TÁSSIA SILVA TAVARES

DEVELOPMENT OF MAGNETIC NANOBIOCATALYSTS FOR ENVIRONMENTAL REMEDIATION

LAVRAS – MG

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

Prof. Dr. Teodorico C. Ramalho
Orientador
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Coorientadora

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DEVELOPMENT OF MAGNETIC NANOBIOCATALYSTS FOR ENVIRONMENTAL REMEDIATION

DESENVOLVIMENTO DE NANOBIOCATALISADORES MAGNÉTICOS PARA REMEDIAÇÃO AMBIENTAL

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

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LAVRAS – MG

2018

DEDICO

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"Não fiz o melhor, mas fiz tudo para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, já não sou o que era antes".

Marthin Luther King

A vida me ensinou...

"A vida me ensinou a dizer adeus às pessoas que amo, sem tira-las do meu coração. Sorrir às pessoas que não gostam de mim, para mostra-las que sou diferente do que elas pensam. Fazer de conta que tudo está bem quando isso não é verdade, para que eu possa acreditar que tudo vai mudar. Calar-me para ouvir e aprender com meus erros, afinal eu posso ser sempre melhor. A lutar contra as injustiças. Sorrir quando o que mais desejo é gritar todas as minhas dores para o mundo. A ser forte quando os que amo estão com problemas. Ser carinhoso com todos que precisam do meu carinho. Ouvir a todos que só precisam desabafar. Amar aos que me machucam ou querem fazer de mim depósito de suas frustrações e desafetos. Perdoar incondicionalmente, pois já precisei desse perdão. Amar incondicionalmente, pois também preciso desse amor. A alegrar a quem precisa. A pedir perdão. A sonhar acordado. A acordar para a realidade. A aproveitar cada instante de felicidade. A chorar de saudade sem vergonha de demonstrar. Me ensinou a ter olhos para "ver e ouvir estrelas", embora nem sempre consiga entendê-las. A ver o encanto do pôr-do-sol. A sentir a dor do adeus e do que se acaba, sempre lutando para preservar tudo o que é importante para a felicidade do meu ser. A abrir minhas janelas para o amor. A não temer o futuro. Me ensinou e esta me ensinando a aproveitar o presente, como um presente que da vida recebi, e usá-lo como um diamante que eu mesmo tenho que lapidar, lhe dando forma da maneira que eu escolher". Charles Chaplin

"Não é sobre chegar no topo do mundo e saber que venceu. É sobre escalar e sentir que o caminho te fortaleceu".

Ana Vilela

"Não é o mais forte que sobrevive, nem o mais inteligente, mas o que melhor se adapta às mudanças". Leon C. Megginson

RESUMO

A Química pode ser considerada como ciência chave na busca por um desenvolvimento sustentável, atuando no aprimoramento de processos para geração cada vez menor de resíduos e efluentes tóxicos. Esse novo caminho a ser delineado pela química é denominado "Química Verde". Dentre os princípios que regem a química verde, o uso de processos catalíticos é de vital importância, portanto, o objetivo dessa pesquisa inclui o desenvolvimento de nanobiocatalisadores magnéticos, os quais apresentam potencial para remediação de fenólicos, compostos tóxicos presentes em águas residuárias. A obtenção desses biocatalisadores foi realizada por meio da imobilização de enzimas oxirredutivas em nanopartículas magnéticas (NPs) de oxidróxido de ferro (δ-FeOOH). As peroxidases utilizadas foram a peroxidase de soja (SP) obtida das cascas de grãos de soja, um resíduo agroindustrial e a peroxidase de raiz forte (HRP) comercial obtida da Sigma-Aldrich. As NPs foram sintetizadas pelo método de co-precipitação e em seguida submetidas à funcionalização química para geração de grupos superficiais de interesse. A peroxidase de soja foi imobilizada nas NPs funcionalizadas, enquanto a imobilização da HRP foi investigada utilizando como suporte as partículas magnéticas não-funcionalizadas. Com a variação de importantes fatores de imobilização (proporção enzima/NPs, pH, temperatura e tempo) foi realizado um *design* estatístico de experimentos (DOE) para otimização do processo de imobilização. A Metodologia de Superfície de Resposta (MSR) foi uma técnica gráfica que possibilitou explorar a influências desses fatores no processo. As estruturas dos materiais foram avaliadas por Difratometria de Raios X (DRX), Espectroscopia de Infravermelho por Transformada de Fourier (IVTF) e Microscopia Eletrônica de Varredura (MEV). Para investigar o potencial catalítico dos nanobiocatalisadores desenvolvidos, avaliou-se a oxidação do poluente ácido ferúlico (AF), uma molécula modelo de lignina. Os resultados mostraram que a imobilização de SP em nanopartículas funcionalizadas (δ-FeOOH-SiO_2-APTES-GLU) é efetiva, com remoção de 93% do AF, e reciclagem em até 10 ciclos. A HRP imobilizada a partir das condições otimizadas resultou em um biocatalisador magnético capaz de remover 82% do AF. A obtenção de catalisadores magnéticos desenvolvidos utilizando enzimas de plantas bem como seus resíduos, mostrou ser uma alternativa que combina propriedades biocatalíticas e magnéticas em um único material, contornando algumas limitações dos processos catalíticos convencionais relacionados à recuperação e regeneração do catalisador.

Palavras-chave: Biocatálise. Química verde. Nanotecnologia. Oxidróxido de ferro. Quimiometria.

ABSTRACT

The chemistry can be considered as a key science for a sustainable development, working on the processes lead to produce less waste, decreasing toxic effluents generation. This new path laid out by the chemistry is denominated "Green Chemistry". Among the principles governing the Green Chemistry, the catalytic processes are vital importance, therefore, the objective of this research includes the development of magnetic nanobiocatalysts, which have a potential for phenolic remediation, toxic compounds present in wastewater. The biocatalysts were obtained immobilizing oxidoreductive enzymes on magnetic nanoparticles (NPs) of iron oxide (δ-FeOOH). Soybean peroxidase (SP) obtained from soybean hulls, an agro industrial residue, and horseradish peroxidase (HRP) commercial peroxidase obtained from Sigma-Aldrich were the peroxidases utilized in the processes. Co-precipitation was the method used to synthesize the NPs. For generating interest groups, the NPs were submitted to chemical functionalization. Soybean peroxidase was immobilized on functionalized NPs, whereas the HRP immobilization was investigated using the magnetic particles non-functionalized as support. With the variation of important immobilization factors (enzyme ratio / NPs, pH, temperature, and time) a statistical *design* of experiments (DOE) was performed to optimize the immobilization process. The Response Surface Methodology (MSR) was a graphical technique that allowed to explore the influences of these factors in the process. The structures of the materials were evaluated by X-ray diffractometry (XRD), Fourier Transform Infrared Spectroscopy (IVTF) and Scanning Electron Microscopy (SEM). To investigate the catalytic potential of the developed nanobiocatalysts, the oxidation of the pollutant ferulic acid (FA), a model molecule of lignin, was evaluated. The results have shown that the SP immobilization on functionalized NPs was effective. Removing 93% of AF, with recycling up to 10 cycles. HRP immobilized from optimized conditions resulted in a magnetic biocatalyst capable of removing 82% of FA. Obtaining magnetic catalysts developed using plant enzymes as well as their residues has proved to be an alternative that combines biocatalytic, and magnetic properties in a single material, overcoming some drawbacks of conventional catalytic processes related to the recovery, and regeneration of the catalyst.

Keywords: Biocatalysis. Green chemistry. Nanotechnology. Iron oxyhydroxide. Chemometrics.

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APRESENTAÇÃO

Esta tese está no formato de artigos, os quais encontram-se na seção Artigo. Os artigos estão formatados de acordo com as revistas científicas em que foram submetidos e/ou publicados. Na primeira parte consta a Introdução, Referencial Teórico e suas respectivas referências. As Considerações finais estão no final desse trabalho.

PRIMEIRA PARTE

1 INTRODUÇÃO

A poluição ambiental é um dos principais problemas que a sociedade enfrenta atualmente. O crescimento populacional, mudanças climáticas e poluição excessiva da água tem contribuído para a escassez da água limpa e segura. O setor industrial é o maior consumidor desse recurso natural limitado, gerando grandes volumes de águas residuárias, contendo diferentes tipos de contaminantes. Os compostos fenólicos são poluentes pertencentes a uma das maiores classes de compostos orgânicos. Esses compostos são frequentemente utilizados nas indústrias, e consequentemente, presente em águas residuárias. De acordo com o CONAMA, Resolução n° 430 de 13/5/2011, o controle dos teores máximos de compostos fenólicos em água é sujeito à análise de “fenóis totais”, portanto, 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}.

Diante disso, novas tecnologias estão sendo exploradas para remoção de contaminantes em águas residuárias. Diferentes tipos de materiais podem ser empregados na remediação ambiental, e uma ampla variedade de abordagens podem ser exploradas para esse fim. Na catálise química, ou seja, utilizando catalisadores sem origem biológica, o princípio da catálise ambiental é bem estabelecido com pesquisas envolvendo a remoção de contaminantes do ambiente.

Atualmente, a biocatálise está implicada em diversos setores, como por exemplo na biorremediação de compostos poluentes. A biocatálise utiliza catalisadores biodegradáveis, as enzimas, e está diretamente associada ao conceito de "química verde”, o qual foi introduzido na década de 90, e está relacionado aos processos sustentáveis.

As peroxidases de plantas são enzimas de grande importância ambiental, capazes de oxidar uma ampla variedade de poluentes orgânicos, os quais geralmente estão presentes em águas residuárias. As peroxidases encontradas na soja e na raiz-forte se destacam na remediação de efluentes contendo corantes, aminas e compostos fenólicos. A soja é um grão abundante no Brasil e a raiz-forte uma planta facilmente encontrada nos países europeus. Portanto, essas enzimas podem ser facilmente obtidas. Por outro lado, a economia na utilização de enzimas nos processos biocatalíticos é um fator importante para o setor industrial, estando dentro dos princípios da "química verde". Assim, a reutilização é uma alternativa, a qual pode ser obtida a partir da imobilização enzimática.

A imobilização de enzima em suporte sólido tem sido um processo inovador, o qual pode resultar na melhoria da estabilidade enzimática e reutilização dos biocatalisadores. Diferentes métodos

podem ser utilizados nesse processo. A adsorção é um dos processos mais simples e tem sido muito abordado. No entanto, na adsorção física, a enzima pode deixar o suporte devido as interações fracas envolvidas na imobilização. Por outro lado, a ligação covalente possibilita uma interação forte entre enzima e suporte, podendo melhorar a estabilidade das enzimas. Geralmente, esta técnica exige a funcionalização do suporte por meio da adição de grupos funcionais que possam favorecer a ligação da enzima ao suporte.

No que se refere a tecnologia enzimática, as nanopartículas despertam grande interesse como suporte para imobilização de enzimas. Tornam-se ainda mais atraentes quando essas partículas são magnéticas, o que permite facilmente a recuperação da enzima a partir do meio reacional, e consequentemente sua reutilização.

As nanopartículas de óxidos de ferro possuem características próprias, como biocompatibilidade, magnetismo e não apresentam toxicidade, o que as tornam promissoras para imobilização de biomoléculas. Os hidróxidos de óxido de ferro III (FeOOH) apesar de apresentarem propriedades atrativas são pouco explorados na tecnologia enzimática. O polimorfo δ-FeOOH é um óxido supermagnético, se destaca pela sua grande área superficial e excelente estabilidade, mas não tem sido relatado como suporte enzimático.

A escolha do suporte é um quesito primordial no processo de imobilização, e esse deve favorecer a ligação da enzima. Por isso, é comum a modificação da superfície dos materiais utilizados como suporte. Isso porque a adição de grupos funcionais na superfície propicia condições para ligação da enzima. O glutaraldeído é um modificador de superfície comumente utilizado. Os resíduos de aminoácidos presente nas enzimas interagem com o aldeído na superfície das partículas.

Dentro desta perspectiva, o presente estudo busca desenvolver nanobiocatalisadores recicláveis, de acordo com os princípios da química verde, visando a remediação de poluentes ambientais. Para isso, tem em vista a imobilização de peroxidases de plantas (peroxidase de soja e peroxidase de raíz forte) em nanopartículas magnéticas de ferroxita (δ-FeOOH) funcionalizadas e não funcionalizadas, e a utilização de uma abordagem quimiométrica para otimização do processo.

2 REFERENCIAL TEÓRICO

2.1 Biorremediação ambiental - Águas residuárias

O desenvolvimento da sociedade nas últimas décadas tem contribuído com a poluição do ambiente, o que causa danos à saúde humana, aos seres vivos e aos ecossistemas. Assim, a recuperação dos locais contaminados, como ar, solo, água, entre outros é necessária. A remediação, ou seja, remediar situações que causam danos ao meio ambiente, é uma técnica promissora (GUERRA et al., 2018). Neste contexto, a contaminação da água é uma das maiores preocupações da atualidade em que a remediação desse recurso limitado torna-se indispensável.

O Brasil é beneficiado com cerca de 12% da disponibilidade de água doce do planeta. Entretanto, a distribuição dos recursos hídricos não é equilibrada. Enquanto apenas 3% dos recursos hídricos estão nas regiões banhadas pelo oceano Atlântico, onde se concentra 45% da população, a Região Hidrográfica Amazônica possui 80% da quantidade de água disponível, mas a demanda é menor, com apenas 5% da população brasileira. A agricultura irrigada é a atividade que mais consome água em todo o mundo. Já no Brasil, o setor industrial nas regiões Sudeste e Sul são os maiores consumidores (ANA, 2018), resultando em grandes volumes de águas residuárias a cada ano.

As águas residuárias provenientes das atividades industriais apresentam diferentes graus de impurezas. A poluição da água ser definida como a presença de produtos químicos tóxicos e agentes biológicos acima dos níveis naturais. Os metais pesados, pesticidas, herbicidas, fertilizantes, derramamentos de óleo, gases tóxicos, efluentes industriais, esgotos e os compostos orgânicos são apenas alguns exemplos dos muitos poluentes encontrados em águas contaminadas (ABOUZEID et al., 2018; BISHOGE et al., 2018; EL KHAMES et al., 2014).

Águas residuárias das indústrias de óleo, azeite e petróleo possuem como principal componente o ácido trans-ferúlico (AF), um composto fenólico recalcitrante, com forte resistência à oxidação. Esse composto também é utilizado frequentemente na indústria farmacêutica, como matéria-prima para formulação de drogas utilizadas no tratamento de doenças como Alzheimer, câncer e diabetes mellitus (KUMAR; PRUTHI, 2014). Devido à sua extensiva utilização, o AF já foi encontrado em rios, lagos e sedimentos marinhos (MANCUSO; SANTANGEL, 2014). Sendo assim, a remediação de águas residuárias é de extrema importância na tentativa de evitar que compostos poluentes como o AF atinjam o meio ambiente.

Sendo a conservação da água um assunto abordado em todo o mundo (ABOUZEID et al., 2018; BISHOGE et al., 2018), sua reutilização é um caminho alternativo para minimizar o problema de

escassez de água no planeta. Entretanto, o tratamento da água contaminada exige a eliminação dos poluentes presentes, o que requer tecnologias de tratamento ambientalmente amigáveis. A biocatálise está dentro desse contexto, uma vez que as enzimas são biocatalisadores sustentáveis que atuam na conversão de compostos orgânicos por meio de condições brandas de reação.

A biocatálise refere-se ao uso de célula microbiana ou enzima livre em conversões catalíticas. A enzima livre, ou seja, fora da célula em que foi produzida, é uma abordagem interessante, e supera a limitação de difusão de substratos, a qual é encontrada quando as enzimas estão nas células (SHELDON; WOODLEY, 2017). Apesar dos benefícios óbvios da biocatálise, os principais obstáculos que dificultam sua exploração são, em muitos casos, os altos custos e baixos rendimentos na purificação de enzimas, limitando a obtenção desses biocatalisadores (ALCALDE et al., 2006).

Por outro lado, a precipitação de proteínas a partir da adição de solventes orgânicos é uma alternativa simples e barata para obtenção de enzimas livres (SILVA et al., 2016a; 2016b; TORRES et al., 2017). Essa técnica leva a formação de agregados proteicos que são mantidos juntos por interações que não afetam a estrutura terciária das enzimas (SHELDON; WOODLEY, 2017). Desse modo, é possível a o thbtenção de biocatalisadores provenientes de plantas, como as peroxidases, enzimas de grande importância na biorremediação ambiental.

2.2 Peroxidases

Peroxidases são enzimas oxidorredutases encontradas em microrganismos, animais e plantas. Todas as heme-peroxidases (E.C. 1.11.1.7) possuem um grupo prostético ferriprotoporfirina IX localizado no sítio ativo. As peroxidases têm encontrado aplicações em diferentes processos industriais devido à sua alta estabilidade em solução, seletividade de substratos e tolerância à ampla faixa de pH e temperatura (GUZIK; HUPERT-KOCUREK; WOJCIESZYNSKA, 2014).

As peroxidases de plantas, como peroxidase de raiz-forte (HRP) e peroxidase de soja (SP) têm despertado grande interesse na remediação de compostos orgânicos (GUZIK; HUPERT-KOCUREK; WOJCIESZYNSKA, 2014; SILVA et al., 2013; SILVA et al., 2015; TORRES et al., 2017). Sendo a HRP e a SP isoenzimas, compartilham características comuns (Tabela 1).

A HRP é obtida a partir da raiz-forte (*Armoracia rusticana*), uma erva perene abundante em regiões temperadas, enquanto a SP está presente na casca da soja (*Glycine max)*, um subproduto do processamento de grãos de soja. Sendo o Brasil o maior produtor de soja (CONAB, 2018), as agroindústrias brasileiras geram grande quantidade de resíduos de cascas de soja. Nesses resíduos concentram-se quantidades significativas de peroxidases de soja (SILVA et al., 2013). Portanto, a

obtenção de peroxidase a partir desse resíduo, além de minimizar o descarte desses no ambiente, é também um meio de baixo custo para obtenção de peroxidases (SILVA et al., 2015; TORRES et al, 2017).

Tabela 1 Comparação das propriedades bioquímicas e estruturais da HRP e SP.

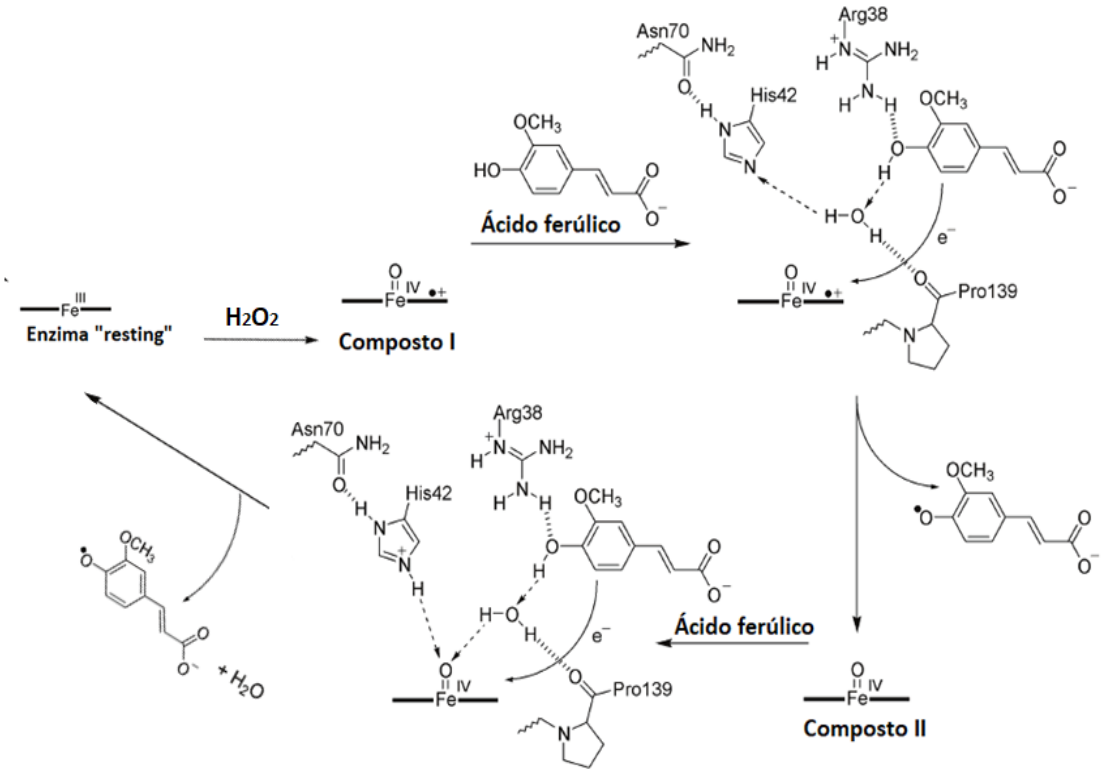
	Peroxidase de raiz-forte (HRP)	**Peroxidase de soja (SP)**
Espécie	Armoracia rusticana	Glycine max
N° de aminoácidos	306	306
Classificação	1.11.1.7	1.11.1.7
Peso molecular	44,100 Da	40,660 Da
Carboidratos	7,580 Da	7,400 Da
Grupo heme	550 Da	550 Da
Ca^{2+}	80 Da	80 Da
Sítios de glicosilação	Asn: 13, 57, 158, 186, 198, 214, 255, 268, 316	Asn: 185, 197, 211, 216
pI	9,0	4,1
pH	(4-8)	(2-10)
Estrutura secundária	13 α-helices 3 β-sheets	13 α-helices 2 β-sheets

Fonte: RYAN; CAROLAN; Ó'FÁGÁIN (2006); PDB: HRP (1ATJ), SP (1FHF).

A HRP é frequentemente utilizada para exemplificar o ciclo catalítico das peroxidases. Essas enzimas atuam oxidando uma molécula de determinado composto orgânico, enquanto catalisa a redução do peróxido de hidrogênio. O ciclo de reações é formado por três etapas, o qual inicia com a oxidação de dois elétrons da porção heme da enzima (Fe^{+3}) pelo H_2O_2, resultando na formação do composto intermediário I (Fe^{+4}=O) e um cátion radical porfirínico (π). O composto I é uma espécie química com alta capacidade oxidante, um estado de oxidação mais alto em comparação com a enzima nativa. Sucessiva redução de um elétron retorna a enzima a sua forma nativa, por meio da formação de um segundo intermediário, o composto II (Fe^{+4}=O) (DERAT; SHAIK, 2006; POULOS, 2014; VEITCH, 2004).

No mecanismo de oxidação de compostos fenólicos e na formação dos compostos I e II, em pH 7, a oxidação das moléculas do substrato leva a desprotonação dos cátions radicais dos compostos I e II, produzindo assim radicais fenoxilo (Figura 1). A oxidação do fenol por HRP é um processo irreversível, o qual ocorre por meio do mecanismo catalítico do tipo ping-pong (GÓMEZ et al., 2005; MAGARIO et al., 2012).

Figura 1 - Mecanismo de oxidação do ácido ferúlico e formação dos compostos intermediários I e II.



Fonte: Adaptado de HENRIKSEN; SMITH; GAIHEDE, (2001); MAGARIO et al., (2012).

No mecanismo proposto para oxidação do ácido ferúlico, bem como outros substratos fenólicos menores, os aminoácidos histidina (His42) e arginina (Arg38) presentes na estrutura da HRP contribuem para a oxidação do substrato. A Arg38 atua na transferência de prótons e se liga ao substrato fenólico. A Arg38 também contribui na formação e liberação de uma molécula de água, a qual é necessária para formação dos compostos intermediários. A molécula de água é o meio para transferência de prótons do substrato para His42 e O ferrilo (Fe^{+4}=O), na redução dos compostos I e II, respectivamente. Quando o composto II é formado, o próton do radical fenoxilo é abstraído por uma base, provavelmente a His42. Então, o composto II oxida a segunda molécula de fenol, formando outro radical fenoxilo pela transferência de um elétron para o ferrilo (Fe^{+4}=O), desse modo a enzima nativa é regenerada (HENRIKSEN; SMITH; GAIHEDE, 2001; MAGARIO et al., 2012).

Os radicais formados durante o processo catalítico reagem entre si, formando moléculas poliaromáticas solúveis, e a maioria delas apresentam características de substrato da enzima, podendo ser utilizadas como substrato em um próximo ciclo enzimático. O ciclo continua até que os polímeros gerados alcançam seu limite de solubilidade e precipitam na solução, podendo ser removido por

processos simples de separação. Desse modo, a demanda de peróxido de hidrogênio (H_2O_2) para a reação global de um substrato aromático é sempre maior do que o previsto pela estequiometria da reação (NICELL, 1994).

As reações de polimerização apresentam grande potencial nos processos de biorremediação. No entanto, a recuperação de enzimas solúveis a partir do meio catalítico é limitada. Diante disso, a facilidade de separação a partir do meio reacional e a reutilização desses biocatalisadores tornou-se quesito importante para os processos enzimáticos, o que pode ser efetivamente alcançados por meio da imobilização de enzimas.

2.3 Imobilização de enzimas

As enzimas são catalisadores biodegradáveis podendo ser obtidas a partir de recursos naturais renováveis (ALCALDE et al., 2006). Os processos enzimáticos são conduzidos em condições brandas de reação. Além disso, as enzimas podem ser destacadas pela sua seletividade por diferentes substratos. Apesar de todas estas vantagens, a aplicação de enzimas é frequentemente limitada pela sua baixa estabilidade operacional e dificuldade de recuperação e reutilização (SHELDON; VAN PELT, 2013).

A imobilização de enzimas é um dos processos mais inovadores das últimas décadas, o qual torna possível superar as limitações das enzimas livres (SHELDON; VAN PELT, 2013; SHELDON; WOODLEY, 2017). A enzima imobilizada é aquela fisicamente ou quimicamente associada a um suporte. Por meio da imobilização é possível converter biocatalisadores homogêneos em biocatalisadores heterogêneos, e assim, possibilitar sua reutilização (DICOSIMO et al., 2013; SHELDON; VAN PELT, 2013; SHELDON; WOODLEY, 2017).

Dentre os diversos fatores envolvidos no processo de imobilização, a escolha do suporte é uma das etapas cruciais. Os suportes são geralmente divididos em orgânicos (quitina, quitosana, celulose e alginato), inorgânicos (sílicas, titânia, hidroxiapatita e óxidos) e sintéticos (polímeros) (JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014). Os critérios para escolha adequada do suporte dependerão das características da enzima e das condições nas quais a enzima imobilizada será utilizada. O custo, a disponibilidade, estabilidade e/ou reatividade do suporte também devem ser considerados. Além disso, a área superficial, o tamanho das partículas e os grupos funcionais presentes na superfície são parâmetros importantes (DICOSIMO et al., 2013; JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014).

De acordo com o suporte enzimático, diferentes métodos de imobilização podem ser realizados (Figura 2), sendo usualmente abordado as técnicas de confinamento (encapsulamento ou

microencapsulamento), adsorção física (interações do tipo Van der Waals e ligações de hidrogênio), adsorção química (ligação covalente), adsorção iônica (interação eletrostática) e reticulação (Figura 2) (JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014; SHELDON; VAN PELT, 2013; SHELDON; WOODLEY, 2017).

O confinamento pode ocorrer por meio da encapsulação da enzima em géis ou fibras (DICOSIMO et al., 2013). No gel, a enzima é confinada nos espaços intersticiais da rede polimérica (interior de micelas), enquanto que nas fibras o confinamento é nas micro cavidades. No micro encapsulamento as enzimas são imobilizadas no interior de pequenas esferas de membranas poliméricas (colágeno, celulose, policarbonato) (DICOSIMO et al., 2013; SHELDON; VAN PELT, 2013). No entanto, as barreiras físicas do suporte são fracas para evitar o vazamento enzimático (REETZ, 2013).

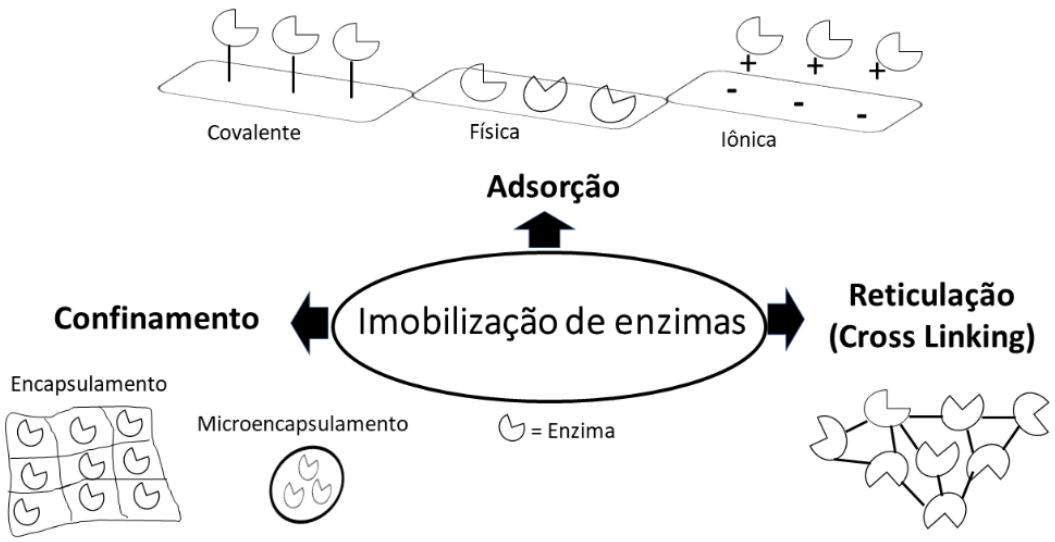
Nos processos envolvendo adsorção física, a interação é fraca, o que é interessante, pois normalmente não altera a estrutura nativa da enzima. Isso impede alterações nos sítios ativos da enzima, permitindo que a enzima retenha sua atividade (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011; JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014). Por outro lado, a adsorção física apresenta limitações em certas condições de utilização. Por exemplo, em meio aquoso a enzima imobilizada por adsorção física pode se desprender do suporte (CANTONE et al., 2013). Essa limitação pode ser contornada nos processos de imobilização envolvendo ligações covalentes.

A ligação covalente possibilita uma forte interação entre enzima e suporte, o que resulta na diminuição da flexibilidade da estrutura da proteína, estando isso associado a melhorias na estabilidade enzimática (GUZIK; HUPERT-KOCUREK; WOJCIESZYNSKA, 2014). As enzimas podem ser imobilizadas covalentemente de forma aleatória ou orientada. Enzimas imobilizadas aleatoriamente podem ter sua estrutura orientada de maneira que limita o acesso do substrato ao sítio catalítico. Por outro lado, a imobilização covalente quando orientada é uma opção a ser considerada (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011).

A explicação sobre a imobilização orientada é encontrada através do mecanismo envolvido no processo. No caso de suportes capazes de imobilizar covalentemente as proteínas por meio de grupos amino primário, a imobilização ocorre principalmente através do aminoácido abundante nas proteínas, a lisina (Lys), levando a uma imobilização enzimática aleatória. Assim, quando em pH abaixo do seu pI (6,02) os grupos aminos da Lys estão carregados positivamente e acima desse pH carregados negativamente. Em pH neutro, a reatividade da Lys externa é significativamente reduzida, enquanto o grupo amino de aminoácidos terminais apresentam uma reatividade superior mesmo em menor quantidade. Já nos casos envolvendo a modificação da superfície do suporte pela adição de grupos

aldeídos, em pH neutro, a imobilização é orientada. Neste caso, as enzimas são imobilizadas através do grupo amino dos aminoácidos terminais (HERNANDEZ; FERNANDEZ-LAFUENTE, 2011).

Figura 2 - Métodos de imobilização de enzimas.

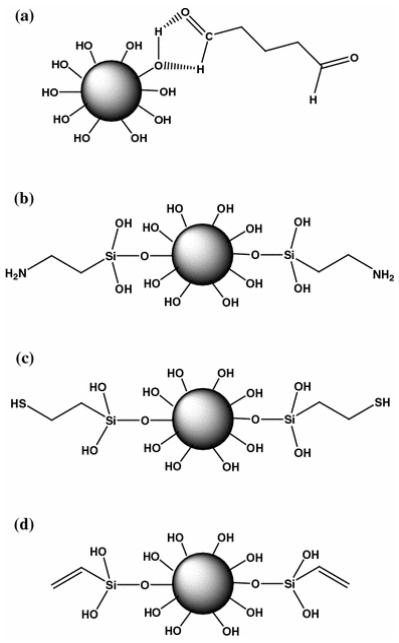


Desse modo, os grupos funcionais presentes na superfície do suporte constituem um dos fatores importantes na imobilização enzimática. Sendo assim, a etapa de funcionalização do suporte antes do processo de imobilização é muito comum (CHANG; TANG, 2014; SILVA et al., 2015). A funcionalização, ocorre por meio da adição de agentes intermediários, ou seja, modificadores da superfície. Geralmente, o agente modificador possui dois grupos reativos, um que permite a interação com o suporte, e outro disponível para interação com a enzima (JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014) (Figura 3).

Os compostos silanos são exemplos de agentes modificadores da superfície de suportes enzimáticos. No processo de funcionalização da superfície, os grupos sofrem hidrólise para grupos hidroxila presentes no suporte (por exemplo suporte de sílica), permitindo a formação de ligações de hidrogênio e ligações covalentes com o suporte. A presença de grupos funcionais na superfície do suporte (-CHO, -SH, -NH) compatíveis com os grupos funcionais das enzimas, possibilita as interações entre enzima - agente modificador - suporte (HOLA et al., 2015; JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014; MIN; YOO, 2014).

Os compostos orgânicos bifuncionais podem ser utilizados como agentes modificadores, atuando como uma ponte entre enzima e suporte. Atualmente, o glutaraldeído $CH_2(CH_2CHO)_2$ é um modificador de superfície frequentemente utilizado nos processos de imobilização: além de possuir dois grupos aldeídos reativos, também contribui como um espaçador entre a enzima e o suporte, deixando os locais ativos da enzima mais acessíveis para os substratos (CHANG; TANG, 2014; JESIONOWSKI; ZDARTA; KRAJEWSKA, 2014).

Figura 3 - Representação dos agentes modificadores de suportes com sílica a) Glutaraldeído; b) 3-aminopropiltrietoxisilano (APTES) c) Mercaptopropiltrietoxisilano (APTMS), d) viniltrimetoxissilano (VTMS).



Fonte: JESIONOWSKI; ZDARTA; KRAJEWSKA, (2014).

A reticulação é outra técnica de imobilização que envolve a reação dos resíduos de aminoácidos das enzimas, geralmente de lisina, com polímeros de glutaraldeído (MIGNEAULT et al., 2004). Inicialmente, as enzimas reticuladas eram produzidas pela mistura da solução enzimática em solução tampão contendo glutaraldeído. Em 1990, os avanços científicos levaram a obtenção de cristais de enzimas reticulados (CLECs). Os CLECs apresentam excelente estabilidade operacional e alta produtividade.

Apesar desse método ser condicionado a necessidade de cristalizar a enzima, um procedimento trabalhoso e dispendioso, o CLEA (Cross-linked Enzyme Aggregates) tornou-se uma interessante classe

de enzima imobilizadas, obtida por uma metodologia que combina a precipitação da proteína e a imobilização (CAO; RANTWIJK; SHELDON, 2000; SHELDON; WOODLEY, 2017). Nessa técnica, a enzima interage covalentemente com o glutaraldeído, podendo levar a melhorias na estabilidade operacional.

Contudo, a utilização de proteínas cristalizadas (CLEAs) é limitada pelo tamanho das partículas, as quais são pequenas (5-50 μm) e podem causar alguns inconvenientes em alguns tipos reatores, enquanto as partículas grandes apresenta limitações de transferência de massa (HICKEY et al., 2007; SHELDON; WOODLEY, 2017). Portanto, são desejadas aquisições de enzimas imobilizadas com facilidade de processamento e com atividade enzimática retida após a imobilização. Isto pode ser obtido com híbridos de partículas ferromagnéticas enzimáticas, os quais podem ser facilmente recuperados magneticamente a partir do meio reacional.

2.4 Nanopartículas de óxidos de ferro como suportes enzimáticos

Nanopartículas de ferro possuem toxicidade baixa e biocompatibilidade (HOLA et al., 2015), características que as tornam promissoras em diversas aplicações biotecnológicas. Diante disso, as partículas de óxido de ferro têm sido frequentemente abordadas como suporte para biomoléculas (HOLA et al., 2015), tendo como alvo o desenvolvimento de processos mais econômicos, específicos, estáveis e reprodutíveis (AGUILAR-ARTEAGA; RODRIGUEZ; BARRADO, 2010).

Usualmente, a imobilização de enzimas em óxidos de ferro está condicionada a modificação da superfície do material, o que proporcionará condições de interação entre suporte e enzima (HOLA et al., 2015; MIN; YOO, 2014). Por meio da adição de compostos silanos na superfície das partículas, os óxidos de ferro adquirem funções adicionais, resultando em um novo material (HOLA et al., 2015; MIN; YOO, 2014), e assim, favorece a imobilização de enzimas em sua superfície.

Diversos estudos (EL-BOUBBOU, 2018; FAN et al., 2017; JIN et al., 2010; TAJIK; NAEIMI; AMIRI, 2017; YAZID; BARRENA; SÁNCHEZ, 2016) têm investigado a modificação da superfície de óxidos de ferro na tentativa de favorecer a imobilização de moléculas. Neste contexto, os hidróxidos de óxido de ferro III (oxidróxidos) não têm sido extensivamente investigados como os demais óxidos de ferro, apesar de apresentar supermagnetismo e características próprias interessantes.

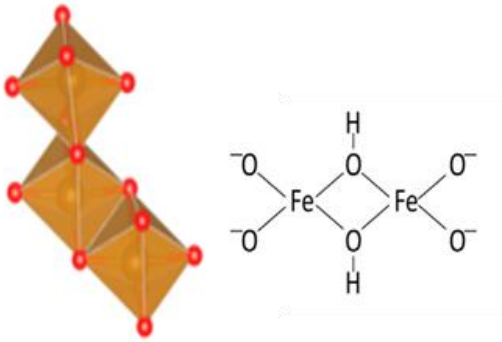
2.4.1 Oxidróxidos de ferro (FeOOH)

Os óxidos de ferro são excelentes suportes para enzimas (HOLA et al., 2015; SILVA et al., 2016b). No entanto, os polimorfos de oxidróxido, como akaganeite (β-FeOOH), goetita (α-FeOOH), lepidocrocita (λ-FeOOH) e ferroxita (δ-FeOOH) são pouco explorados. A unidade estrutural básica dos óxidos de ferro é um octaedro, no qual cada átomo de Fe é circundado por seis átomos de O ou por ambos íons O^- e OH^-, dispostos espacialmente de maneiras especificamente diferentes. Os íons formam camadas que são compactadas hexagonalmente (hcp), como em goetita (α-FeOOH), ou compactadas cúbicas (ccp), como em lepidocrocita (λ-FeOOH) (SCHWERTMANN; CORNELL, 2007).

A ferroxita (δ-FeOOH) apresenta uma estrutura constituída por planos contendo O e OH em empacotamento hexagonal (hcp) com íons Fe^{+3} nos interstícios octaédricos (SCHWERTMANN; CORNELL, 2007; OKAMOTO, 1968). De acordo com Sestu et al. (2015), a unidade atômica representada na Figura 4 está presente em todos oxidróxidos de ferro, podendo ser observada geometricamente como dois octaedros de compartilhamento de aresta com os átomos de Fe nas cavidades e O nos vértices conectando com outras unidades similares.

No entanto, é conhecido que goetita (α-FeOOH), lepidocrocita (λ-FeOOH) e akaganeite (β-FeOOH) possuem ângulos da interação Fe-O-Fe diferentes, dando origem a conectividade da rede estrutural com células unitárias com densidades diferentes. Desse modo, cada oxidróxido apresenta um volume de células unitárias, sendo a goetita (α-FeOOH) 34,7$Å^3$, lepidocrocita (λ-FeOOH) 37,3$Å^3$ e akaganeite (β-FeOOH) 42,3 $Å^3$. O volume da célula unitária da ferroxita (δ-FeOOH) apresenta cerca de 34 $Å^3$, sendo esse valor semelhante ao observado para a goetita (α-FeOOH). Portanto, essa fase do oxidróxido pode ser considerada referência para a estrutura da ferroxita (SESTU, et al., 2015).

Figura 4 – Unidade atômica dos oxidróxidos (FeOOH)

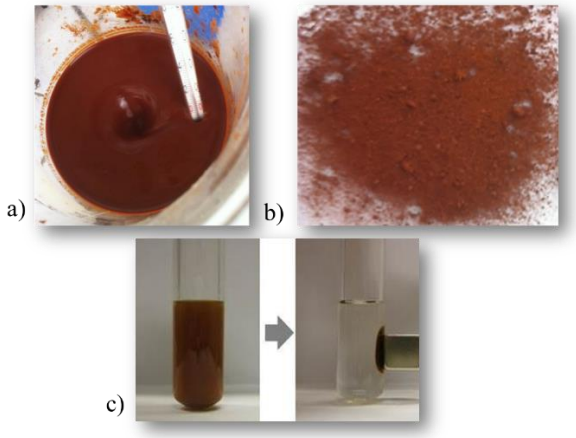


Fonte: SESTU, et al. (2015).

A síntese da ferroxita (δ-FeOOH) ocorre a partir da oxidação rápida do hidróxido ferroso $Fe(OH)_2$ em solução de hidróxido de sódio, resultando na formação de um precipitado castanho-escuro,

o δ-FeOOH (Figura 5) (SCHWERTMANN; CORNELL, 2007). A ferroxita é uma das fases dos oxidróxidos que apresenta uma área superficial grande (101 m^2 g^{-1}), excelente estabilidade e supermagnetismo (DA SILVA et al., 2017).

Figura 5 – Síntese da ferroxita (δ-FeOOH): a) Solução de δ-FeOOH; b) Nanopartículas de δ-FeOOH; c) Separação magnética de δ-FeOOH em meio aquoso.



A presença de sítios de Fe vagos na estrutura da ferroxita, como também a substituição de alguns oxigênios por H_2O e/ou OH^- está associado à sua cristalinidade (SCHWERTMANN; CORNELL, 2007). De acordo com as condições de síntese, a fase amorfa desse óxido pode ser observada por meio da difração de raio-X, isso por que, uma quantidade menor de Fe^{3+} formado afeta sua cristalinidade (GOTIC; POPOVIC; MUSIC, 1994). Nesse caso, a alcalinidade forte no meio reacional de síntese é um fator importante para a formação desse oxidróxido a partir do $Fe(OH)_2$. Após a síntese, o tamanho da partícula pode ser calculado a partir da equação de Scherrer (1) (KLUG; ALEXANDER, 1962), utilizando os espectros de raio-X, em que D é o tamanho do cristalito em nm, λ = 0,15406 nm é comprimento de onda de raio X; Θ é o angulo de difração de pico; e β é a largura da linha na metade máxima da intensidade calculada em radianos.

$$D = \frac{0{,}9\,\lambda}{\beta cos\theta} \qquad (1)$$

O infravermelho (IV) é outra técnica interessante, por meio da qual é possível observar os grupos funcionais presentes no óxido de ferro ‘puro’ e funcionalizado. O espectro de IV do δ-FeOOH

sintetizado já foi analisado em diferentes estudos (CARLSON e SCHWERTMANN, 1980; GOTIC; POPOVIC; MUSIC, 1994; KULAL, et al., 2011).

Foram observadas bandas a 2900 cm^{-1} característica de alongamento OH (H_2O), bandas fracas em 1115, 910, 790 e 670 cm^{-1}, atribuídas aos modos de flexão Fe-OH. Também as bandas a 430 e 300 cm^{-1}, características de vibrações da interação Fe-O (CARLSON e SCHWERTMANN, 1980). Outros estudos verificaram uma banda intensa e larga a 3386 cm^{-1}, a qual pode ser atribuída aos modos de alongamento das moléculas de água superficial ou devido as ligações de H na superfície do óxido, provenientes dos grupos OH (GOTIC; POPOVIC; MUSIC, 1994). Considerando que a banda em 1637 cm^{-1} é decorrente do modo de flexão da H_20 ou vibrações Fe-OH, a qual ocorrem em 1636 - 1112 cm^{-1} (KULAL, et al., 2011).

Diante do exposto, as nanopartículas de ferroxita como suporte para biocatalisadores é uma proposta interessante para obtenção de nanobiocatalisadores magnéticos e de baixo custo, que podem ser facilmente recuperados utilizando um campo magnético. Isso é possível devido à combinação de compostos orgânicos e inorgânicos, que possibilita a produção de novos materiais com propriedades complementares, denominados materiais híbridos. A combinação adequada dos componentes pode levar à formação de materiais que apresentam propriedades que normalmente não são encontradas em uma única substância (MARTÍNEZ-CABANAS et al., 2016; SU, 2017).

Neste sentido, a otimização do processo de imobilização é interessante para obtenção de nanobiocatalisadores cataliticamente eficientes. A otimização do processo pode ser realizada utilizando uma abordagem quimiométrica por meio de um *design* estatístico de experimentos, o qual possibilita a melhor exploração dos resultados, além de minimizar a quantidade de experimentos quando comparado com uma abordagem tradicional.

2.5 Abordagem quimiométrica para análise de dados

A quimiometria é a aplicação de ferramentas matemáticas e estatísticas em dados químicos, que possibilita planejar e/ou obter a condição ótima do processo. Com a chegada da quimiometria no Brasil na década de 70, a realização de experimentos estatisticamente planejados cresceu rapidamente em vários campos de pesquisa, especialmente na Química, Engenharia Química, Engenharia de Alimentos e Biotecnologia (BARROS NETO; SCARMINIO; BRUNS, 2006).

O *design* estatístico de experimentos (DOE) é uma abordagem eficiente para otimizar processos químicos, e tem sido uma ferramenta utilizada em diversas pesquisas (WEISSMAN; ANDERSON, 2014). O DOE é um planejamento detalhado para realização de um experimento. Utilizando o DOE, os

pesquisadores podem aprimorar as condições ótimas de reações, realizando um número mínimo de experimentos (LUNDSTEDT et al., 1998; WEISSMAN; ANDERSON, 2014).

A utilização do DOE nos processos químicos pode ser destacada em relação aos princípios da "Química Verde", uma vez que esse conceito tem como princípio minimizar a quantidade de reagentes envolvidos na otimização dos processos, como um meio de gerar menor quantidade de resíduos químicos (LEAHY et al., 2013; WEISSMAN; ANDERSON, 2014).

A otimização do processo pela abordagem tradicional, ou seja, variando um fator de cada vez, apresenta inconvenientes, como um nível de significância inferior, envolve uma série de repetições e maior número de experimentos, uma vez que não possibilita a interação dos fatores analisados (WEISSMAN; ANDERSON, 2014). O DOE muda o viés da investigação e, muitas vezes, leva a uma condição a qual não foi proposta anteriormente. É ainda mais significativo na utilização do DOE a capacidade de detectar rapidamente como as interações entre os fatores podem afetar o processo (WEISSMAN; ANDERSON, 2014).

As etapas fundamentais para elaborar o DOE incluem a escolha dos fatores que afetam o processo e a escolha da variável que será medida na resposta do processo (variável dependente). Supondo que o interesse de um planejamento seja utilizar n fatores, e admitindo dois níveis (-1 e +1), nesse caso, o fatorial é 2^n. Os sinais – e + correspondem aos valores mínimo e máximo dos fatores considerados. A interação dos fatores é obtida pela multiplicação simples dos sinais atribuídos aos fatores (CHEN et al, 2015; LUNDSTEDT at al., 1998).

Os resultados dos experimentos, incluindo as interações, são analisados para verificar a influência significativa dos fatores no processo. Essas análises podem ser através da Análise de Variância (ANOVA), e os resultados apresentados pela Metodologia de Superfície de Resposta (MSR).

A metodologia da superfície de resposta (MSR) é um conjunto de técnicas estatístico-matemáticas úteis na modelagem e na análise em aplicações em que a resposta de interesse é influenciada por diversas variáveis (fatores), cujo objetivo é otimizar a resposta (CHEN et al., 2015; LUNDSTEDT et al., 1998). O pacote RSM para R (R Development Core Team, 2009) dispõe de várias funções as quais facilitam os métodos de superfície de resposta. Além disso, a interpretação de um experimento fatorial para análise de MSR envolve várias etapas: 1) Determinar quais termos mais contribuem para a variabilidade na resposta; 2) Determinar se a associação entre a resposta e o termo é estatisticamente significativa; 3) Determinar quão bem o modelo se ajusta aos seus dados; 4) Determinar se o modelo atende às suposições da análise.

Na imobilização enzimática, a definição das variáveis é uma etapa crucial, a qual deve obedecer alguns critérios pré estabelecidos, os quais envolvem a estabilidade da enzima bem como a reatividade

dos grupos funcionais externos da proteína. É bem conhecido que a estabilidade enzimática é influenciada principalmente pela temperatura e pH. A temperatura influencia na estrutura tridimensional das proteínas. Por outro lado, a escolha do pH envolvido no processo deve considerar a estabilidade da enzima no meio reacional e o ponto isoelétrico (pI), uma vez que as condições devem favorecer a interação dos grupos amino da proteína com o suporte enzimático. Além disso, a capacidade da enzima imobilizada reter a atividade catalítica também está associada a proporção de enzima e suporte envolvido na reação. Portanto, o conhecimento prévio das propriedades da enzima e do suporte, como também das possíveis influências dos fatores envolvidos na imobilização enzimática são fundamentais para otimização do processo utilizando a abordagem quimiométrica.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 - Soybean Peroxidase Immobilized on δ-FeOOH as New Magnetically Recyclable Biocatalyst for Removal of Ferulic acid

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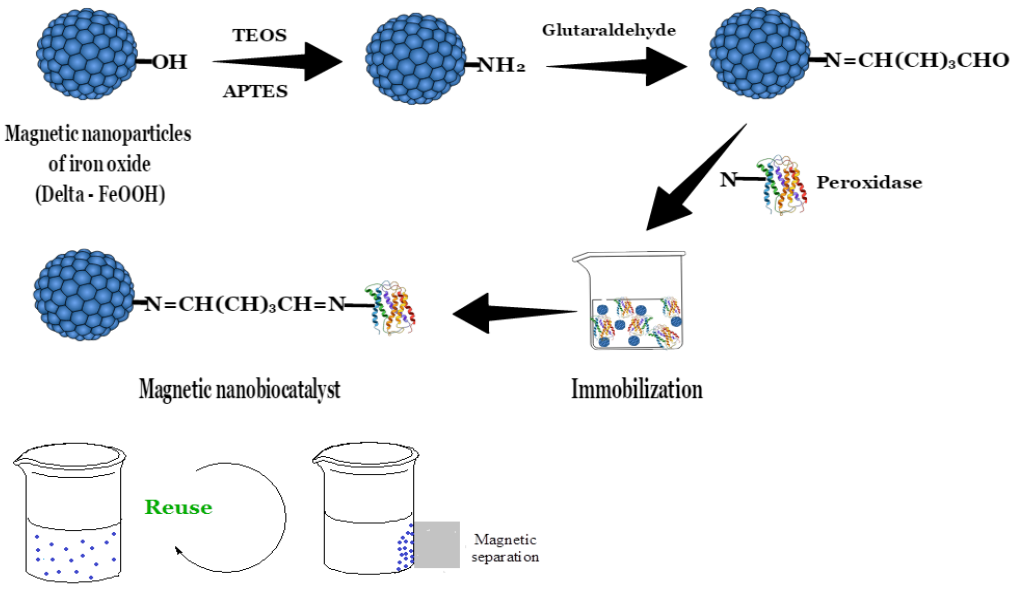
Abstract

A significant enhancement in the catalytic performance due to enzymes immobilization is a great way to enhance the economics of biocatalytic processes. The soybean peroxidase (SP) immobilization under ferroxyte and the ferulic acid removal by the enzyme free and immobilized were investigated. The immobilization via silica-coated ferroxyte nanoparticles was effective, and immobilization yield of 39%. The scanning electron microscopy (SEM) images showed significant changes in the materials morphology. Substantial differences were observed in the particles' Fourier Transform Infrared (FTIR) spectra. The magnetic catalyst revealed a better performance than the free enzyme in the ferulic acid conversion, presenting a good Vmax/Km ratio when compared with the free enzyme. The reuse evaluated by ten cycles exhibited excellent recycling, remaining constant between the sixth and seventh cycles. The use of magnetic nanocatalyst becomes possible to eliminate the high operational costs, and complicated steps of the conventional enzymatic processes. Thus, a viable industrial route for the use of the enzyme as catalyst is possible.

Keywords: Biocatalyst; Iron oxide; Bioremediation; Wastewater.

Graphical abstract



Introduction

The industrial sector stands out as one of the major consumers of water throughout the world, which results in large volumes of waste water [1] rich in various types of organic compounds, such as phenol. Thus, organic pollutants significantly contribute to environmental pollution, being considered a wide problem today and the object of different studies [2, 3]. Therefore, wastewater treatment is of great importance and the pollutant removal has been widely studied by different techniques such as oxidation electrochemistry [4], solar photocatalysis [5] and biodegradation [6, 7]. However, these methods have some drawbacks related to catalysts stability and reuse [8]. Furthermore, in treatment using microorganisms, their growth is inhibited in the presence of high pollutant concentrations [7]. Considering this, there is a need to find alternatives that can minimize the impact that these contaminants cause to the environment, as well as the oxidation of these pollutants to an efficient system and at the same time viable.

In this context, enzymes as catalysts should be highlighted. Since that, this class of catalysts shows their own characteristics, and their applications are becoming very interesting in different fields. In the

environmental proposal, plant peroxidases (E.C. 1.11.1.7) such as turnip, horseradish and soybean seed hulls, stand out with excellent potential for pollutant removal [3, 9]. While on the other hand, the agroindustry generates large quantities of residues from the soybean hulls. Thus, it is possible to obtain the soybean peroxidase from a low cost and still recycle the industrial waste. Peroxidases have a complex catalytic mechanism, involving the two electrons oxidation of the heme fraction, besides intermediate compounds formation [11]. This enzyme acts on the oxidation of a broad spectrum of aromatic compounds such as phenols, biphenols and anilines by catalyzing the oxidation in the presence of hydrogen peroxide to phenoxy radicals, resulting in the formation of water-insoluble polymers which can be separated by coagulation and sedimentation [10, 11].

Unfortunately, the use of enzymes as biocatalysts has some drawbacks, including limited natural resources, the cost to obtainment, and limitations for their large scale use for environmental purposes which can greatly restrict their practical applications [12]. In this context, immobilization is one of the most recent and innovative techniques that enable the production of these biocatalysts in industrial scale and with higher productivity [13]. Immobilization processes enable to the overcome the limitations of the free enzyme. It is possible to improve the molecules stability, activity, and selectivity of the enzymes. Also, this may lead to increased resistance against inhibition and consequently, the formation of the products with more efficiency [14–19].

Different techniques are used for the immobilization, highlighting physical methods such as adsorption, encapsulation, entrapment and chemical methods including electrostatic interactions, immobilization via chelating with a metal and covalent attachment. The encapsulation, entrapment, physical adsorption, and electrostatic interactions are commonly used. However, these techniques have some drawbacks, such as enzyme leakage, the nonspecific protein binding and the loss of the enzyme in the process. Covalent attachment is the most effective approach to prevent detachment of the enzyme from the support during operation [19].

In face of this feature, several studies have investigated the enzymatic supports, e.g., Hydroxyapatite-encapsulated γ-Fe_2O_3 [20], core–shell structured Fe_3O_4–MC such as M-41 nanocomposites [21], Fe_3O_4/poly (styrene-methacrylic acid) magnetic microsphere [22] magnetic chitosan microspheres [23]. On the other hand, feroxyhyte (δ-FeOOH) is a poorly exploited oxide. Ferroxyte structure is similar to that of hematite (α-Fe_2O_3). However, it should be kept in mind that feroxyhyte presents high stability, high specific surface area as well as supermagnetism properties [24]. In addition, ferroxyte synthesis is simple, and viable economically, making it more advantageous for technological application.

Based on our previous knowledge about ferroxyte [25–28], it is worth pointing out that this oxide has interesting characteristics, being promissory as enzymatic support. Also, from the ferroxyte, it is possible to obtain a magnetic biocatalyst, that can be recovered by a simple magnetic separation and reused [29, 30]. Additionally, the soybean hull is a byproduct of the soybean food industry, and the SP has the potential of being a cost effective alternative for wastewater treatment. Since SP was reported less susceptible to inactivation than HRP [31], the SP extract was chosen for immobilization studies. In this work, SP has been immobilized onto silica-coated ferroxyte nanoparticles (δ-FeOOH–SiO_2–APTES), for the obtainment of a magnetic biocatalyst. The oxidation of the ferulic acid, a model pollutant, and the biocatalyst reuse has been evaluated. In these applications, the enzyme immobilization is significant for the enhancement of catalytic activity and stability.

Materials and methods

Chemical reagents

Ferulic acid (FA), tetraethoxysilane (TEOS), 3-aminopropyltriethoxysilane (APTES) and Catalase (EC 1.11.1.6 ≥ 10.000 units per mg protein) were obtained from Sigma–Aldrich (St. Louis, MO, USA) and ammonia (30%) was obtained from Merck. All other chemicals were of analytical grade and purchased from Sigma–Aldrich.

Hybrid material synthesis

Synthesis and functionalization of δ-FeOOH

The synthesis of δ-FeOOH was carried out according to the modified procedure described by Chagas and collaborators [24]. It essentially consists of precipitating $Fe(NH_4)_2(SO_4)_2.6H_2O$ solution with NaOH followed by fast oxidation with H_2O_2. This leads directly to the product δ-FeOOH. For the silica coating, a mixture of ethanol (200 mL) and distilled water (20 mL) was added to 2 g of δ-FeOOH particles and the resulting dispersion was sonicated for 1 h. Next, 5 mL of ammonium hydroxide and TEOS (6 mL) were added to the reaction solution. The resulting dispersion was mechanically stirred continuously for 3 h at room temperature. To afford NH_2-modified magnetic silica particles, 4 mL of APTES was then added and the mixture was agitated for 12 h. The NH_2-modified magnetic silica particles were collected by vacuum filtration, followed by washing with water and ethanol, and then dried overnight in an oven

at 60 °C [32]. 100 mL of glutaraldehyde solution (1.6% v/v) was then added to 2 g of silica-coated magnetic particles; the mixture was stirred at 25 °C for 3 h. Finally, the suspension was filtered under vacuum, washed several times with phosphate buffer (0.1 mM, pH 7.0) and distilled water. The support obtained was dried at 60 °C for 24 h in an oven. The steps for support preparation involve the functionalization of magnetic particles and immobilization of enzyme process.

Formulation and immobilization of the soybean peroxidase

The enzyme was extracted from soybean seed hulls. The crude SP extract was obtained according to Silva and collaborators [3]. The enzymatic preparation was used in a lyophilized form. For immobilization of SP, 2 g of functionalized oxide was added to 20 mL of enzymatic solution (2 g of SP in 20 mL of citrate phosphate buffer 0.1 mM, pH 6.0) and stirred continuously for 5 h at 4 °C. Aliquots were taken at specific time intervals to determine the residual enzyme activity and the maximum immobilization yield. The immobilization yield (IY) or relative activity (%) was calculated from the difference between the enzyme activity before immobilization and the enzyme activity in the supernatant after immobilization, divided by enzyme activity before immobilization. Activity measurements of free and immobilized SP were determined based on the rate of guaiacol oxidation for the formation of tetraguaiacol (ε = 26.6 $mM^{-1}cm^{-1}$) [33]. One unit of peroxidase activity at pH 6.0 represents the oxidation of 1 μmol of guaiacol/minute.

Characterization of materials

X-ray Powder Diffraction (XRD)

The X-ray Powder Diffraction (XRD) spectra data were collected using a Shimadzu XRD-6000 system with Cu Kα radiation (λ= 0.15406 nm), scanning step of 0.02, count time of 6s and 2θ range from 10 to 85o.

Scanning Electron Microscopy (SEM)

The materials morphology was determined with Scanning Electron Microscopy (SEM). The Scanning Electronic Micrographs (SEM) were obtained using an LEO 440 with an Oxford detector with an electron beam operating at 15 kV.

Fourier transform infrared Spectroscopy (FTIR)

The surface groups present in the materials were characterized by Fourier transform infrared spectroscopy and spectra were obtained from a PerkinElmer Spectrum 2000 Spectrometer, with spectral range from 400 to 4000 cm-1.

FA removal by free and immobilized soybean peroxidase

The removal assays were carried out in glass reaction flasks at 30 °C, at a constant pH (citrate phosphate buffer 0.05 mM; pH 6.0), in a reaction medium containing 0.8 mL H_2O_2 (2.0 mM). The reaction mixture was stirred continuously for 30 min [34]. The FA solution 1.0 mM was used in the oxidation assay with the free enzyme and immobilized enzyme under same conditions. The control was carried out in the absence of H_2O_2 to evaluate the adsorption material. At the end of the reaction, the FA residual concentration was measured by the Folin and Denis colorimetric method [35]. The hybrid catalyst was removed from the reaction medium by magnetic separation and was reused in the sequential degradation assays, under the experimental conditions used in the original reaction, totalizing ten cycles. To investigate the permanency of the enzyme on the support, we measured the enzyme activity of the supernatant during hybrid catalyst reuse and we observed if there was active enzyme leaching from the support under the reaction conditions. The assay degradations using free enzyme as a catalyst were stopped by adding 0.1 mL of catalase solution (1.2 mg of the commercial enzyme in 1.0 mL of 0.1 mM, phosphate buffer, pH 7.0) [5]. The experiments were performed triplicate and the derivations are represented in the form of a bar in all graphs presented.

Determination of enzyme kinetic parameters

Removal assays using FA as substrate were carried out to determine the kinetic parameters K_m and V_{max} (Michaelis-Menten constant and maximum reaction rate, respectively) for the free, and immobilized enzyme The magnetic biocatalyst (300 mg, which corresponds to 0.26 U) was dispersed into 0.05 mM phosphate buffer, pH 6.0 (2.4 mL) with addition of 3.0 mL of FA solution (1 mM) and 0.8 mL from H_2O_2 (2 mM). The same conditions (enzymatic solution 0.26 U in a total volume of 6.2 mL) were used for the free enzyme. The reaction mixture was kept under stirring for 30 min in a water bath at 30 °C. Measurements were performed with different ferulic acid concentrations. K_m and

V_{max} were transformed to Lineweaver–Burk plots and values were calculated from the slopes and intercepts of the curves.

Results and Discussion

Preparation of magnetic biocatalyst

For the preparation of the biocatalyst, the δ-FeOOH nanoparticles were coated with silica by hydrolysis and condensation of tetraethoxysilane (TEOS) and to introduce amino groups (3-aminopropyl) triethoxysilane (APTES) was used [34]. The silica is frequently used to stabilize oxide particles. It improves particles stability, and makes possible the addition of reactive functional groups [32]. Thus, the aldehyde groups added may react with the amino group of the amino acid residues of the enzyme. This way, the peroxidase was covalently immobilized onto δ-FeOOH–SiO_2–APTES.

The SP immobilization was carried out from 1 to 5 h (Fig. 1). The use of δ-FeOOH nanoparticles as support for SP was not still reported. However, it deserves much attention due to its many interesting properties, like magnetism, non-toxicity, biocompatibility and easy preparation. The feroxyhyte was investigated in relation to its potential as a magnetic support for SP immobilization (activity 30.40 U mL^{-1}) in the reaction medium. The immobilization yield increased with time. It obtained 39% immobilized peroxidase at 5 h. In only 3 h of reaction, the yield was 37%, remaining constant until 4 h; other studies reported an obtainment of only 25% in this time [36]. This suggests that these results are promising. Nevertheless, the immobilization processes should be optimized, since the enzyme immobilization depends on the support type and method used. As such, enzyme immobilization studies are very important. The immobilized enzyme can be easily separated, which allows its reuse and ease of process control [37].

Previous works have shown long time processes [32, 38–40]; until even over 10 h for enzyme immobilization [18, 32]. In our study, the enzymatic immobilization occurred for only 3 h. The immobilization process with ferroxyte support was considered efficient. However, biocatalyst efficiency and reuse for pollutant removal are parameters that should be considered.

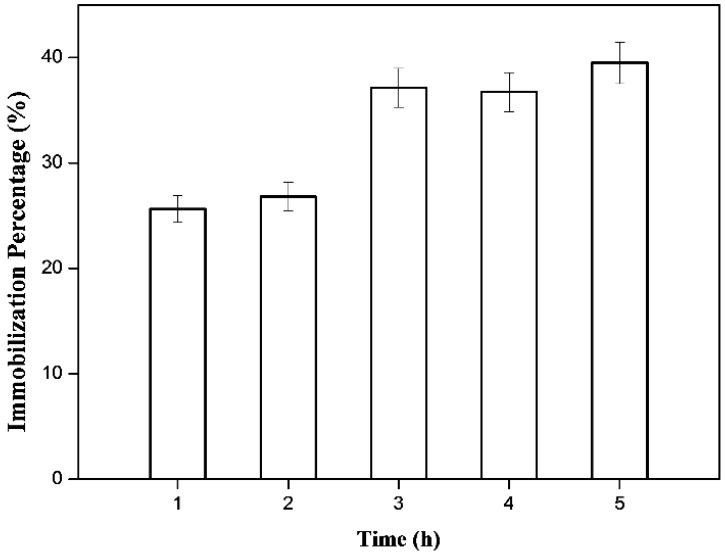


Fig.1 Immobilization percentage of SP on support upon 5 hours activation. Reaction conditions: X g of δ-FeOOH-SiO_2-APTES-GLU; enzyme initial load – SP X; pH 6.0, - 4 ^oC

Characterization of materials

X-ray diffraction analysis

The XRD diffraction patterns for the materials are shown in Fig. 2. The patterns showed that all samples presented reflections corresponding to planes (100) (101) (102) (110), corresponding to the δ-FeOOH phase (JCPDS file # 13–87). The average crystalline sizes were estimated from the peak width at half height of the strongest reflection (100) plane, after correction for instrumental broadening effects, using the Scherrer Equation [41]. The average crystalline size of δ-FeOOH was 15 nm (Fig. 2a). However, after the silanization reaction, the average crystalline size was approximately 19 nm (Fig. 2b–d). The diffractogram showed after functionalization a halo in the region of 2θ between 20° and 30°, resulting from the characteristic halo pattern of an amorphous phase. This is due to the amorphous silica diffraction peaks formed around the δ-FeOOH particles. Although the crystallite size increased after this process, the strategies used for the preparation of hybrid materials (silanization reaction) were not sufficient for the phase change of δ-FeOOH.

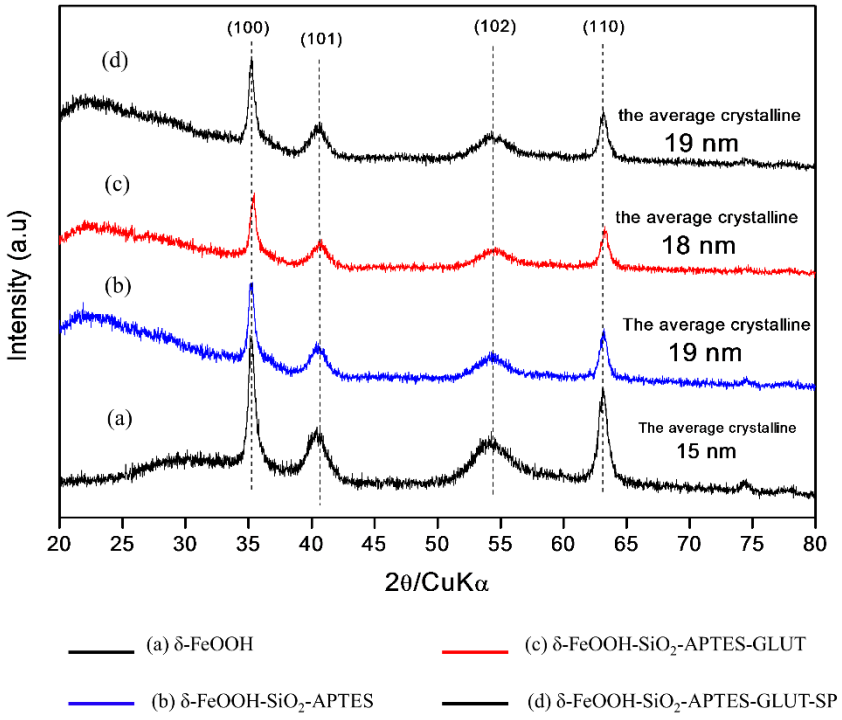


Fig.2 X-ray diffraction (XRD) of δ-FeOOH, δ-FeOOH-SiO_2-APTES; δ-FeOOH-SiO_2-APTES-GLUT and δ-FeOOH-SiO_2-APTES–GLUT-SP

Scanning electron microscopy (SEM)

The morphology of the materials was characterized by SEM (Fig. 3). The SEM images of materials, after the functionalization and immobilization process (Fig. 3B, C, D), show that there are significant changes in the materials morphology compared with the pure δ-FeOOH. SEM images show that the FeOOH–SiO_2–APTES, FeOOH–SiO_2–APTES–GLUT and FeOOH–SiO_2–APTES–GLUT–SP materials are composed of uniformly dispersed particles with a nearly spherical shape, approximately 300 nm in diameter. This spherical shape could be attributed to the preparation process for functionalization of the material. Similar results were obtained by Yamaura and collaborators [42], in which the authors observed that after being coated with TEOS by a silanization reaction there was the formation of nanospheres on the iron oxide surface, indicating that the functionalization process was successful. After the SP immobilization, there is a certain degree of deformation of the nanospheres formed in (B) and (C), suggesting the presence of the enzyme in the magnetic support.

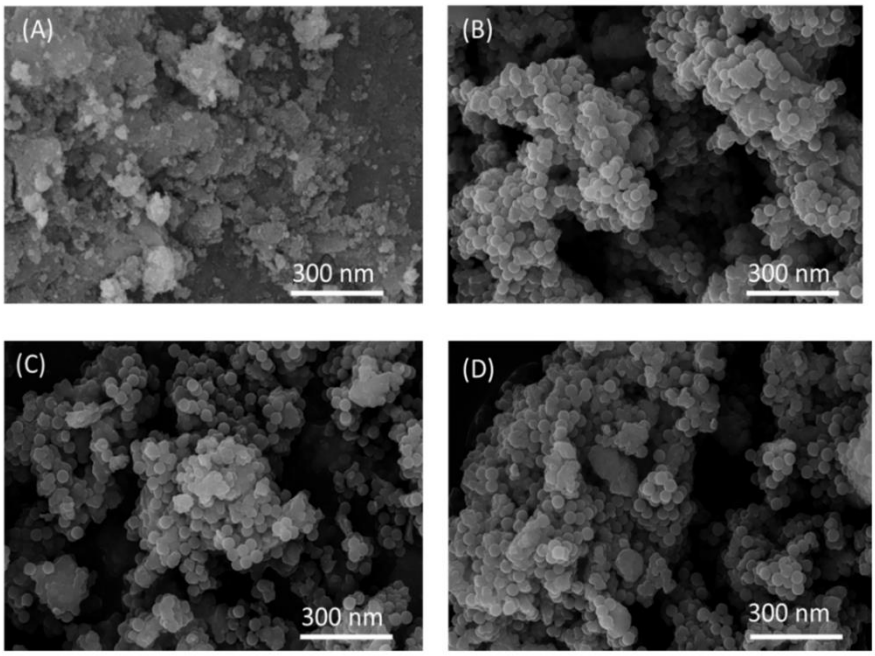


Fig.3 SEM image: (A) Pure δ-FeOOH particles (B) FeOOH-SiO_2-APTES (C) FeOOH-SiO_2APTES-GLUT (D) FeOOH-SiO_2-APTES-GLUT-SP

Spectroscopy Fourier transform infrared (FTIR)

The surface groups present in the materials were characterized by Fourier transform infrared Spectroscopy and spectra were obtained from a PerkinElmer Spectrum 2000 Spectrometer, with spectral range from 400 to 4000 cm^{-1}. The FTIR spectra show the substantial differences in the structures of the δ-FeOOH compared to δ-FeOOH–Enzyme. The δ-FeOOH spectrum shows bands at 826, 1103, 1395 and 1762 cm^{-1}. The absorption bands of iron oxyhydroxides arise from Fe–OH and Fe–O vibration. There are OH bending bands in the 826–1395 cm^{-1} region, which match exactly with the δ-FeOOH spectrum reported in previous literature. A disperse band due to bulk hydroxyl stretch is observed in the 1395 cm^{-1} region [43]. The band located at 1762 cm^{-1} can be assigned to carbonyl (C=O) vibrations. However, after the immobilization process (Fig. 4) the band at 1395 cm^{-1} disappears indicating the formation of the Si–O–Fe bond, evidence that the δ-FeOOH had been silanized. In addition, there are bands at 472 and 954 cm^{-1} attributed to a deformation mode (Si–O–Si) bond and Si–OH groups, respectively [44, 45]. The broad band at 1645 cm^{-1} can be ascribed to the NH_2 bending mode of free NH_2 [42].

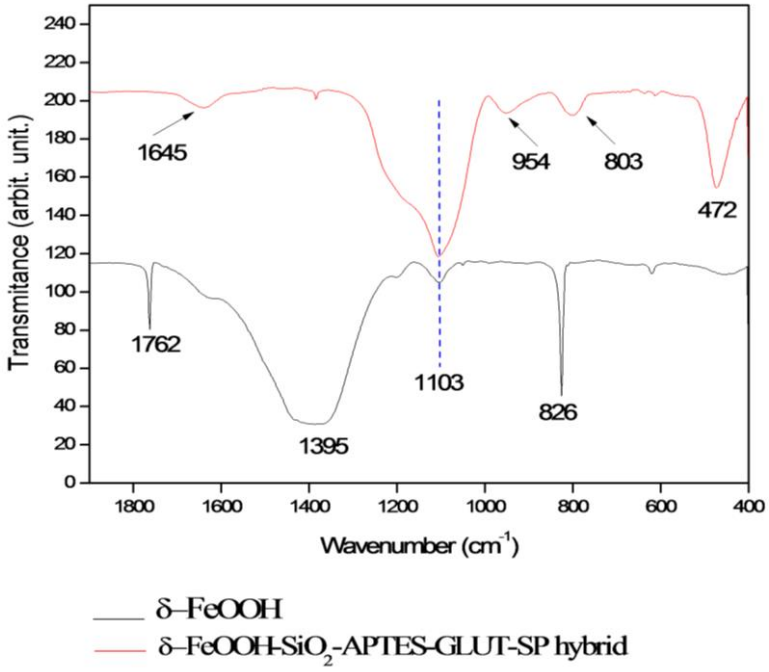


Fig.4 FTIR spectra of δ-FeOOH and FeOOH-SiO_2-APTES-GLUT-SP

Ferulic acid removal

To assess the potential of the free enzyme and the hybrid material prepared in the FA removal (Fig. 5), degradation assays were performed under the same conditions. The free enzyme (activity of 0.26 U) presented 56% FA removal, while the immobilized enzyme (300 mg - which corresponds to an enzyme activity of 0.26 U) presented better performance, 93% FA removal, indicating that the hybrid material is highly efficient for FA transformation. The immobilized SP without H_2O_2 (control sample) showed a slight (16%) ferulic acid removal, probably due to adsorption of the hybrid. Therefore, we can conclude that pollutant degradation is governed by the peroxidase activity.

It is well known that the peroxidases are able to catalyze the oxidative polymerization of phenolic compounds. In the catalytic process of the peroxidase, radicals that react with each other are formed, forming insoluble polymers that precipitate in the solution [11]. These products can be removed by simple processes of separation. However, the formation of insoluble polymers may compromise the performance of the enzyme [46], even more than free enzyme have some limitations, as low operational stability. This drawback could be overcome by the enzyme immobilization [32].

Indeed, immobilized enzymes may have their activity committed after immobilization, due to the diffusion limitations and conformational changes. However, one of the properties that are generally considered in the immobilization is the enzyme stability [47]. The enzyme stability may decrease after immobilization, if the support is not able to establish desired interactions with the enzyme.

Faced with these features, the high potential of the immobilized SP for FA removal can be attributed to the improvement of the enzymes stability due to immobilization. Nevertheless, the explanation for the stabilizations may be diverse. The nanoparticles have important aspects that determine the biocatalysts efficiency, as large surface area and minimal diffusion limitation [48]. In addition, the immobilization processes from the reactions between amino and aldehyde groups to produce a Schiff base are well known. From this feature, it is possible to have covalent binding between enzymes and support [32], which causes rigidity in the structure of the immobilized enzyme. This stiffness can contribute to an increase of the enzymatic stability by reductions of the conformational changes involved in enzymatic inactivation [49].

The results indicated that the use of the magnetic catalyst obtained is advantageous in the pollutant removal processes, since the immobilized enzyme removed 60% more pollutant than the free enzyme. Another attractive advantage of the enzyme immobilization in magnetic particles is the easy separation and reusability of the support. Most of the enzymes used in industry are too expensive, so the enzymatic process becomes viable only if the catalyst can be reused [37].

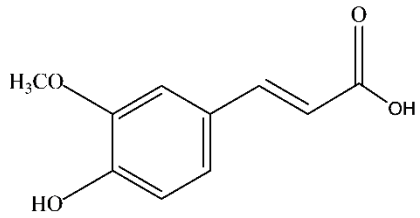


Fig. 5 Structure of trans-Ferulic acid (FA)

Reusability of biocatalysts

Biocatalysts should be used efficiently, and their capacity to perform a same function repeatedly is considered an important property. Investigations were carried out to assess repeated usability of immobilized SP. There was not observed active enzyme leaching from the support, under the reaction conditions and time of reuse. Therefore, it was concluded that the interaction between the enzyme and

the support surface was strong enough since the FA degradation by magnetic catalyst was very considerable.

According to Fig.6, there was a decrease in the enzymatic activity with the increased cycles number. In the first cycle, δ-FeOOH/ SiO_2-APTES-SP showed a high capacity for FA removal (93%). Subsequent applications resulted in a decrease initial removal capacity of the FA over time, dropping from the second cycle 7%, 21%, 39%, and 50%, respectively, and remaining practically constant between the sixth and seven cycles. The immobilized enzyme activity decline can probably be attributed to the accumulation of the reaction products produced during the enzymatic reaction on the support, which may cover the enzyme and affect the reaction of the next cycle [11,28].

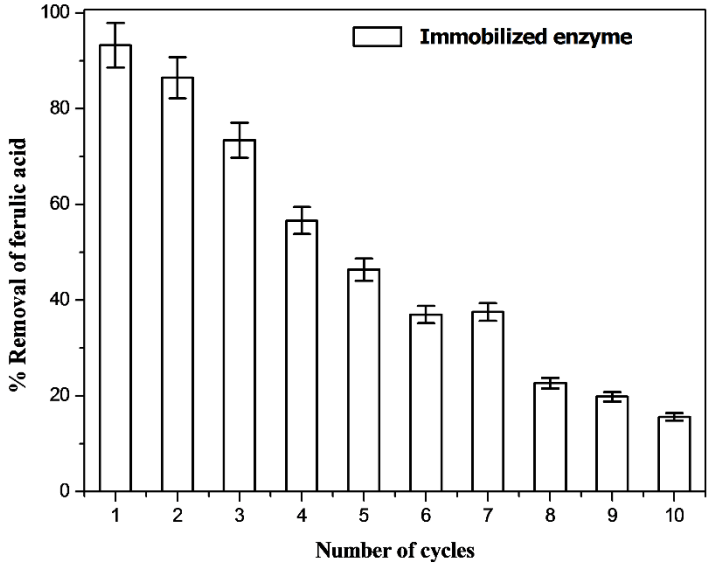


Fig.6 Removal of 2.13 mM ferulic acid with repeated applications of immobilized enzyme (δ-FeOOH-SiO_2-APTES/SP), pH 6.0, at 30 °C for 30 minutes-each cycle

However, besides highly efficient and stable, a support used for industrial applications must also be produced at affordable costs. The use of δ-FeOOH as a support for an enzyme with the environmental application has proved to be a promising approach. The separation and reuse of iron oxides are very simple, effective and economical, while also being environmentally friendly.

Determination of enzyme kinetic parameters

In catalytic process, the study of Michaelis–Menten kinetics parameters is one of the best ways to understand enzyme kinetics. The Michaelis (K_m) constant is an important parameter for evaluating enzyme efficiency and signifies the extent to which the enzymes have access to the substrate molecules; lower K_m value indicate high affinity between enzyme and substrate [50]. For an enzyme-catalysed process, the reaction rate (v) and the concentration of the substrate ([S]) will be derived from the Michaelis–Menten equation, in which the basic parameters can be determined from the Lineweaver–Burk plots according to Eq. 1.

$$\frac{1}{v} = \frac{Km}{Vmax} \times \frac{1}{[S]} + \frac{1}{Vmax} \quad (1)$$

Table 1 presents the kinetic parameters of free and immobilized peroxidase for ferulic acid substrate. According to the results, the free and immobilized enzyme showed distinct kinetics. The Km value of the immobilized enzyme is 1.6-fold lower (10.85 mM) than for the free enzyme (17.25 mM) for the ferulic acid oxidation in the presence of the hydrogen peroxide. The hydrogen peroxide is a substrate that activates the enzymatic reaction of the peroxidase. The ideal concentration of this substrate is an important factor for success in the removal of phenolic compounds. Based on the peroxidase known mechanism, a minimum H_2O_2/ phenol molar ratio of 1:2 is required for the complete conversion [51]. While small amounts of peroxide limit the enzymatic reaction rate, its excess inhibits enzymatic activity, because there is a formation of polymers produced in the catalytic process [46, 51]. Therefore, the peroxide concentration standardized was derived from the previous studies [3, 24], was sufficient to promote the enzymatic action without affecting the catalytic performance of the free and immobilized enzymes, and thus, the study of the kinetics parameters.

Table 1 Kinetic parameters of free and immobilized (δ-FeOOH/SiO_2-APTES-SP) in the presence of FA

Catalyst	K_m (mM)	V_{max} (mM/min)	V_{max}/K_m (min^{-1})
Free enzyme	17.25	0.417	0.024
Immobilized enzyme	10.85	0.348	0.032

Figure 7 shows that the apparent Km value of the immobilized SP with FA as substrates, in the presence of H_2O_2 (17.25 mM) is only slightly greater than that of free SP (10.85 mM), which indicates

that the affinity of the immobilized enzyme for the substrate is close to that of the free SP for the substrate FA. The results indicated an affinity of the hybrid catalyst toward the substrate, which can be explained by the positive effect of immobilization, due to the reduction of the steric hindrance, and then facilitating the substrates diffusion [52]. Previous studies attributed higher Km to possible conformational changes. Which it may be due to the better interaction between substrate and enzyme [49]. Also, from the V_{max}/K_m, it was possible to compare the catalytic efficiency in each enzyme–substrate system. The V_{max}/K_m ratio for the immobilized enzyme (0.032 min^{-1}) was greater than for the free enzyme (0.024 min^{-1}), indicating a good catalytic efficiency of the immobilized enzyme for this model pollutant, corroborating with previous studies [34]. The results showed that the use of on δ-FeOOH–SiO_2–APTES as support, may lead to an improvement in the catalytic performance of the enzyme. However, important considerations about some supports and their possible mechanisms have been reported [53, 54], and should be considerate. Thus, the enzymatic immobilization has been associated with catalytic efficiency improvements [55, 56]. In addition, previous works have investigated the use of nanoparticles on the peroxidase immobilization, reporting an enhanced stability, corroborating with data obtained in this present study [34, 57, 58].

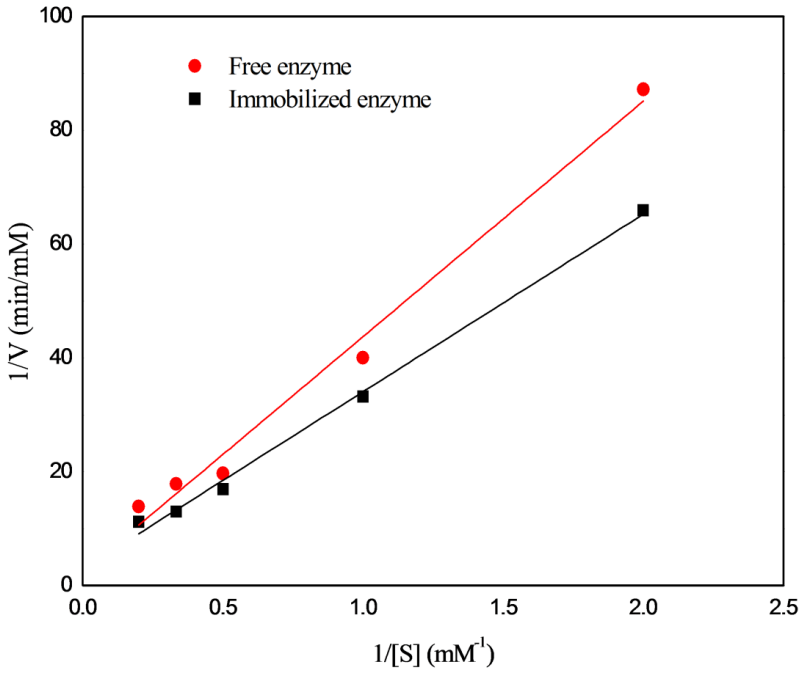


Fig.7 The Lineweaver–Burk plots of free and immobilized peroxidase

Conclusion

Enzyme immobilization is an attractive form of reuse and stabilization of biocatalysts. In this work, a magnetic biocatalyst was prepared via immobilization of soybean peroxidase on magnetic nanoparticles. The immobilization process until 3 h was sufficient to obtain the immobilized enzyme with excellent catalytic performance in the FA removal, a pollutant model. Besides this feature, the magnetic catalyst showed its promising reuse, which is a very attractive aspect for a real application considering the high cost of the enzyme. We strongly feel, then, that our study might be helpful for exploring the potential of immobilized enzyme in the removal of other phenolic pollutants. Biotechnological solutions targeting industrial waste problems are vital to addressing and circumventing the impact of industry on the environment. In this context, the biocatalytic processes can provide several relevant improvements.

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Compliance with ethical standards

Conflict of interest The respective authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; membership, employment, consultancies or patent-licensing arrangements), or non-financial interest (such as personal, affiliations, or beliefs) in the subject matter or materials discussed in this manuscript.

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ARTIGO 2 - Δ-FeOOH as Support for Peroxidase: Low-cost and Magnetically Recoverable Nanocatalyst Obtainment for Pollutant Removal via a Chemometric Approach

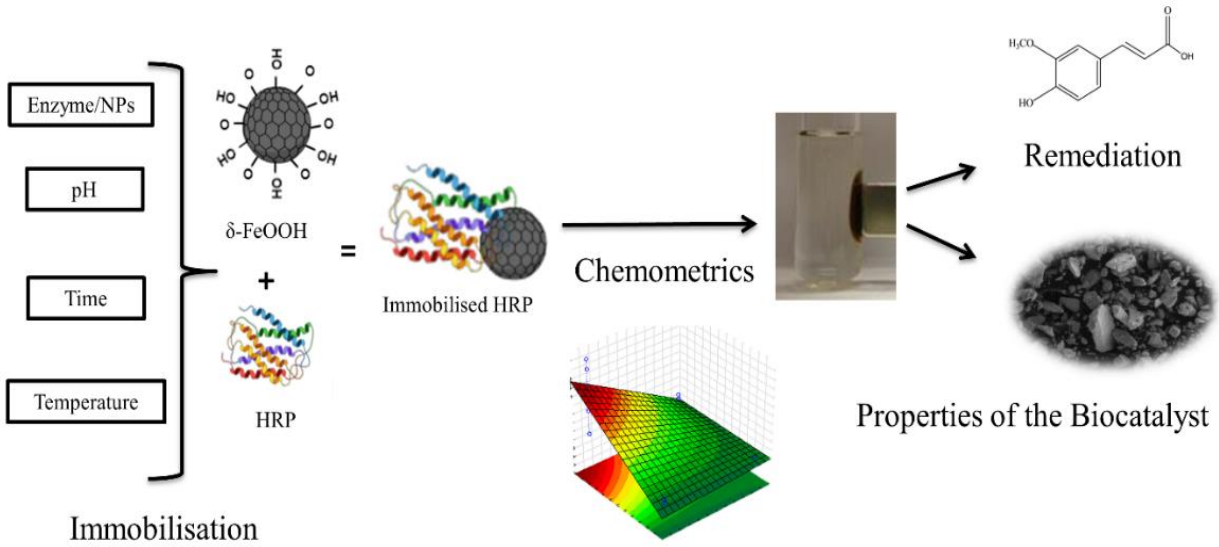
Artigo submetido a Revista Applied Nanoscience

Abstract

Due to their high surface area, stability and functional groups on the surface, iron oxide hydroxide nanoparticles have attracted attention as enzymatic support. In this work, a chemometric approach was performed, aiming at optimization of the horseradish peroxidase (HRP) immobilisation process on δ-FeOOH nanoparticles (NPs). The enzyme/NPs ratio (X1), pH (X2), temperature (X3), and time (X4) were the independent variables analyzed and immobilised enzyme activity the response variable (Y). The effects of the factors were studied using a factorial design at two levels (-1 and 1). The biocatalyst obtained was evaluated for the ferulic acid (FA) removal, a pollutant model. The materials were characterized by X-ray powder diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), and Scanning Electron Microscopy (SEM). The SEM images indicated significant changes in material morphology. The independent variables X1 (-0.57), X2 (0.71), and X4 (0.42), presented significance effects estimate. The variable combinations resulted in two significance effects estimate, X1*X2 (-0.57), and X2*X4 (0.39). The immobilised HRP by optimized conditions (X1 = 1/63 (enzyme/NPs ratio); X2 = pH 8 and X4 = 60°C, and 30 minutes) showed high efficiency for FA oxidation (82%).

Keywords: Bioremediation; Horseradish Peroxidase; Iron oxide hydroxide; Immobilisation.

Graphical abstract



1. Introduction

Nanomaterials have attracted much attention due to their unique properties and potential applications in several areas such as environmental, medicine and pharmacology (Hola et al., 2015; Xu et al., 2014; Zhang et al., 2008). Magnetic iron oxide nanoparticle, when its surface chemistry is appropriate, can be used for numerous applications. A number of approaches (e.g. immunoassay, drug delivery detoxification of biological fluids, magnetic resonance imaging (MRI) contrast enhancement, and hyperthermia), including molecules immobilisation have been described as its applications (Hola et al., 2015; Zhou et al., 2013).

On the other hand, the enzymes play an important role in several fields, and the immobilisation has been considered one of the most innovative processes, being able to obtain from this process, the enzyme with good catalytic activity, improve the stability, and reuse (Guzik et al., 2014). The peroxidase can be highlighted in several areas of studies, as medicine, life sciences, biotechnology, cancer therapy, biosensor systems, bioremediation (Krainer and Glieder, 2015), and protein immobilisation (Ma et al., 2013; Silva et al., 2016; Tavares et al., 2017; Zhu et al., 2011). The Horseradish peroxidase (HRP), is a powerful oxidoreductase to remove organic pollutants (Chang et al., 2015; Cheng et al., 2006; Kermad et al., 2013; Lai e Lin, 2005). It acts on the oxidation of compounds in the presence of hydrogen peroxide

to phenoxy radicals, resulting in the formation of water-insoluble polymers, which can be separated by sedimentation (Nicell, 1994).

In this context, different techniques of enzymatic immobilisation are investigated in order to obtain success in the process. The immobilisation by covalent bond has been an approach commonly studied (Cao et al., 2016). Unfortunately, this technique requires agents of functionalization, consequently several steps are needed to obtain the immobilised enzyme (Chang and Tang, 2017).

Iron oxides particles have become widely recognized as support for molecules. Alternatively, the ferric oxyhydroxide can be considered an innovate material, due to their unique characteristics. This material has a high surface area, super magnetism, and excellent stability (Chagas et al., 2013). In addition, its structure is composed of the hydroxide anion (OH-), making it even more advantageous as support for enzymes immobilisation.

The functionalized iron oxide nanoparticles are frequently reported (Barbosa et al., 2012; Hola et al., 2015; Silva et al., 2016; Tavares et al., 2018). Nevertheless, immobilisation processes can be more interesting and less dispendious without the need to make functionalization steps. Which is possible when the support has functional groups able to interact with the enzyme, resulting in quicker, and economically viable processes.

The current work aimed to optimize the immobilisation process. We have undertaken a systematic chemometric study involving the HRP immobilisation onto nanoparticles. Important factors of the immobilisation process (temperature, pH, time, and enzyme/NPs ratio) were investigated. The effect of the components was studied using a factorial design at two levels. In studying these factors, the immobilised enzyme activity was the response measured. The obtained biocatalyst was evaluated in the ferulic acid oxidation, a model pollutant.

2. Materials and methods

2.1. Chemical reagents

Ferulic acid (FA) and horseradish peroxidase (EC 1.11.1.7 ≥ 146 units per mg protein) well as the other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Δ-FeOOH nanoparticles synthesis

The δ-FeOOH nanoparticles were synthesized according to the modified procedure described by Tavares and collaborators (Tavares et al., 2018). It consists of $Fe(NH_4)_2(SO_4)_2.6H_2O$ solution precipitation by NaOH solution, and oxidation by H_2O_2. This leads directly to the product δ-FeOOH.

2.3. Characterization of materials

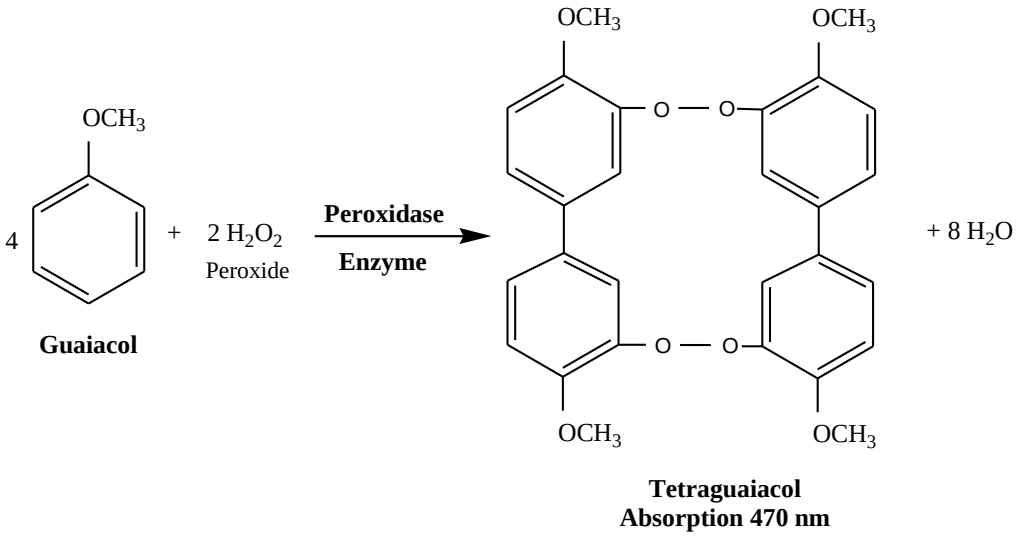
X-ray Powder Diffraction (XRD) was used to identify crystal structure and to determine the average particles size. It was used a Shimadzu XRD-6000 system with Cu Kα radiation (λ= 0.15406 nm), with the scanning step of 0.02, count time of 6s, 2θ range from 10 to 80º Scherrer equation was used to determine average particle size, after the correcting for instrumental broadening effects. From the Scanning Electron Microscopy (SEM) (LEO 440 with an Oxford detector with an electron beam operating at 15 kV) was determined the materials morphology. The materials surface groups were determined by Fourier transform infrared spectrophotometry (FT-IR Spectrometer, PerkinElmer Spectrum 2000 Spectrometer) with spectral range from 400 to 4000 cm^{-1}.

2.4. HRP immobilisation

The enzymatic solution of HRP (0.8 mg/mL; pH 4.0 citrate-phosphate buffer, and pH 8.0 Tris-HCl buffer) was added to the nanoparticles and left at the desired temperature and time, under constant stirring. The suspensions were submitted to an external magnet for 3 min to separate the particles. Next, the particles were washed with buffer (3 mL), distilled water (3 mL), and dried at 25 °C for 24 h. The enzymatic activity was measured from the washing waters to verify if the enzyme was coming out of the support. Then, the biocatalyst obtained was stored at 4 °C. Aliquots were taken at the end of each time to determine the residual enzyme activity and the maximum immobilisation yield.

2.4.1. Enzymatic activity

The activity measurements were conducted according to the classical assay described by Khan and Robison. The activity was expressed as units (U). One unit of peroxidase activity represents the oxidation of 1 μmol of guaiacol per minute. The assays were performed using 1.5 mL of guaiacol 1% (v/v), 0.4 mL of H_2O_2 0.3% (v/v), and 1.2 mL of 0.05 $molL^{-1}$ phosphate buffer pH 7.0. Adding the enzymatic solution (0.1 mL) at room temperature, and when for the immobilised enzyme (20 mg of particles) at 30 °C. The assay described is based on the rate of guaiacol oxidation for the formation of tetraguaiacol (ε = 26.6 $mM^{-1}cm^{-1}$), according to the Equation 1 (Khan e Robinson, 1994).



Equation 1 Biocatalytic oxidation of guaiacol catalyzed by horseradish peroxidase (HRP).

Organic compounds oxidation by the peroxidase occurs from the catalytic cycle (Fig. 1). Firstly, the native peroxidase [heme (Fe^{3+})] binding to the H_2O_2, resulting in a heterolytic cleavage between the oxygen atoms by electron transfer. A water molecule is released, and an intermediary enzyme state (I) is formed, comprising an oxyferryl species (Fe^{4+}=O) and a porphyrin radical cation. Next reaction step, the enzyme state (I) oxidizes the first organic compound molecule, releasing a radical product, and the enzyme state (II), an oxyferryl species (Fe^{4+}=O). Finally, the reactive enzyme state (II) is reduced by a second organic compound molecule, converting the enzyme back to the initial form. Both enzyme states (I and II) are powerful oxidants (Veitch, 2004).

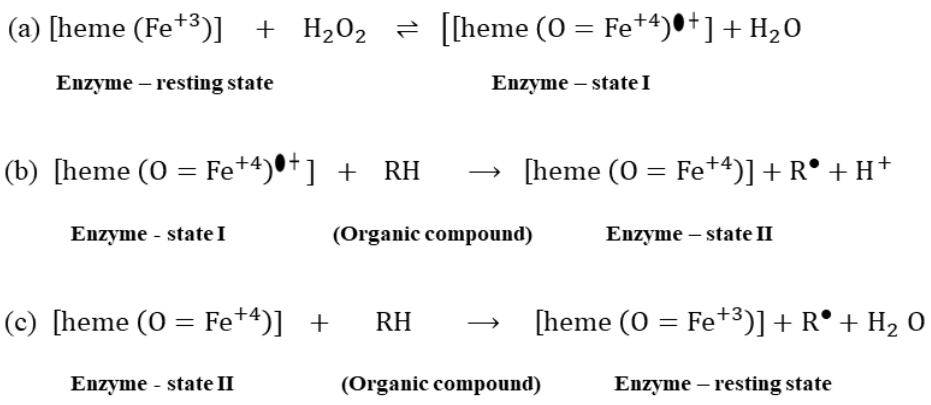


Fig. 1 The catalytic cycle of the horseradish peroxidase (HRP).

2.4.2. Process Optimization strategy – HRP immobilisation

It is known that the success of the enzymatic immobilisation depends on several factors, thus, a statistical study that considered different process conditions was conducted herein. The effects of the independent variables were evaluated at 95% significance by using a statistical software. The factorial design was developed using two levels, -1 and 1 (Table 1S). The independent variable X1 was the enzyme/NPs ratio (mg), with the lower level 1/63 (100 mg of NPs and 1.6 mg of protein) and the greater 1/313 (500 mg of NPs and 1.6 mg of protein). X2 was the variable pH, and the levels 4 and 8. The reaction time (min), levels 30 and 180 min was the variable X3. The variable temperature (X4) was using the levels 25 °C and 60 °C. Analyzing the effects of the fractional design on the responses, enabled to establish conditions to obtain the immobilised HRP without the need costly steps.

After the immobilisation, the Immobilisation Yield (IY) and the Immobilisation Efficiency (IE) of the process were calculated. IY and IE are terms often used to determine the success of enzyme immobilisation. These values can be obtained according to Equations 1 and 2. The immobilised activity was determined by measuring the total residual enzyme activity (remaing in the enzyme solution) after immobilisation, and by subtracting this activity from the total starting activity. Regarding the IE, it is considered the activity of the immobilised enzyme, denominated observed activity (Sheldon and Pelt, 2013).

$$\%\ IY = \frac{100x(Immobilised\ activity)}{starting\ activity} \qquad (1)$$

$$\%\ IE = \frac{100x\big(observed\ activity(particles)\big)}{immobilised\ activity} \qquad (2)$$

2.5. Ferulic acid oxidation

The biocatalyst performance was evaluated in the FA removal. The magnetic biocatalyst (800 mg of particles, which corresponds to activity 1.48U) obtained from the best immobilisation condition, was dispersed into at a constant pH (citrate phosphate buffer pH 7.0) with the addition of 3.0 mL of FA solution (1 mM), and 0.8 mL from H_2O_2 (2 mM). The FA removal assays were carried out in glass reaction flasks, and the mixture was kept under stirring for 30 minutes at 30 °C (Tavares et al., 2018). Two experiments controls were carried out, one in the absence of the enzyme, and the other in the absence of hydrogen peroxide. At the end of the reaction, the immobilised enzyme was removed from the medium by magnetic separation, and the enzymatic activity measured from the reaction medium, in

order to verify if the enzyme was coming out of the support. The FA residual concentration was measured by the Folin and Denis colorimetric method (AOAC, 2012). The removal percentage (%) was calculated from the difference between initial and remained concentration of FA, after the reaction.

3. Results and Discussion

3.1. Characterization of materials

The XRD results indicated that the particles of δ-FeOOH were synthesized as a dominant phase. It presenting the reflections of the planes (100) (101) (102) (110) which are consistent with the reported data at the International Centre for Diffraction Data (JCPDS N° 13-0087) for this oxide (Fig. 2A c). However, goethite (α-FeOOH) phase was also observed for this material. It being evident for δ- FeOOH-HRP material (Fig. 2A b). In addition, an amorphous halo was observed, centered on $2\theta = 23°$ (Fig. 2A).

In order to know the chemical bonds present in the materials before and after immobilisation, they were characterized by FTIR within the wavelength range 500-4000 cm^{-1} (Fig. 2B). The spectral regions at 3380 cm^{-1} are attributed to O-H vibration modes, at 2930 cm^{-1} for the alkyl ($-CH_2$) chains (Szymanski, 1962). The free enzyme sample has presented peaks at 1655 and 1545 cm^{-1} that can be assigned to the amide I (α-helix structure) (Goormaghtigh et al., 2006). It can also be observed absorption at 1400 cm^{-1}, attributed to several modes of NH (amide III) (Barth, 2007; Goormaghtigh et al., 2006). Aliphatic amines (C-N stretching vibration) may be observed at the region 1076 cm^{-1}. Also, C-H deformation was displayed at around 600 cm^{-1}. The OH characteristic of oxide hydroxide was displayed at 3400 cm^{-1}. Bending vibrations Fe-OH may be related to the peaks around at 1636 and 1112 cm^{-1}. The peaks at 890-454 cm^{-1} correspond to the metal-oxygen (Fe-O) (Kulal et al., 2011). However, vibrations Fe-OH (1112 cm^{-1}) was not displayed after immobilisation, indicating a possible interaction between enzyme and the support after immobilisation process.

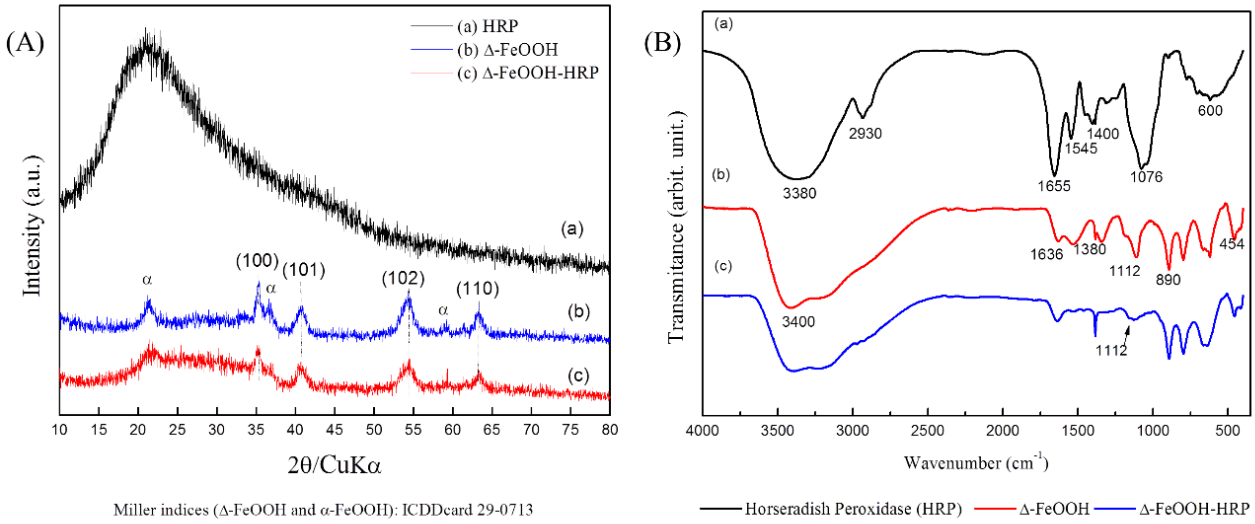


Fig. 2 (A) X-ray diffraction (XRD): (a) Horseradish Peroxidase (HRP) (b) δ-FeOOH-HRP (c) δ-FeOOH; (B) FTIR spectra of (a) HRP; (b) δ-FeOOH and (c) δ-FeOOH-HRP.

The morphological changes of the materials were analyzed by SEM. The SEM images of HRP, pure δ-FeOOH, and biocatalyst (δ-FeOOH-HRP) are shown in Fig. 3. The SEM micrographs (Fig. 3b) show that the nanoparticles are structured in irregular compact blocks, and the enzyme is probably entrapped in these structures (Fig. 3c and d).

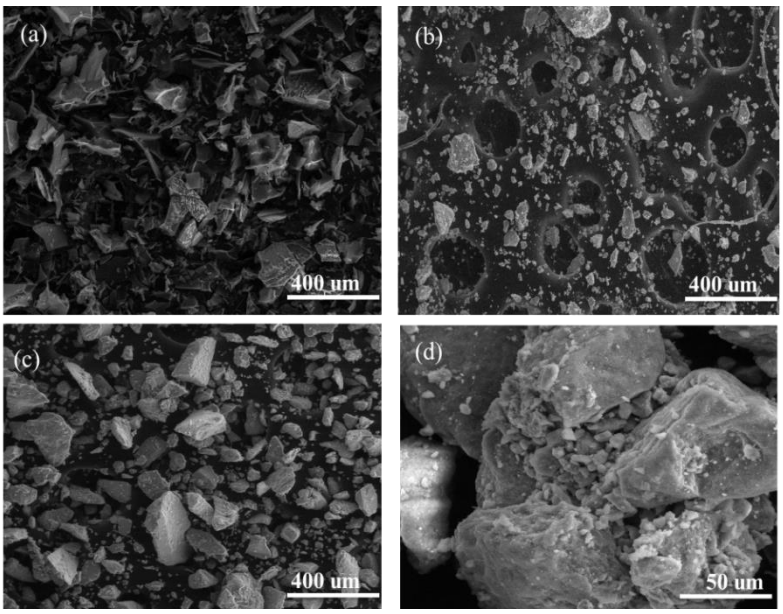


Fig. 3 SEM image: (a) Horseradish Peroxidase (HRP); (b) Δ-FeOOH particles; (c and d) Δ-FeOOH-HRP.

3.2. Immobilisation process HRP - Process Optimization strategy

The immobilisation experiments were conducted successfully. Regarding as to how the HRP has interacted on the surface of the δ-FeOOH, it known that -OH (hydroxyl) groups and O (oxygen) are available on its structure. Being δ-FeOOH surface positively charged (Da Silva et al., 2017), the interaction between HRP and NPs will more likely occurs with HRP residues charged negatively. Therefore, the parameters variation for the immobilisation process is an important step to achieve the enzyme stabilization, enabling the interaction of the support to ligands provided by a combination of amino acid side-chain of the enzyme. The parameter: enzyme/NPs ratio (X1), pH (X2), time (X3) and temperature (X4) were considered to define the best conditions for immobilisation process, associated with the immobilised enzyme with best performance catalytic.

The effects of the independent variables were analyzed (Table 1). The independent variables that showed significant effects estimate were the variables X1 (-0.58), X2 (0.71) and X4 (0.42). In the combination of the variables there were two significant effects estimate, X1*X2 (- 0.57) and X2*X4 (0.39). The results showed that the change from the X1 level -1 (1/63 enzyme/NPs) to level 1 (1/313 enzyme/NPs) decreased 58% the immobilised HRP activity.

Although the change in variable X2 and X4 from level -1 (pH 4.0 and 25 °C) to level 1 (pH 8.0 and 60 °C) increased the activity in 71% and 42%, respectively and in the combination of the X2 (pH 8.0) and X4 (60 °C) the change caused 39% of increase in the activity. These effects can be observed in the surface response (Fig. 4) for the changes interaction in the variables X1 and X2, and their influences under the enzymatic activity (Y), the dependent variable (further details are available in the supplementary materials).

Also, the response surface (Fig. 4) showed the effects of the results influenced by the changes in variables X1 and X4. It could be observed that when the variable X1 is positive (1), and the variable X2 is positive (1) the results (Y) is lower. When the variable X1 is negative (-1), and the variable X2 is positive (1) the result assumed the higher value. By analyzing the response surfaces was possible to find the best immobilisation parameters inside the experimental conditions. The optimized experiment was X1 = 1/63 (enzyme/NPs), X2 = pH 8, and X4 = 60 °C (Fig. 4 and Table S1: experiments 10 and 14). The variable X3 (Time) does not change with the changing to level -1 from 1, consequently, its values were not significant. This way, 30 minutes is the time recommended.

When using a lower enzyme dose (1/313 enzyme/NPs at pH 8.0). It was obtained a higher immobilisation yield (with the exception in pH 4.0 at 60 °C). That is because the excess of enzymes may result in an agglomeration of those on the surface of the support. However, the process conducted with pH 4.0 at 60 °C, and lower enzyme dose (experiments 11 and 15) presented low immobilisation

(29% and 52%, respectively) when compared to greater enzyme dose (experiments 9 and 13), presenting 86% and 100% of immobilisation yield, respectively (Fig. 5A).

Although a higher immobilisation was observed, considering the lower dose enzyme (1/313 enzyme/NPs), the activity was not found in the immobilised. That can be attributed, to the inaccessible substrate to surface, or pores of the support with higher enzyme layers (Sheldon and Pelt, 2013). In addition, the immobilised enzyme activity was not found at pH 4.0, probably due to the decrease in the stability of the three-dimensional structure at pH below 4.5 (Chattopadhyay and Mazumdar, 2000). The immobilised HRP from processes (2, 6, 10 and 14) involving the greater dose enzyme (1/63 enzyme/NPs) and pH 8.0 presented catalytic activity, displaying better activity when obtained at 60 °C than at 25 °C.

Table 1 Effects Estimate from each independent variable of the Factorial Design 2^4 evaluated at 95% of the coefficient limit.

Factor	**Effects Estimate**
Mean/Interc.	0.371250
X1	-0.578250
X2	0.712750
X3	-0.022750
X4	0.422000
X1*X2	-0.566000
X1*X3	0.038500
X1*X4	-0.257750
X2*X3	-0.035000
X2*X4	0.392250
X3*X4	0.113250

X_1: enzyme/NPs; X_2: pH; X_3: time (minutes); X_4: temperature (°C).

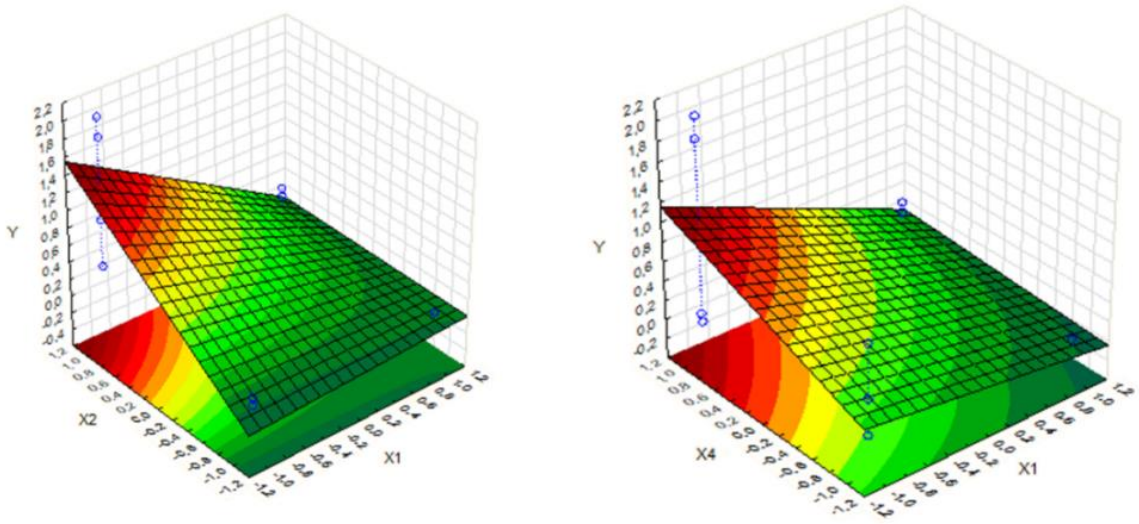


Fig. 4 Response Surface of Factorial Design a) Variable X1 vs X2 b) Variable X1 vs X4.

Nevertheless, under certain reaction conditions (Fig. 5B) the immobilisation processes resulted 0% of immobilisation efficiency, or none enzymatic activity was found in the immobilised enzyme. It is possible to obtain an immobilisation yield of 100%, and lowest, or 0% immobilisation efficiency. It occurs when all of the enzymes in solution is immobilised, but no activity is found in the immobilised enzyme, because the enzyme was deactivated or became inaccessible for some reason upon immobilisation (Sheldon and Pelt, 2013).

A good immobilisation yield and the best activity of the immobilised HRP were obtained at 60 °C. The influence of temperature may lead to changes in the structure of the enzymes. Regardless of pH, the higher temperature can result in HRP unfolding phase. Thus, the enzyme tertiary structure can be affected at around 45 °C but without depletion of the heme group, which occurs at the highest temperature (74 °C) (Chattopadhyay and Mazumdar, 2000). The interaction between HRP and support (δ-FeOOH) was directly influenced by higher temperatures, without compromising the activity after immobilisation.

After process optimization, the immobilised horseradish peroxidase displayed 1.9 U/g of activity. Those values are highest than reported for immobilised soybean peroxidase (0.87 U/g) on δ-FeOOH (added aldehyde group) (Tavares et al., 2018), indicating that δ-FeOOH, without the functionalization steps, is a promising support for peroxidase. Although it is common in some cases, a certain enzyme amount comes out of the support, after immobilisation (Sheldon and Pelt, 2013). In the present study, after immobilisation process, and at the end of oxidation reaction, no enzyme was observed leaching

from the support. Therefore, it could be concluded the strong interaction between the enzyme and the support.

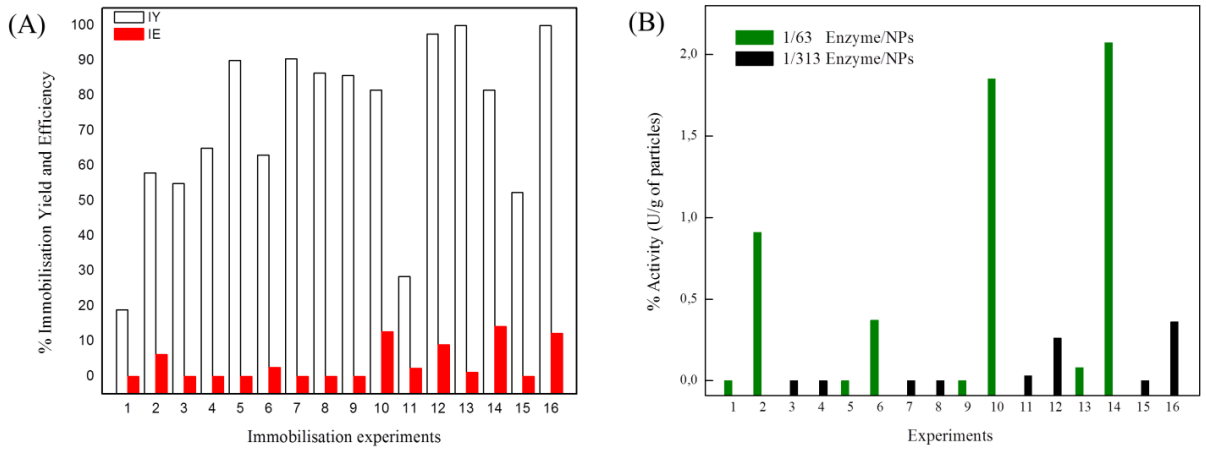


Fig. 5 (A) Immobilisation yield (IY); Immobilisation efficiency (IE) and (B) Activity (U/g of nanoparticles) of HRP on ferroxyte Δ-FeOOH under different processes conditions: pH 4.0 (odd-number experiments), pH 8.0 (even-number experiments) and temperature 25 °C (experiments 1 until 8), 60 °C (experiments 9 until 16).

3.3. Ferulic acid oxidation

The peroxidase showed potential of 82% in the FA removal (800 mg of immobilised HRP - 1.48U). The controls without peroxidase, as that made in the absence of hydrogen peroxide, did not result in any removal of the pollutant, confirming that only the peroxidase acted in the FA oxidation. This indicates that the NPs bonded to HRP do not block the site where the FA is going to bond. Usually, the methoxy oxygen of FA (Fig. 6) is hydrogen bonded to the arginine 38 residue at the substrate binding site (Henriksen et al., 1999).

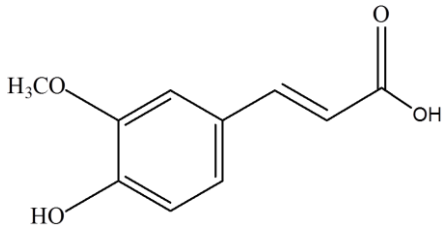


Fig. 6 Structure of trans-Ferulic acid (FA).

The FA oxidation is initiated from the peroxidase catalytic mechanism (Fig. 7), which includes the transfer of electrons. The first state of the modified enzyme (I) is generated from the reaction between H_2O_2, and the Fe^{+3} resting state, comprising Fe^{+4}, and porphyrin radical cation. The enzyme stage (I) has two oxidation equivalents, above the resting state. Then, the modified enzyme oxidizes the ferulic acid, resulting the other modified enzyme state (II), a Fe^{+4} no porphyrin radical cation. The ferulic acid oxidation in the two reduction steps, leads to the radical species generation. It can result in the oxidized molecules to react to each other, resulting in products as dimers, trimers, and higher oligomers (Veitch, 2004; Silva et al., 2015).

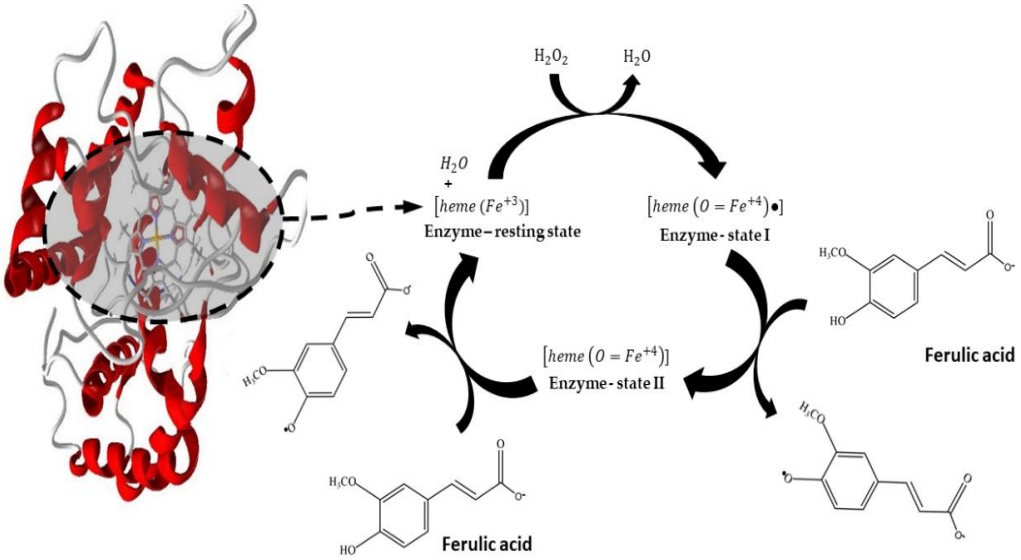


Fig. 7 Peroxidases catalytic cycle involving the ferulic acid oxidation.

The immobilised enzyme conformation, as well as the diffusion of the substrate, seem to be favorable to the catalytic performance since the substantial removal of pollutants has been achieved (Brenda and Batista-Vieira, 2006; Es et al., 2015; Xie et al., 2009). Besides overcoming the limitations of the free enzyme, the peroxidase immobilised on δ- FeOOH, after addition of aldehyde groups (δ-FeOOH-SiO_2-APTES-GLU), showed a good efficiency to remove the AF (Tavares, 2018).

Faced with this, δ-FeOOH may be highlighted as an excellent support for peroxidases. This mainly can be assigned to its large surface area, and functional groups on its surface, theses enable its interaction with the amino acid residues of the HRP, and do not compromise the interaction between FA and immobilised HRP. Therefore, the acquisition of an efficient nanobiocatalyst, from a support with attractive characteristics, cheap synthesis, and without functionalization steps, becomes the process more attractive to industrial applications.

4. Conclusion

In the present work, a magnetic biocatalyst was obtained via peroxidase immobilisation onto the iron oxide. The enzyme successfully interacted to the ferroxyte nanoparticles, without the need to use silica coatings and the addition of functional groups. The optimized process allows the obtainment of a reusable biocatalyst, in the absence of costly steps. Besides that, the biocatalyst showed good catalytic activity, which makes this process more interesting for industrial applications than free enzymes. Future studies will be done to evaluate its performance in the removal of other organic pollutants. Based on the results, we conclude that HRP immobilised on ferroxyte can be considered a promising catalyst for environmental bioremediation.

Conflicts of Interest: The respective authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; membership, employment, consultancies or patent-licensing arrangements), or non-financial interest (such as personal, affiliations, or beliefs) in the subject matter or materials discussed in this manuscript.

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Supplementary Materials

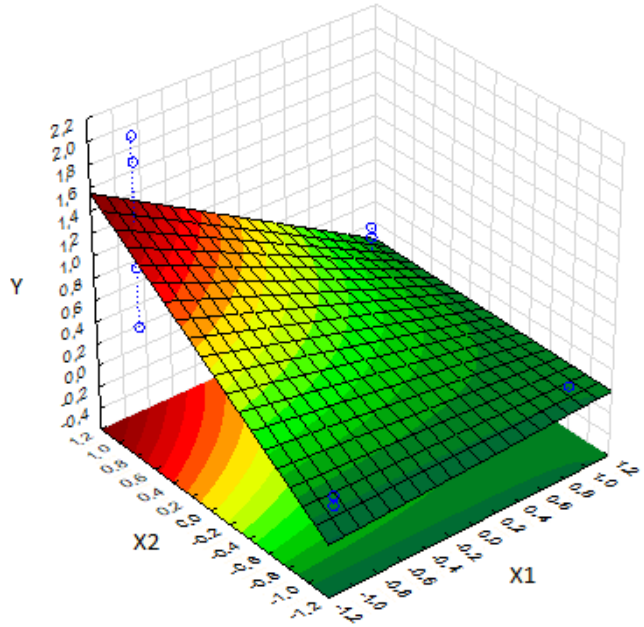


Fig.S1 Response Surface of Factorial Design between Variable X1 vs X2

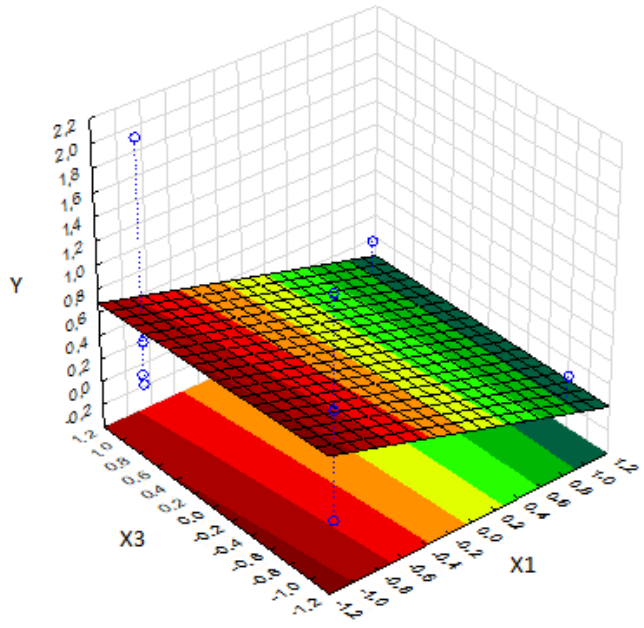


Fig.S2 Response Surface of Factorial Design between Variable X1 vs X3

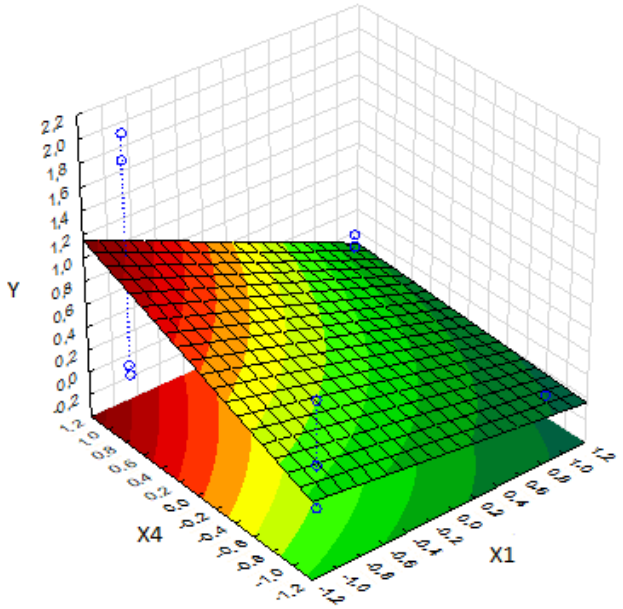


Fig.S3 Response Surface of Factorial Design between Variable X1 vs X4

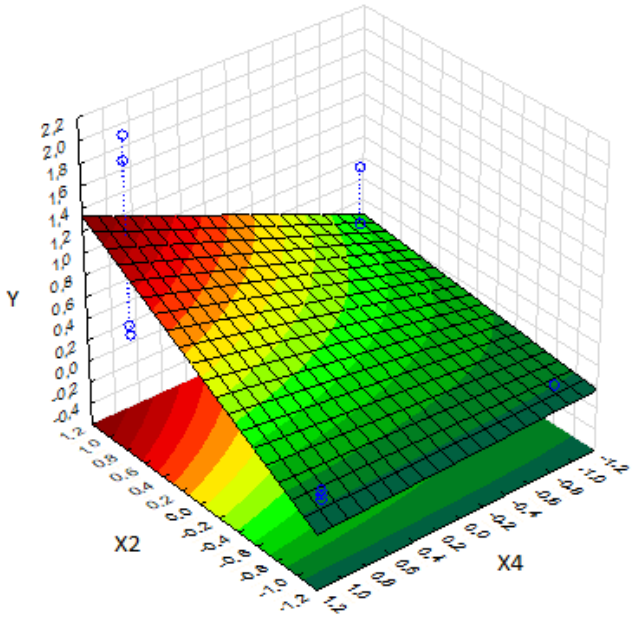


Fig.S4 Response Surface of Factorial Design between Variable X2 vs X4

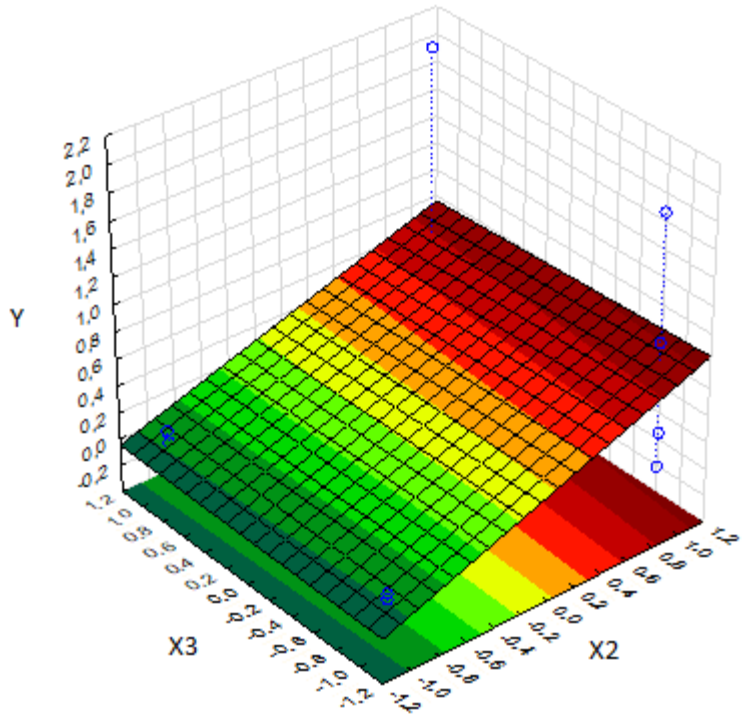


Fig.S5 Response Surface of Factorial Design between Variable X2 vs X3

Table S1 - Experiments matrix of 24 fractional factorial.

Experiments	(X1)a	(X2)b	(X3)c	(X4)d
1	-1 (1/63)	-1 (4.0)	-1 (30)	-1 (25)
2	-1 (1/63)	1 (8.0)	-1 (30)	-1 (25)
3	1 (1/313)	-1 (4.0)	-1 (30)	-1 (25)
4	1(1/313)	1 (8.0)	-1 (30)	-1 (25)
5	-1(1/63)	-1 (4.0)	1 (180)	-1 (25)
6	-1(1/63)	1 (8.0)	1 (180)	-1 (25)
7	1(1/313)	-1 (4.0)	1 (180)	-1 (25)
8	1(1/313)	1 (8.0)	1 (180)	-1 (25)
9	-1(1/63)	-1 (4.0)	-1 (30)	1 (60)
10	-1(1/63)	1 (8.0)	-1(30)	1 (60)
11	1 (1/313)	-1 (4.0)	-1 (30)	1 (60)
12	1(1/313)	1 (8.0)	-1 (30)	1 (60)
13	-1(1/63)	-1 (4.0)	1(180)	1 (60)
14	-1(1/63)	1 (8.0)	1 (180)	1 (60)
15	1(1/313)	-1 (4.0)	1 (180)	1 (60)
16	1(1/313)	-1 (8.0)	1 (180)	1 (60)

a X1: enzyme/NPs ratio. b X2: pH. c X3: time (minutes). d X4: temperature (°C).

Table S2 - ANOVA from each independent variable of the factorial. Design 2^4 evaluated at 95% of the coefficient limit.

ANOVA; Var.:Var5; R-sqr=0.94646; Adj:0.83937					
Factor	SS	df	MS	F	p
X1	4.57285	1	4.572854	28.46786	0.003101
X2	3.00985	1	3.009845	18.73750	0.007509
X3	0.00466	1	0.004659	0.02900	0.871449
X4	1.60302	1	1.603016	9.97942	0.025123
X1 by X2	2.88367	1	2.883671	17.95202	0.008193
X1 by X3	0.01103	1	0.011027	0.06865	0.803770
X1 by X4	1.38496	1	1.384965	8.62197	0.032395
X2 by X3	0.01334	1	0.013342	0.08306	0.784755
X2 by X4	0.59801	1	0.598012	3.72287	0.111565
X3 by X4	0.11545	1	0.115449	0.71872	0.435259
Error	0.80316	5	0.160632		
Total SS	15.0000	15			

Table S3 - Effect estimates from each independent variable of the Factorial. Design 2^4 evaluated at 95% of the coefficient limit.

Effect Estimates							
Factor	Effect	Std.Err.	t(5)	p	-95% Cnf.Limt	+95% Cnf.Limt	Coeff.
Mean/Interc.	-0.000000	0.100197	-0.00000	1.000000	-0.25757	0.257565	-0.000000
X1	1.069212	0.200395	5.33553	0.003101	0.55408	1.584343	0.534606
X2	-0.867445	0.200395	-4.32868	0.007509	-1.38258	-0.352314	-0.433723
X3	-0.034128	0.200395	-0.17030	0.871449	-0.54926	0.481003	-0.017064
X4	0.633051	0.200395	3.15902	0.025123	0.11792	1.148182	0.316526
X1 by X2	-0.849069	0.200395	-4.23698	0.008193	-1.36420	-0.333938	-0.424534
X1 by X3	-0.052504	0.200395	-0.26200	0.803770	-0.56764	0.462627	-0.026252
X1 by X4	0.588423	0.200395	2.93632	0.032395	0.07329	1.103554	0.294211
X2 by X3	0.057755	0.200395	0.28820	0.784755	-0.45738	0.572886	0.028877
X2 by X4	-0.386656	0.200395	-1.92947	0.111565	-0.90179	0.128475	-0.193328
X3 by X4	0.169889	0.200395	0.84777	0.435259	-0.34524	0.685020	0.084944

CONSIDERAÇÕES FINAIS

A obtenção de um biocatalisador de baixo custo foi possível utilizando resíduos do grão de soja (casca) como fonte de peroxidase, por meio da precipitação das proteínas com um solvente orgânico (acetona). O solvente foi reciclado, podendo ser reutilizado no processo. A enzima apresentou alta atividade catalítica e excelente potencial na remediação de poluentes fenólicos.

A peroxidase de soja foi imobilizada em nanopartículas de ferroxita (δ-FeOOH), um oxidróxido de ferro pouco explorado como suporte para biomoléculas. As partículas foram revestidas com sílica, e sua superfície modificada com adição de grupos aldeídos. As técnicas de caracterização confirmaram o sucesso da síntese e funcionalização das nanopartículas. A peroxidase de soja imobilizada exibiu excelente performance na remoção do ácido ferúlico em meio aquoso, com melhor desempenho que a enzima livre.

Tanto a peroxidase de soja quanto a peroxidase de raiz forte são isoenzimas, portanto, o processo de imobilização em nanopartículas de ferroxita, na ausência de agentes intermediários, foi otimizado utilizando peroxidase de raiz forte purificada. Uma abordagem quimiométrica, o DOE (*design* estatístico de experimentos) possibilitou uma melhor análise da influência dos fatores no processo de imobilização, o que envolveu a realização mínima de experimentos quando comparados com o planejamento de experimentos tradicional. A Metodologia de Superfície de Resposta foi utilizada para avaliar a relação entre os fatores envolvidos no processo e a variável resposta.

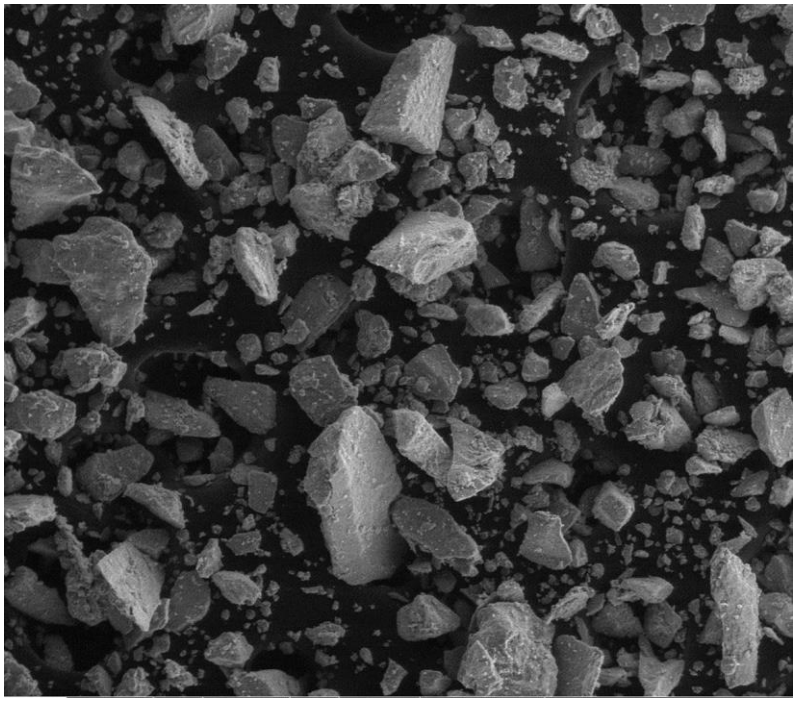
Os resultados promissores das nanopartículas de ferroxita "pura" como suporte enzimático, levou a um novo projeto, o qual foi realizado durante o doutorado sanduíche na Universidade de Hradec Králové, República Tcheca. Essas pesquisas envolveram a ferroxita como suporte para colinesterases, enzimas de grande importância na desintoxicação de compostos organofosforados. Neste projeto, a butirilcolinesterase foi obtida por meio da Tecnologia do DNA recombinante após o cultivo de células humanas. A enzima foi imobilizada em nanopartículas de ferroxita, resultando em um excelente scavenger (eliminador) de compostos organofosforados.

A tecnologia desenvolvida apresenta várias vantagens em relação a outros processos. A ferroxita como um suporte enzimático supermagnético possibilita a obtenção de biocatalisadores "verdes" para remediação ambiental, os quais são de baixo custo e recicláveis. Além disso, o oxidróxido de ferro apresenta uma superfície que favorece a interação de enzimas sem a necessidade de agentes intermediários, o que é muito interessante para imobilização de enzimas de importância toxicológica.

ANEXO

Figura a ser publicada (E-book /Editora UFLA)

Figura selecionada no concurso “Ciência em Imagem” para integrar o e-book a ser publicado pela Editora UFLA.



O princípio da “Química verde” envolve o desenvolvimento de tecnologias sustentáveis gerando o mínimo de resíduos possíveis. Neste princípio, foi desenvolvido um catalisador biodegradável e magnético (Figura acima) preparado a partir de plantas (enzima peroxidase de raiz-forte) e nanopartículas magnéticas de óxido de ferro. O catalisador foi utilizado para remoção de contaminantes em água, removendo mais de 80% de um poluente (ácido ferúlico) frequentemente descartado nas águas residuais das indústrias de óleo, petróleo, papel e farmacêuticas.

Artigos para publicação

Methyl orange and Rhodamine B Oxidation by a Super Magnetic Biocatalyst based on Peroxidase Immobilised on δ-FeOOH Nanoparticles

Artigo á ser submetido na revista Journal of Environmental Management

Abstract

In recent years, wastewater remediation by biocatalysts has been considered a very attractive alternative, since the use of enzyme involves sustainable processes. In this work, it was prepared a nanocatalyst from the peroxidase immobilisation onto iron oxide nanoparticles. The electrophoresis, protein concentration, and enzymatic activity remaining have been used to evaluate the enzyme immobilisation. The materials were characterized before and after immobilisation. The magnetic catalyst obtained, it was used for color removal of dyes in the aqueous medium. It was investigated the following parameters variations: reaction time, peroxide concentrations, methyl of orange and rhodamine B concentrations. Also, the storage stability was observed. Repeated cycles were performed in the catalyst reuse. The protein concentrations corroborated the enzymatic activity results, presenting immobilisation yield of 80%. In only 30 minutes, the immobilised enzyme was able to remove 75% and 34% of color, methyl orange, and rhodamine B, respectively. The immobilised enzyme displayed better performance than free enzyme in color removal from the rhodamine B. Peroxidase immobilised as free peroxidase removed 75% of the methyl orange color. The color removal decreased in the second cycle of the reuse for both dyes, removing until the second, and the third cycle the rhodamine B, and methyl orange color respectively.

Keywords: Dyes; Bioremediation; Horseradish; Ferroxyte, Immobilisation.

Hybrid Scavenger against Organophosphate based on Magnetic Nanoparticles and Recombinant Human Butyrylcholinesterase

Artigo á ser submetido na revista Bioconjugate Chemistry

Abstract

Organophosphorus compounds (OPs) persistent overuse global has led to the accumulation of these compounds in food and the environment, causing a major hazard to agricultural laborers, and consumers. In another hand, hybrid nanomaterials attract much attention because of their unique properties and potential applications. The development of a magnetic scavenger for organophosphate compounds, it is approached in this work. It was based on the human butyrylcholinesterase (BChE) immobilisation on ferroxyte nanoparticles. The protein was obtained from the kidney cells humans, involving the Recombinant DNA Technology. In three-weeks human cells were cultivated. Then, proteins expression was followed by three-days. The purification of the protein was carried out by affinity chromatography. The evaluation of the purification process was performed by determining the electrophoresis, protein concentration, and enzymatic activity. The immobilisation process was investigated at 5°C, 23°C, and 37°C for 2 hours. The evaluation of the process was performed by determining the protein concentration, enzymatic activity, and western blotting. For evaluation of the organophosphate scavenger, paraoxon-ethyl, and chlorpyrifos were used as model compounds. The scavenger process was carried out by the enzymatic inhibition of the free and immobilised enzyme, determining the inhibition constant (Ki). 100% yield of the immobilisation process was obtained, displaying activity of 0.60 ΔU/mg of particles. Similar values of the inhibition constant (Ki) was obtained for the paraoxon-ethyl inhibitor, involving the free enzyme (Ki = 0.16 μM), and immobilized enzyme (Ki = 0.18 μM). Chlorpyrifos inhibitor has shown a better binding affinity to immobilised enzyme (Ki = 2.09 μM), when compared to free enzyme (Ki = 1.90 μM). The immobilised recombinant BChE presented excellent performance, inhibiting more than 90% of the compounds in the conditions investigated.

Keywords: Recombinant protein; Cholinesterase; Iron oxide; Immobilisation.

Artigos publicados

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Review article

Insights into the pharmaceuticals and mechanisms of neurological orphan diseases: Current Status and future expectations

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ARTICLE INFO

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Amyotrophic lateral sclerosis
Duchenne muscular dystrophy
Spinal muscular atrophy and familial amyloid polyneuropathy

ABSTRACT

Several rare or orphan diseases have been characterized that singly affect low numbers of people, but cumulatively reach ~6%–10% of the population in Europe and in the United States. Human genetics has shown to be broadly effective when evaluating subjacent genetic defects such as orphan genetic diseases, but on the other hand, a modest progress has been achieved toward comprehending the molecular pathologies and designing new therapies. Chemical genetics, placed at the interface of chemistry and genetics, could be employed to understand the molecular mechanisms of subjacent illnesses and for the discovery of new remediation processes. This review debates current progress in chemical genetics, and how a variety of compounds and reaction mechanisms can be used to study and ultimately treat rare genetic diseases. We focus here on a study involving Amyotrophic lateral sclerosis (ALS), Duchenne Muscular Dystrophy (DMD), Spinal muscular atrophy (SMA) and Familial Amyloid Polyneuropathy (FAP), approaching different treatment methods and the reaction mechanisms of several compounds, trying to elucidate new routes capable of assisting in the treatment profile.

1. General introduction

In general, a rare or orphan disease (OD) is a health framework which is characterized by affecting a low percentage of people. In this sense, a disease considered rare somewhere, or in a specific group of people, could be an ordinary disease anywhere else; therefore, there is no universal definition of rare disease to date (Richter et al., 2016; Sardana et al., 2011).

A rare disease is defined as any illness or condition that reaches less than 200 000 people inside the United States, and fewer than 50 000 patients in Japan. The European Commission on Public Health, in turn, describes rare diseases as those of low predominance (1 in 2000 people) (Sardana et al., 2011). So far, in the medicine field, a disease is defined as a change in the health condition, possessing a unique pattern of

Abbreviations: ALS, Amyotrophic lateral sclerosis; DMD, DuchenneMuscular Dystrophy; SMA, SpinalmuscularAtrophy; FAP, FamilialAmyloid Polyneuropathy; OD, Orphandisease; ORD, Officeof Rare Diseases; NIH, NationalInstitutes of Health; sALS, SporadicALS; fALS, FamilialALS; CNS, Centralnervous system; iGluRs, Ionotropicglutamate receptors; AMPA, α-amino-3-hydroxy-5-isoxazolepropionate; NMDA, *N*-methyl-o-aspartate; NOS, NOsynthase; EAATs, Excitatoryamino-acid transporters; AD, Alzheimer's disease; PD, Parkinson's disease; OS, Oxidative stress; ROS, Reactive oxygen species; RNS, Reactive Nitrogen Species; NO-, Nitric Oxide; endothelial NOS, namely eNOS; inducible NOS, iNOS; neuronal NOS, nNOS; H_2O_2, Hydrogenperoxide; CSF, Cerebrospinalfluid; HNE, Hydroxy-2-nonenal; TBARS, Thiobarbituricacid-reactive substances; MDA, Malondialdehyde; IsoP's, Isoprostanes; MYPT, Myosinlight chain phosphatase; DCPs, Decappingscavenger enzyme; ETC, Electrontransport chain; FDA, Foodand Drug Administration; NMDA or AMPA receptors, Ionotropicglutamate receptors; GLT, Glialglutamate transporter; JAK, Januskinase; hnRNPs, Heterogeneousnuclear ribonucleoproteins; FAP, Familialamyloid polyneuropathy; TTR, Transthyretin; ATTR, TTRamyloidosis; RBP, Retinol-bindingprotein; ATTRwt, Wild-typeTTR amyloidosis; NSAID, Non-steroidalanti-inflammatory drug; siRNAs, InterferingRNAs; OXPHOS, Oxidativephosphorylation; RPPX, Dexpramipexole; MPCs, Muscleprogenitor cells; CS, Corticosteroids; MLPA, Multiplexligation-dependent probe amplification; CNV, Copynumber variation; STAT, SignalTransducer and Activator of Transcription; ROCK, Rho-associatedprotein kinase; LIMK, Limkinases; CGH, Comparativegenomic hybridization; AAVs, AdenoAssociated Viruses; EDL, Extensordigitorumlongus; HDAC, Histonedeacetylase; TSA, TrichostatinA; Aas, Aminoacids; TCA, Tricarboxylicacid; nNOS, Neuronalnitric oxide synthase; SERCA1, Sarcoplasmicreticulum (SR) calcium ATPase 1; SMN1, TheSurvival motor neuron 1; pre-mRNA, Precursormessenger RNA; CREB, cAMPresponse element-binding protein

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

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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 carbon activated (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.

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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

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ARTICLE

Immobilization of soybean peroxidase on silica-coated magnetic particles: a magnetically recoverable biocatalyst for pollutants removal.

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In this work we investigated the enzymatic degradation of ferulic acid, a model pollutant by free and immobilized soybean peroxidase. With the aim of developing greener catalysts we proposed the synthesis of a magnetic catalyst prepared via immobilization of soybean peroxidase onto a magnetic nanosupport by covalent attachment. The immobilization of soybean peroxidase was carried out using the magnetite nanoparticles modified with amino groups as support. The magnetite particles were characterized before and after chemical modification by XRD, SEM and TEM analysis. The characterization data indicated that the Fe_3O_4-SiO_2 nanoparticles were successfully synthetized. The high immobilization yield was obtained in only 1 hour of reaction (89.23%). The resulting nanobiocatalyst (enzyme load 5.25 U) was able to remove 99.67 ± 0.10% of ferulic acid in comparing to 57.67 ± 0.27% for free enzyme in the same reaction conditions. The immobilized peroxidase could easily be separated and reused under magnetic field. On the basis of these results, we concluded that the prepared magnetic nanoparticles can be considered a high-performance nanocatalyst for environmental remediation.

Introduction

Concerning environmental issues, the development of highly efficient catalysts constitutes one important approach to minimize the effect of pollutants in wastewaters. In this context, the use of enzymes, which are versatile and specific catalysts, has constantly increased over time due to peculiar properties of this class of proteins.[1]

Peroxidases, like horseradish peroxidase (HRP) and soybean peroxidase (SP) are efficient catalyst for removal of phenols from industrial wastewaters.[2-6] These enzymes are able to catalyse the oxidation of phenols in the presence of H_2O_2, producing free radicals. The generated free radicals react with each other in a non-enzymatic process to form polymers, which can be easily separated from aqueous medium.[3-4]

Ferulic acid, a phenolic compound, is considered a high-priority environmental pollutant due to its high resistance to conventional biological oxidation. Ferulic acid is a lignin model compound and can be found in many industrial effluents and residues, including those produced in wine-distilleries, olive oil processing industries, pulp paper processing and others.[7-8]

The short catalytic lifetime of the enzyme is one significant drawback in enzymatic technology, mainly to phenolic compounds degradation, due to the inactivation of the enzyme induced by the polymerization process.[9] The enzyme immobilization is an effective way to overcome this limitation. Besides improving stability and promoting significant enhancement in the overall process efficiency, the biocatalyst can be reused and recovered at the end of the process and be used in continuous processes.[1]

The immobilization strategies include encapsulation or entrapment, physical adsorption and covalent attachment.[10-11] The most popular enzyme immobilization method is the physical adsorption. This method is characterized by its simplicity, because previous functionalization of the carrier surface is typically not required. Denaturation and/or deactivation of the enzyme can be avoided and, thus, retention of catalytic activity of the immobilized enzyme is very high.[12-15] Moreover, the enzyme stability is significantly improved since this process makes use of the physical interactions generated between the carrier and enzyme that include van der Waals forces, ionic interactions and hydrogen bonding. The binding are rather weak and, what is important, typically are does not change the native structure of the enzyme. This prevents the active sites of the enzyme from disturbing and allows the enzyme to retain its activity. Physical bonding is generally too weak to keep the enzyme fixed to the carrier and is prone to leaching of the enzyme. Besides that, the physical adsorption is

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