protection effect of the support exerted on the enzyme and/or by magnetic recovery of the SP-MAC from the reaction medium, which favors a low weight loss of the catalyst (Zheng et al., 2017).

The decrease in SP-MAC performance after the first cycle may be related to the accumulation of final products of the reaction on the surface of the immobilized biocatalyst,

since according to previous work experiments leaching of enzyme was not observed using the same soybean peroxidase and activated carbon as support (Torres et al., 2017).

Thus, the catalyst developed can be used in different industrial applications, such as in the food, pharmaceutical, wastewater treatment, textile, and other industries, due to the oxidative and selective character of the enzyme incorporated in the carbon. In addition, the adsorptive and magnetic properties of AC contribute to the process of removal of pollutants.

Conclusion

The immobilization of enzymes circumvents problems related to their stability and the incorporation of magnetic nanoparticles facilitates their removal from the reaction medium by the application of a magnetic field. In this study, a magnetic composite was successfully formed with activated carbon.

The synthesis method of magnetite by co-precipitation over an activated carbon suspension was effective for the synthesis of a magnetic composite. A reduction of approximately 78% in the specific surface area of the carbon was observed after the synthesis of the composite, which was already expected due to the higher proportion of magnetite compared to the activated carbon. Despite this decrease, 100% immobilization of soybean peroxidase was achieved in the magnetic composite obtained after optimization of the immobilization parameters. The best conditions were: relation support:enzyme 1.0:0.05 g, time of equilibrium of 7 h, pH 3.0 (citrate buffer phosphate 0.1 mol L^{-1}) and temperature of 20 to 50 °C.

The presence of the magnetite in the synthesized composite was extremely efficient to assist in the removal of immobilized biocatalyst from the reaction medium through the application of a magnetic field, in addition to facilitating the reuse process. The synergism between adsorptive property of activated carbon and the magnetic property of magnetite, besides the high catalytic activity of the enzyme immobilized to the final support, are significant characteristics for the synthesis of stable biocatalysts that can be applied in several areas of the technology.

Acknowledgments

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