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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

MARIANA PAOLA CABRERA

IMOBILIZAÇÃO DE ENZIMAS EM SUPORTES MAGNÉTICOS

Recife
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Tese apresentado ao Programa de Pós-Graduação em Ciências Biológicas para obtenção do título de Doutora em Ciências Biológicas pela Universidade Federal de Pernambuco.

Orientador: Prof. Dr. Luiz Bezerra de Carvalho Júnior

Co-orientador: Prof. Dr. Fernando Soria

Co-orientador: Prof. Dr. David Fernando Morais Neri

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pelo grande exemplo de mãe, amiga e mulher.

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A Deus, pela vida.

“O dia mais belo: hoje
A coisa mais fácil: errar
O maior obstáculo: o medo
O maior erro: o abandono
A raiz de todos os males: o egoísmo
A distração mais bela: o trabalho
A pior derrota: o desânimo
Os melhores professores: as crianças
A primeira necessidade: comunicar-se
O que traz felicidade: ser útil aos demais
O pior defeito: o mau humor
A pessoa mais perigosa: a mentirosa
O pior sentimento: o rancor
O presente mais belo: o perdão
O mais imprescindível: o lar
A rota mais rápida: o caminho certo
A sensação mais agradável: a paz interior
A maior proteção efetiva: o sorriso
O maior remédio: o otimismo
A maior satisfação: o dever cumprido
A força mais potente do mundo: a fé
As pessoas mais necessárias: os pais
A mais bela de todas as coisas: O AMOR!”

Madre Tereza de Calcutá

Resumo

O uso de enzimas imobilizadas em aplicações industriais permite o desenvolvimento de processos de produção com alta produtividade, fácil separação do produto e reutilização do biocatalisador. Alguns grupos de enzimas apresentam muitas vantagens quando imobilizadas em suportes insolúveis em água, devido à melhoria de suas propriedades catalíticas e estabilidade ou pela síntese de baixo custo. Tendo em vista tais vantagens, enzimas de aplicabilidade industrial como α-L-ramnosidase e invertase foram imobilizadas em diferentes suportes magnéticos. A α-L-ramnosidase de *Aspergillus terreus* foi imobilizada covalentemente nos seguintes suportes ferromagnéticos: polietileno tereftalato (Dacron-hidrazida), polisiloxano/álcool polivinílico (POS/PVA) e quitosana. A atividade da enzima imobilizada em Dacron-hidrazida (0,53 nkat/μg de proteína) e POS/PVA (0,59 nkat/μg de proteína) foi significativamente maior do que a encontrada para o derivado de quitosana (0,06 nkat/mg de proteína). Os perfis de pH e temperatura para todas as enzimas imobilizadas não mostraram diferença em relação à enzima livre, exceto o derivado de quitosana que apresentou maior temperatura máxima. O derivado enzimático Dacron-hidrazida mostrou melhor desempenho que a enzima livre para hidrolisar a naringina 0,3% (91% e 73% após 1 h, respectivamente) e na síntese de ramnósidos (0,116 e 0,014 mg narirutina após 1 h, respectivamente). Além disso, minerais como argilas e terra de diatomáceas foram utilizados para produzir compósitos com partículas de magnetita. Esses compósitos foram caracterizados por meio de diferentes técnicas físico-químicas a fim de elucidar suas propriedades estruturais, morfológicas e magnéticas. Três tipos de materiais foram sintetizados: argila montmorilonita magnética (mMMT), terra de diatomáceas magnética (mTD) e terra de diatomáceas magnética revestida com polianilina (mTD-PANI). Os compósitos magnéticos foram tratados com 3-aminopropiltrietoxisilano ou polianilina, disponibilizando grupamentos químicos para ocorrência de ligação covalente entre a matriz e a biomolécula. Após funcionalização dos suportes e ativação com glutaraldeído, os materiais foram utilizados como matriz para imobilização covalente de invertase. A invertase imobilizada em mMMT apresentou igual pH ótimo, maior temperatura máxima e estabilidade térmica quando comparada com a enzima livre, e manteve 91% da sua atividade inicial após 7 ciclos consecutivos de reutilização. No estudo da hidrólise de sacarose pela mTD-invertase, foi realizado um planejamento fatorial completo 2^4, sendo observadas como melhores condições experimentais para este processo: pH 4,5; temperatura de 45°C; concentração de sacarose 0,25 M e concentração de invertase 0,05 mg mL^{-1}. A mTD-invertase mostrou bom desempenho quanto à termoestabilidade, estabilidade de armazenamento, tempo de prateleira e reuso quando comparada à enzima livre. A mTD-PANI-invertase apresentou igual pH ótimo e temperatura máxima e maior termoestabilidade que a enzima livre, e manteve 55% da sua atividade inicial após 10 ciclos consecutivos de reutilização. Portanto, os resultados mostraram que os compósitos magnéticos produzidos a partir de materiais orgânicos e inorgânicos (minerais de baixo custo e altamente disponíveis na natureza) são matrizes promissoras para a imobilização covalente de α-L-ramnosidase e invertase, bem como para a imobilização de outras biomoléculas.

Palavras-chaves: compósitos, partículas magnéticas, imobilização, α-L-ramnosidase, invertase

Abstract

The use of immobilized enzymes in industrial applications allows the development of production processes with high productivity, easier separation of the product and reuse of the biocatalyst. Some groups of enzymes have many advantages when immobilized on water insoluble supports due to improvement in their catalytic properties and stability or by low cost synthesis. In view of these advantages, enzymes of industrial applicability as α-L-rhamnosidase and invertase were immobilized on different magnetic supports. The α-L-rhamnosidase from *Aspergillus terreus* was covalently immobilized on the following ferromagnetic supports: polyethylene terephthalate (Dacron-hydrazide), polysiloxane/polyvinyl alcohol (POS/PVA) and chitosan. The activity of immobilized enzyme on Dacron-hydrazide (0.53 nkat/μg of protein) and on POS/PVA (0.59 nkat/μg of protein) was significantly higher than that found for the chitosan derivative (0.06 nkat/μg of protein). The activity–pH and activity–temperature profiles for all immobilized enzymes did not show difference compared to the free enzyme, except the chitosan derivative that presented higher maximum temperature. The Dacron-hydrazide enzyme derivative showed better performance than the free enzyme to hydrolyze 0.3% narigin (91% and 73% after 1 h, respectively) and synthesize rhamnosides (0.116 and 0.014 mg narirutin after 1 h, respectively). In addition, minerals such as clays and diatomaceous earth were used to produce composites with magnetite particles. These composites were characterized by different physico-chemical techniques to elucidate their structural, morphological and magnetic properties. Three types of materials were synthesized: magnetic montmorillonite clay (mMMT), magnetic diatomaceous earth (mDE) and magnetic diatomaceous earth coated with polyaniline (mDE-PANI). The magnetic composites were treated with 3-aminopropyltriethoxysilane or polyaniline, providing chemical groups for the covalent bonding between the matrix and biomolecule. After supports functionalization and activation with glutaraldehyde, materials were used as matrix for covalent immobilization of invertase, a model enzyme. The immobilized invertase on mMMT presented equal optimum pH, higher maximum temperature and thermal stability compared to the free enzyme, and retained 91% of its initial activity after seven consecutive cycles of reuse. In the sucrose hydrolysis study by mDE-invertase was carried out a complete factorial design 2^4, being observed as best experimental conditions for this process: pH 4.5, temperature of 45°C, 0.25 M sucrose concentration and 0.05 mg mL^{-1} invertase concentration. The mDE-invertase showed good performance as regards the thermal stability, storage stability, shelf life and reuse when compared to the free enzyme. The mDE-PANI-invertase showed equal optimum pH and maximum temperature and higher thermal stability than the free enzyme, and retained 55% of its initial activity after ten consecutive cycles of reuse. Therefore, the results showed that the magnetic composite produced from organic and inorganic materials (low cost minerals and highly available in nature) are promising matrices for the covalent immobilization of α-L-rhamnosidase and invertase, as well as for the immobilization of other biomolecules.

Keywords: composites, magnetic particles, immobilization, α-L-rhamnosidase, invertase

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Lista de Abreviaturas, Siglas e Símbolos

AAO/PANI	Óxido de alumínio anódico - polianilina
AAO/PEI	Óxido de alumínio anódico - polietilenimina
APTES	3-aminopropiltrietoxisilano
CM - celulose	Carboximetil - celulose
DEAE-celulose	Dietilaminoetil - celulose
EC	Enzyme Commission
EDS	Espectroscopia por dispersão de energia
Fe_3O_4/PANI	Magnetita - polianilina
fcc	Rede cúbica unitária de face centrada
kDa	Quilo daltons
mDacron	Dacron magnético
meq	Miliequivalente
MMT	Montmorilonita
mPOS/PANI	Polisiloxano – polianilina magnético
mPOS/PVA	Polisiloxano – álcool polivinílico magnético
PANI	Polianilina
PET	Polietileno tereftalato
POS	Polisiloxano
POS/PVA	Polisiloxano – álcool polivinílico
PVA	Álcool polivínilico
PVA/PANI	Álcool polivinílico - polianilina
RASE	α-L-ramnosidase
Silicone/PANI	Silicone - polianilina
TD	Terra de diatomáceas
TEOS	Tetraortosilicato
χ	Susceptibilidade relativa

Sumário

Introdução

A biotecnologia industrial é uma área da tecnologia que influencia cada vez mais o setor químico, permitindo uma conversão mais eficiente das matérias-primas mediante os processos biotecnológicos, em uma ampla variedade de produtos químicos, muitos dos quais não podem ser obtidos diretamente por via sintética. Estes produtos incluem químicos finos, produtos farmacêuticos, bio-corantes, bio-plásticos, aditivos alimentares, pesticidas e bio-combustíveis líquidos, como o bioetanol e o biodiesel. A biotecnologia industrial incentiva a integração de disciplinas como a bioquímica, microbiologia, genética molecular e a tecnologia de processos, para desenvolver produtos e processos úteis baseados em microrganismos, células animais ou vegetais, suas organelas ou enzimas como biocatalisadores.

A imobilização de um biocatalisador permite seu reuso de maneira econômica e o desenvolvimento de bioprocessos contínuos. Os biocatalisadores podem ser imobilizados utilizando enzimas isoladas ou células inteiras. Um fator que determina a aplicação de uma enzima num processo tecnológico é seu custo. Quando são utilizadas em sua forma nativa, depois da reação retém atividade que contamina o produto, e sua eliminação pode envolver um custo extra de purificação. Assim o processo de recuperação não é uma vantagem e geralmente a enzima é descartada. Esta desvantagem pode ser eliminada mediante o uso de enzimas sob a forma imobilizada, processo que pode ser efetuado confinando fisicamente a enzima a algum material orgânico e/ou inorgânico, com retenção da sua atividade catalítica, podendo ser usada repetidamente e continuamente (Wingard, 1972). A invertase é uma enzima modelo, pois foi a primeira enzima a ser estudada e imobilizada em carvão vegetal e alumina (Nelson e Griffin, 1916). A imobilização geralmente estabiliza a estrutura da enzima, de modo que permite seu uso sob condições ambientais extremas de pH, temperatura e solventes orgânicos, e permite assim sua aplicação em meios não aquosos. O custo financeiro do uso das enzimas nativas e solúveis em água na catálise de inúmeras reações na indústria tem sido reduzido com suas imobilizações em materiais insolúveis em água, tais como: celulose, náilon, cerâmica, poliacrilamida, entre outros.

Óxidos de ferro têm recebido um crescente interesse nas áreas da nanociência e nanotecnologia, devido às suas propriedades magnéticas, elétricas e físico-químicas únicas que se obtém conforme morfologia e tamanho das partículas. Estes óxidos apresentam especial importância devido a suas aplicações em pigmentos, como agentes anticorrosivos, catalisadores e em processos de tratamento de águas residuais (Dai et al., 2005). As partículas magnéticas também têm sido de grande interesse nas áreas da biotecnologia e ciências biomédicas, principalmente na tecnologia enzimática que se tornou muito popular. A magnetita ($FeO.Fe_2O_3$ ou Fe_3O_4) preparada pelo método de co-precipitação de sais de cloreto ferroso (Fe^{+2}) e cloreto férrico (Fe^{+3}) têm sido uma das propostas mais usadas. Materias magnéticos são utilizados como matrizes para a imobilização de enzimas e apresentam como principais vantagens: a fácil remoção da mistura por aplicação de um campo magnético externo e a relativa simplicidade para a preparação desses materiais. Diversas aplicações incluem o uso de partículas magnéticas, tais como: imobilização de enzimas (Neri et al., 2011; Soria et al., 2012; Maciel et al., 2012), isolamento de células (Haik et al., 1999), imunoensaio (Richardson et al., 2001), adsorção e purificação de proteínas (Abudiab e Beitle, 1998), separação de ácidos nucléicos (Levison et al., 1998), e liberação de drogas (Rusetski e Ruuge, 1990). Diversos materiais têm sido utilizados para preparar matrizes magnéticas tais como polímeros sintéticos: Dacron (Oliveira et al., 1989; Carneiro Leão et al., 1991), POS/PVA (Coêlho et al., 2002); e biopolímeros: celulose (Safarik et al., 1999), nitrocelulose (Tanyolac e Ozdural, 2000), vidro poroso (Bruce et al., 2004), quitina (Safarik et al., 1993).

Nesta tese de doutorado foram desenvolvidos materiais magnéticos baseados em óxidos de ferro e minerias de baixo custo e altamente disponível na natureza, tais como argilas e terra de diatomáceas. Estrategias de funcionalização dos materias magnéticos foram feitas também com o intuito de produzir novos materiais. Para a síntese dos compóstiso magnéticos e caracterização dos derivados imobilizados foram realizados planejamentos de experimentos como um método estruturado e sistemático de experimentação.

Capítulo 1

Revisão da literatura

2.1 Imobilização de enzimas

Em inícios da década de 1960, a tecnologia enzimática despontou como área de investigação, com a imobilização de enzimas para utilização em processos químicos. O mercado mundial das enzimas divide-se em três segmentos: enzimas empregadas na indústria de alimentos, enzimas técnicas e enzimas empregadas na produção de ração animal. Destes três grupos, destacam-se as enzimas destinadas aos setores de alimentos, que são empregadas basicamente na produção de xarope de açúcar invertido e de compostos aromatizantes; e as enzimas técnicas, que são utilizadas na formulação de detergentes, produção de papel e celulose, manufatura de couros e produção de fármacos. Este último grupo é o principal mercado consumidor de enzimas, detendo aproximadamente 50% do total das enzimas comercializadas. De acordo com "Freedonia Group" o mercado global de enzimas industriais foi de U$ 5,1 bilhões de dólares em 2008 (46% Estados Unidos) e a projeção é de que o crescimento anual será de aproximadamente 6,3%, com estimativa de movimentação de U$ 7,0 bilhões de dólares para 2013 (Mendes et al., 2011).

A imobilização é definida como o processo pelo qual se restringem, completamente ou parcialmente, os graus de liberdade de movimento de enzimas, organelas, células, etc. por união a um suporte (Arroyo, 1998). Este processo proporciona um complexo insolúvel em um meio especializado onde os fluidos podem passar facilmente, transformando substrato em produto numa reação enzimática controlada e facilitando a remoção do catalisador e produto. A imobilização depende do tipo de suporte, método de ativação e imobilização. A escolha da matriz (propriedades químicas e magnéticas, tamanho da partícula e distribuição, porosidade) é um fator chave que influencia na qualidade da imobilização e na conquista das aplicações finais (Korecká et al., 2005).

As enzimas imobilizadas têm grande importância devido a sua ampla variedade de aplicações em tecnologia dos alimentos, biotecnologia, biomedicina e também na química analítica (Varavinit et al., 2001). A **Figura 1** mostra as vantagens e desvantagens da imobilização de enzimas.

VANTAGENS	DESVANTAGENS
❖ Maior estabilidade; ❖ Fácil separação enzima-produto; ❖ Reator enzimático; ❖ Reutilização do derivado.	❖ Alteração da conformidade; ❖ Grande heterogeneidade; ❖ Menor atividade enzimática; ❖ Biocatalizador mais caro que enzima nativa.

Figura 1. Vantagens e desvantagens da imobilização de enzimas.

As enzimas podem ser imobilizadas por diferentes métodos (**Figura 2**) e em geral, costuma-se classificar os métodos de imobilização da seguinte forma (Norouzian, 2003):

1. Adsorção sobre um suporte inerte;
2. Aprisionamento dentro de uma rede polimérica (sintética ou não sintética);
3. Reticulação (“Cross-linking”) da proteína com um agente bifuncional;
4. Ligação covalente a um suporte insolúvel reativo.

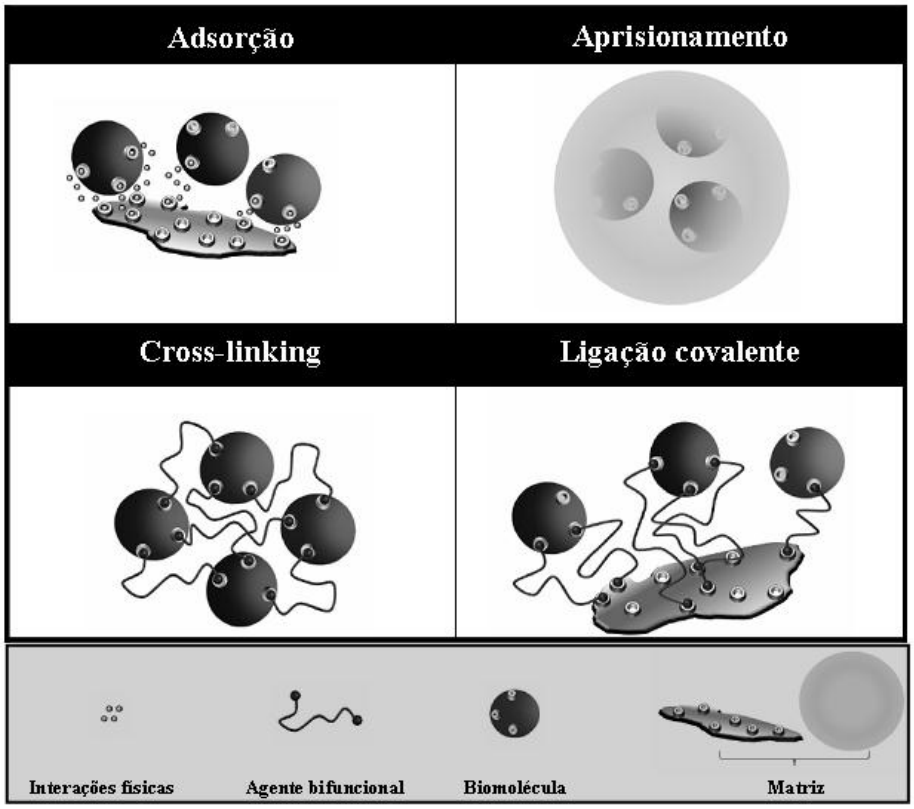

Figura 2. Métodos de imobilização de enzimas. *Fonte: Bickerstaff, 1997.*

2.1.1. Imobilização covalente

A metodologia da imobilização por ligação covalente consiste na ativação dos grupos químicos do suporte/enzima para que seja estabelecida a ligação covalente. Esta técnica é amplamente investigada e a mais interessante do ponto de vista industrial. A natureza do suporte pode ter um efeito considerável sobre a atividade expressa pela enzima e a cinética aparente. O suporte ideal deve ser de baixo custo, inerte, insolúvel, permeável, ter estabilidade química, mecânica e térmica, elevada área superficial e rigidez, características hidrofílicas/hidrofóbicas, tamanho de partículas e forma adequados, ser resistente ao ataque microbiano e regenerativo (Norouzian, 2003).

As vantagens da metodologia por ligação covalente são:

1. Manipulação simples do derivado imobilizado;
2. Carga da enzima se mantém constante após a imobilização;
3. Maior resistência à desativação pelo efeito da temperatura, solventes orgânicos ou pH, por ter estabilizada sua estrutura terciária;
4. Diminuição da lixiviação da enzima imobilizada ao suporte durante o uso do derivado enzimático.

Os suportes inorgânicos são de interesse para imobilização de enzimas por sua durabilidade, alta resistência mecânica e baixo custo. Para a preparação de um biocatalisador em um suporte inorgânico, antes da união com a enzima, é necessário um pré-tratamento de sua superfície, como a silanização. Os métodos de silanização podem ser realizados em meios aquosos e não aquosos (orgânico). A silanização aquosa tem a vantagem de formar uma capa fina de silano no suporte, a silanização orgânica produz uma capa mais grossa. Os silanos disponíveis comercialmente incluem epoxisilanos, aminosilanos, cianosilanos, fenilsilanos e, dentre eles, o mais usado é o 3-aminopropiltrietoxisilano (APTES). Depois da silanização, é necessário efetuar modificações químicas no suporte (ativação) para trocar os grupos funcionais que posteriormente se utilizarão na união com a proteína. Geralmente utiliza-se para ativar o suporte um reativo bifuncional, como por exemplo, o glutaraldeído. As proteínas contêm grupos reativos (amino, sulfidril, carboxil e grupos aromáticos) que podem ser utilizados para formar ligações covalentes com o suporte (**Figura 3**).

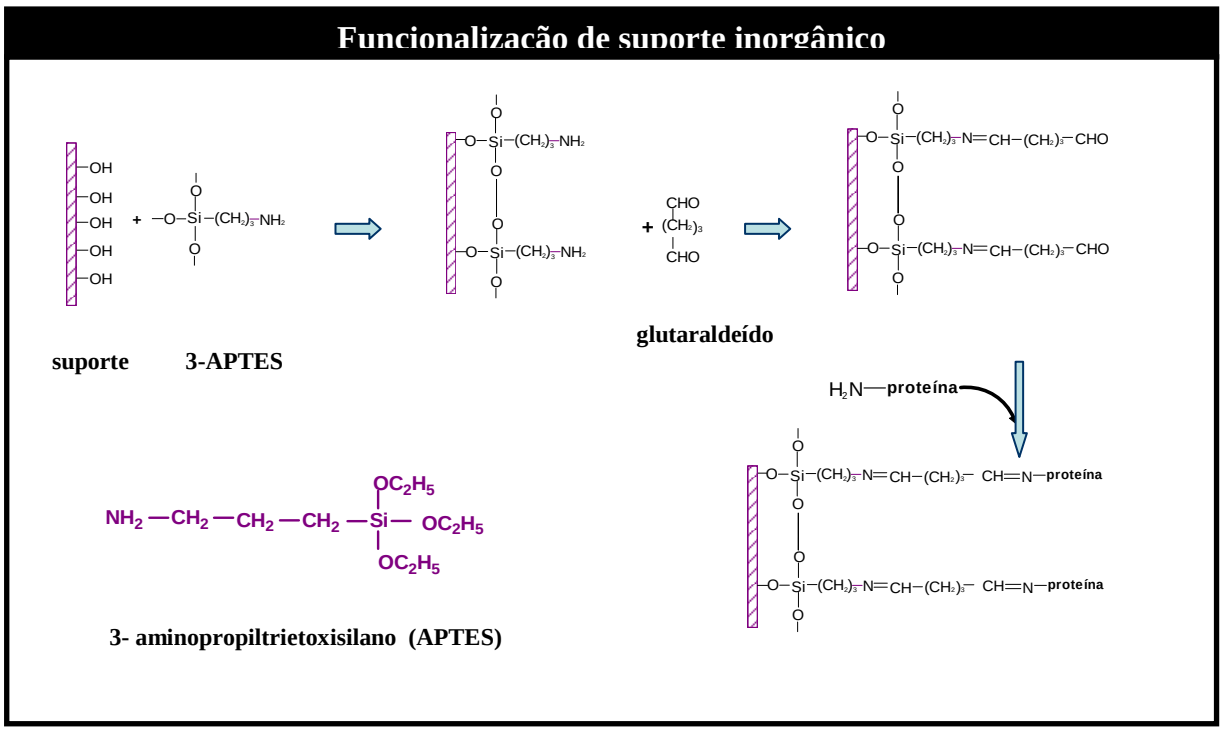

Figura 3. Mecanismo de funcionalização de um suporte inorgânico com o agente aminosilano APTES e glutaraldeído seguido da ligação da proteína.

O grupo de pesquisa "Imobilização de biomoléculas" (IMOBIO) do Laboratório de Imunopatologia Keizo Asami (LIKA/UFPE), liderado pelo Prof. Luiz Bezerra de Carvalho Júnior, vem trabalhando com diferentes suportes de natureza orgânica e inorgânica, compósitos magnéticos e não magnéticos, com uma ampla gama de aplicações, como por exemplo, matrizes para a imobilização de biomoléculas (enzimas, anticorpos, antibiótico) e matrizes de afinidade (purificação de proteínas). As **Tabelas 1** e **2** mostram a contribuição de pesquisadores da UFPE e do grupo IMOBIO na procura de novos materiais com propriedades destacáveis para a imobilização de biomoléculas.

Tabela 1. Suportes sólidos propostos para a imobilização de biomoléculas por pesquisadores da UFPE.

Suporte	Biomolécula Imobilizada	Referência Bibliográfica
Poliuretano	Invertase	Cadena et al., 2011
Poliuretano, filme plástico, mDacron	Invertase	Cadena et al., 2010
Silício poroso	Urease	Diniz et al., 2007
Poliacrilamida, mDacron	Lipase	Knight et al., 2000
Membrana nylon	Lipase	Bruno et al., 2004
Contas de vidro	Ascorbato oxidase	Marques e Lima Filho, 1992
	Ascorbato oxidase	Marques et al., 1994
	Lipase	Nadruz et al., 1994
	Invertase e glicose oxidase	Leite et al., 1995
	Lectina (Cratylia mollis)	Souza et al., 2003
PANI	Glicose oxidase	Parente et al., 1992
	Glicose oxidase	Leite et al., 1994

Tabela 2. Suportes sólidos propostos para a imobilização de biomoléculas pelo grupo de pesquisa IMOBIO.

Suporte	Biomolécula Imobilizada	Referência Bibliográfica
Dacron	α-amilase	Carvalho Jr et al., 1987
	Ascorbato oxidase	Carvalho Jr et al., 1989
	Amiloglicosidase	Oliveira et al., 1989
	Antígeno (F1A)	Montenegro et al., 1991
	Antígeno (F1A)	Montenegro et al., 1993
	Xantina oxidase	Barbosa et al., 1995
Dacron, poliacrilamida	Glicose oxidase	Carvalho Jr et al., 1986
Dacron/PANI	Antígeno (F1A)	Coêlho et al., 2001
mDacron	Amiloglicosidase	Carneiro Leão et al., 1991
	Albumina de soro humano	Carneiro Leão et al., 1994
	Albumina de soro humano	Pinheiro et al., 1999
	Tripsina	Amaral et al., 2006
	β-galactosidase	Neri et al., 2011
mDacron, mPOS/PVA e quitosana magnética	α-ramnosidase	Soria et al., 2012
POS/PVA	Anticorpo (anti S-100)	Melo Jr et al., 2007
	Anticorpo (anti S-100)	Melo Jr et al., 2008
	Anti-galectina-3	Melo Jr et al., 2010
mPOS/PVA	β-galactosidase	Neri et al., 2008
	β-galactosidase	Neri et al., 2009
mPOS/PANI	β-galactosidase	Neri et al., 2009
PVA/PANI	Tripsina	Caramori et al., 2011
	Peroxidase (HRP)	Caramori et al., 2012
Fe_3O_4/PANI	β-galactosidase	Neri et al., 2011
AAO/PEI e AAO/PANI	Tripsina	Oliveira et al., 2008
Silicone/PANI	Xantina oxidase	Nadruz Jr et al., 1996
PVA/Glutaraldeído	Xantina oxidase, α-amilase e amiloglicosidase	Araujo et al., 1996
Gliptal	Amiloglicosidase	Jordão et al., 1996
Fucana sulfatada	Antibiótico	Araújo et al., 2004
Exopolissacarídeo celulósico	Tripsina	Cavalcante et al., 2006
Celulose	Tripsina	Monteiro et al., 2007
Carboximetilcelulose-azida e quitosana	Xantina oxidase	Carvalho Jr. e Medeiros, 1981
Levana magnética	Tripsina	Maciel et al., 2012

2.2 Matrizes

Os suportes sólidos utilizados para a imobilização de enzimas podem ser divididos em dois grupos, segundo a sua natureza.

- Suportes inorgânicos: subdivididos em *naturais* (argilas como a bentonita, pedra-pomes, sílica) ou *materiais manufaturados* (óxidos de metais e vidro de tamanho de poro controlado, vidro não poroso, alumina, cerâmicas, gel de sílica);
- Suportes orgânicos: subdivididos em *polímeros naturais* (polissacarídeos: celulose, amido, dextrana, agarose, alginato, quitina e quitosana; proteínas fibrosas (colágeno e queratina) e *polímeros sintéticos* (poliolefinas: poliestireno; polímeros acrílicos: poliacrilamidas, poliacrilatos; outros tipos: álcool polivinílico, poliamidas).

2.2.1 Dacron

O polietileno tereftalato (PET) também conhecido como Dacron é um dos materiais sintéticos mais amplamente utilizados no mundo. Em geral, o polietileno tereftalato é conhecido como poliéster, e no segmento de embalagens, como PET. Este material possui propriedades termoplásticas, isto é, pode ser reprocessado diversas vezes pelo mesmo ou por outro processo de transformação. Quando aquecidos a temperaturas adequadas, esses plásticos amolecem, fundem e podem ser novamente modelados. Sua alta cristalinidade e elevado ponto de fusão são responsáveis pela sua resistência e excelente fibra, adequada para a formação de filmes. O Dacron é um polímero biocompatível, de natureza antimicrobiana, inerte, hidrofóbico e com resistência mecânica (Irena et al., 2009; Phaugat et al., 2010).

2.2.1.1 Estrutura

O Dacron é um polímero de estrutura lineal insolúvel em água produzido pela condensação entre o ácido tereftálico e o etileno glicol **(Figura 4**).

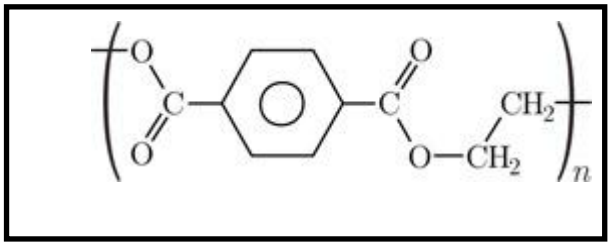

Figura 4. Estrutura do poliéster Dacron (onde n > 15.000). *Fonte: Sanaee et al., 2010.*

Este poliéster tem sido proposto como suporte para a imobilização covalente de biomoléculas. Embora o Dacron não apresente grupos funcionais adequados para a imobilização de biomoléculas, este problema pode ser contornado com modificações químicas realizadas na superfície do material. Para isto, aminas primárias são frequentemente introduzidas por aminólise induzida termicamente, e a reação ocorre entre uma amina orgânica com as ligações éster ao longo da cadeia polimérica. As aminas mais frequentemente utilizadas são hidrazina, etilenodiamina e 1,6-diaminohexano (Irena et al., 2009). Na **Figura 5** pode se observar a síntese e funcionalização do Dacron hidrazida para a imobilização de enzima. Primeiramente, é realizada a hidrazinólise parcial do Dacron com hidrazida e metanol, depois a ativação do suporte com o glutaraldeído para finalmente unir covalentemente a enzima.

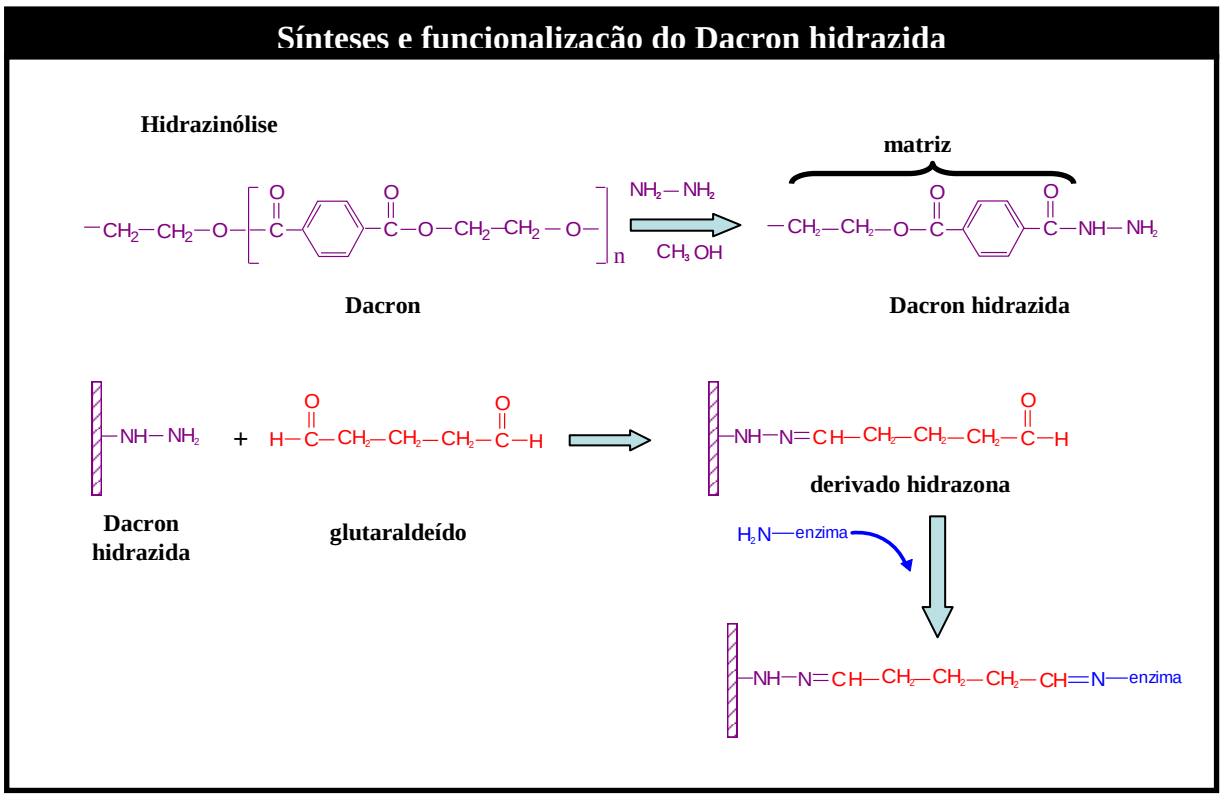

Figura 5. Mecanismo de sínteses e funcionalização do Dacron hidrazida para a imobilização de enzima.

2.2.1.2 Aplicações

A principal aplicação do Dacron é na produção de recipientes para refrigerantes, mas este material representa um problema ambiental desde que se trata de um plástico não biodegradável. A ideia de fazer reciclagem deste material contribui ao meio ambiente, embora o Dacron reciclado não possa ser usado para a produção destes recipientes porque as temperaturas envolvidas não são altas o bastante para garantir a esterilização do produto. Os produtos finais deste polímero reciclado incluem embalagens para armazenar produtos não alimentícios, fibras para tecidos e carpetes, filmes, folhas e outros produtos comerciais.

O Dacron é também usado em fibras, pois é mais forte que o algodão e a celulose, e se mistura facilmente com fibras de algodão. Tecidos feitos com essas fibras são mais resistentes ao enrugamento. Além disso, este poliéster forma um polímero claro utilizado em filmes fotográficos e transparências. Outra aplicação industrial do poliéster é a fabricação de fios, cordas e filtros, e também são misturadas com fios de aço na fabricação de pneus. O Dacron possui aplicações médicas, pois suas fibras fortes podem ser usadas para reparar cirurgicamente tecidos danificados (vasos sanguíneos, tendões). Enxertos vasculares sintéticos realizados de Dacron ou Teflon expandido são amplamente utilizados para substituir artérias obstruídas no homem (Wissink et al., 2001).

2.2.2 POS/PVA

O material híbrido polisiloxano-álcool polivinílico (POS/PVA) é um compósito formado pela policondensação entre o polisiloxano (POS) e álcool polivinílico (PVA), e apresenta características desejáveis como suporte para a imobilização de enzimas, devido a sua grande área de superfície, alta porosidade, estabilidade térmica, óptica e química. Este material também é particularmente atrativo para a fabricação de biossensores, pois possui rigidez física, inércia química e elevada estabilidade fotoquímica e térmica (Lima-Barros et al., 2002).

2.2.2.1 Estrutura

O POS/PVA (**Figura 6**) é sintetizado pela técnica sol-gel, processo que envolve a transição de um sistema da fase líquida "sol" (coloidal) para a fase sólida "gel". As matérias-primas utilizadas na preparação do "sol" são geralmente sais inorgânicos metálicos ou compostos orgânicos metálicos, tais como alcóxidos metálicos. O alcóxido metálico mais estudado é o tetróxido de silício ou tetraortosilicato (TEOS).

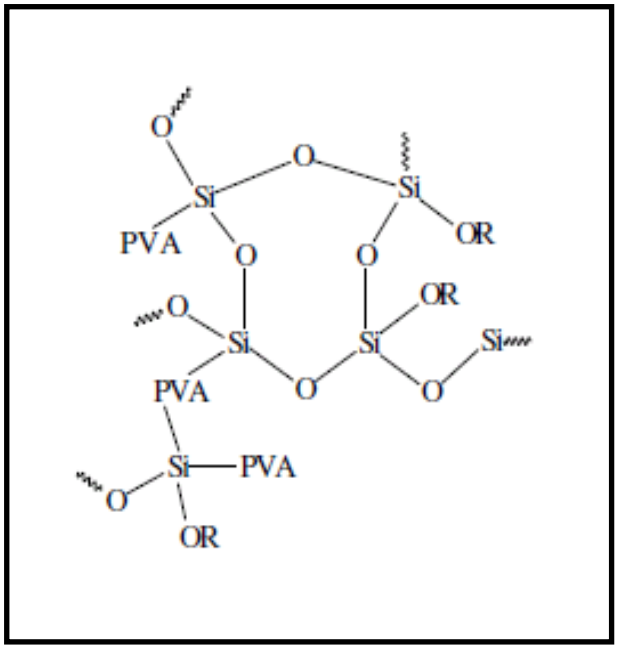

Figura 6. Estrutura do POS/PVA (R = grupos etil). *Fonte: Santos et al., 2008.*

A síntese deste suporte começa com a hidrólise de um alcóxido de silício formando um produto hidroxilado e um álcool correspondente. O segundo passo é a condensação entre um grupo alcóxido não hidrolisado e uma hidroxila, ou entre duas hidroxilas apenas, formando uma mistura coloidal (sol). O último passo envolve a policondensação entre os componentes dessa mistura

coloidal e uma rede adicional (PVA) resultando numa matriz híbrida porosa (Ingersoll e Bright, 1997). Para a imobilização da enzima é necessária a ativação do compósito. Existem diferentes métodos para incorporar um grupo químico na superfície do suporte, o agente bifuncional mais comum é o glutaraldeído (**Figura 7**).

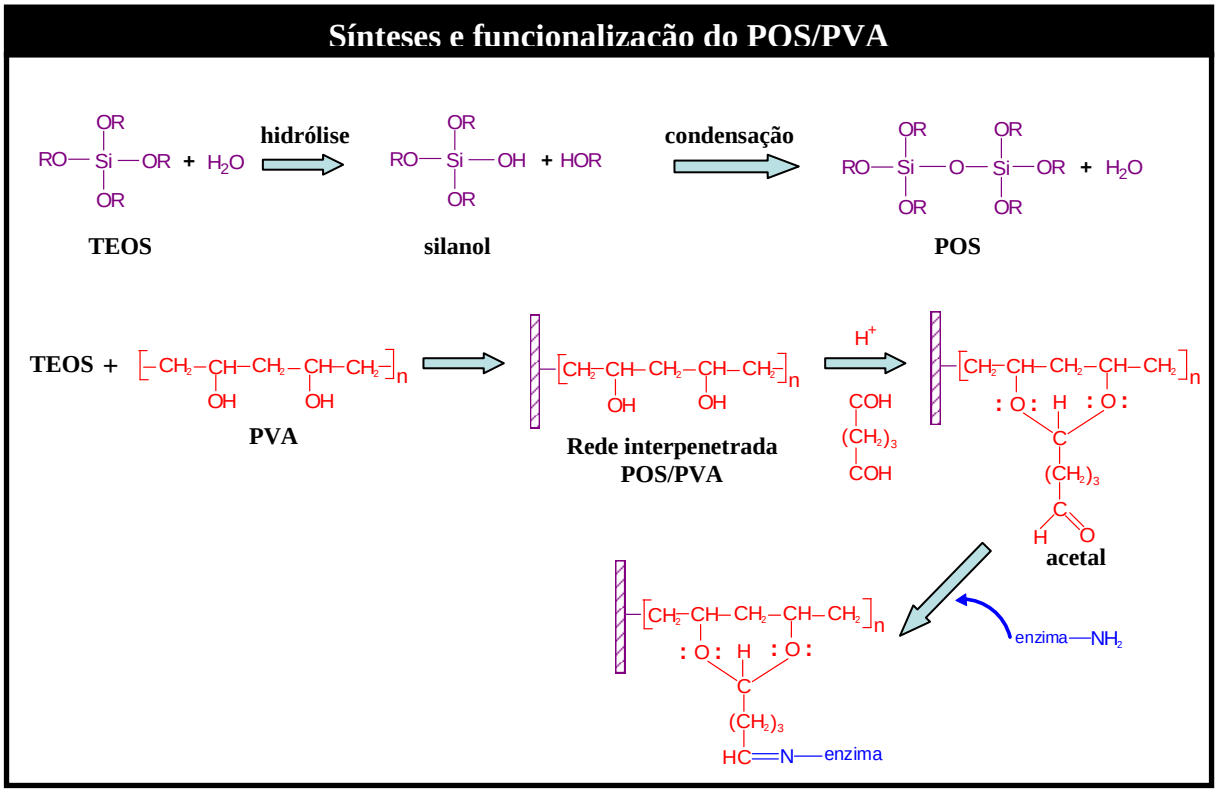

Figura 7. Mecanismo de sínteses e funcionalização da matriz híbrida POS/PVA para a imobilização de enzima.

2.2.2.2 Aplicações

O compósito POS/PVA tem sido utilizado em diversas aplicações: imobilização de anticorpos (Melo-Junior, et al., 2007, 2008, 2010), enzimas (Neri et al., 2008, 2009; Soria et al., 2011) e como fase sólida para ensaios quimiluminescentes (Coêlho et al., 2002).

Em nosso grupo de pesquisa, muitos estudos têm sido feitos sobre a imobilização de biomoléculas (enzimas, anticorpos, proteínas) e demonstrou-se que a escolha do suporte sólido pode influenciar fortemente nas propriedades do derivado imobilizado. Assim vários materiais compósitos envolvendo o polisiloxano e álcool polivinilico foram propostos como fase sólida, especialmente para a imobilização covalente de enzimas. Dentre estes materiais o mPOS/PVA, mPOS/PANI, PVA/PANI, PVA/Glut já foram citados na **Tabela 2**.

2.2.3 Quitosana

O polímero quitosana produzido pela desacetilação da quitina, um componente importante nos artrópodes e casca de crustáceos, como lagostas e caranguejos, apresenta propriedades biológicas e químicas significativas, tais como biocompatibilidade, biodegradabilidade, bioatividade e não toxicidade, bem como propriedades antibacterianas e policatiônicas, inerte, hidrofílico, excelente capacidade de formação de filme e elevada permeabilidade de água (Ravi Kumar, 2000). A desacetilação da quitina é realizada em meio alcalino via processo termoquímico, normalmente com NaOH (40-50% p/p) a 110-115°C. Os principais fatores que afetam o grau de desacetilação e as características da quitosana são: temperatura e tempo de reação, concentração da solução do álcali, razão quitina/álcali, tamanho das partículas da quitina e presença de agentes que evitam a despolimerização (Mendes et al., 2011).

2.2.3.1 Estrutura

A quitosana possui uma estrutura molecular quimicamente similar à da celulose, diferenciando-se somente nos grupos funcionais. Os grupos hidroxilas (-OH) estão presentes na estrutura geral desses biopolímeros, mas a principal diferença entre eles é a presença de grupos amino ($-NH_2$) na estrutura da quitosana (**Figura 8**). Este biopolímero pode facilmente se dissolver em soluções de ácidos fracos diluídos, devido à protonação de seus grupos amino, sendo o ácido acético o solvente mais empregado. Agentes reticulantes, tais como glutaraldeído, etilenoglicol diglicidil éter, tripolifosfato, ácido sulfúrico e epicloridrina, são usados para aumentar a sua estabilidade química e a resistência mecânica (Mendes et al., 2011).

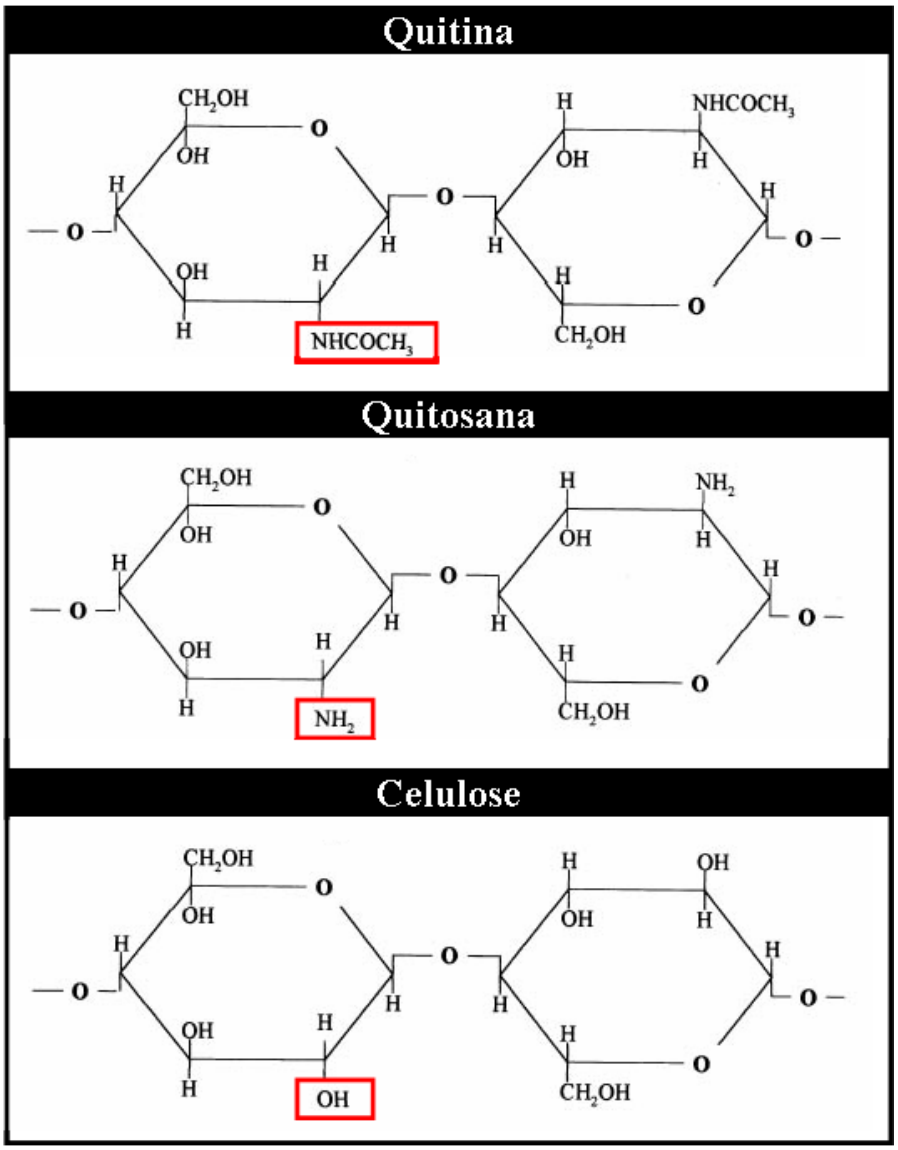

Figura 8. Estruturas dos biopolímeros quitina, quitosana e celulose.

O biopolímero quitosana pode ser modificado fisicamente, sendo uma das vantagens mais interessantes a sua grande versatilidade em ser preparado em diferentes formas, tais como pós, flocos, microesferas, nanopartículas, membranas, esponjas, colmeias, fibras e fibras ocas (Laranjeira e Valfredo, 2009).

A quitosana é uma matriz ideal para imobilização de enzimas. Ela pode ser usada na forma de membrana em gel, contas ou em pó. Os grupos hidroxila e amino presentes na quitosana favorecem o processo de imobilização por adsorção e ligação covalente (Singh et al., 2011). Uma modificação química é necessária para a imobilização de enzimas. A ativação da quitosana é geralmente realizada com o glutaraldeído (**Figura 9**).

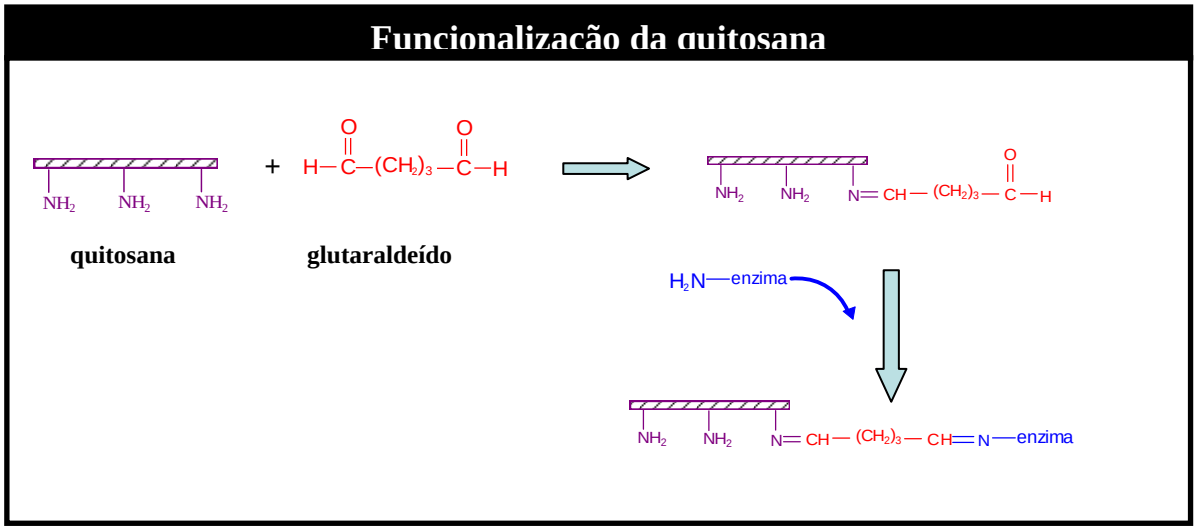

Figura 9. Mecanismo de funcionalização da quitosana para a imobilização de enzima.

2.2.3.2 Aplicações

Este biopolímero tem sido utilizado em muitas aplicações industriais e biomédicas, incluindo tratamento de águas residuais (remoção de íons de metais pesados, floculação/coagulação de corantes e proteínas, processos de purificação de membrana), indústrias de alimentos (anticolesterol, conservante, material de embalagem, aditivo para a ração animal), agricultura (revestimento de sementes e fertilizante, liberação controlada de agroquímicos), indústria de celulose e papel (tratamento de superfície, papel fotográfico), cosméticos e de higiene pessoal (hidratante, creme para o corpo, loção de banho), suporte cromatográfico, engenharia de tecidos, analgésico, matriz para sistema de liberação controlada de fármacos e imobilização de enzimas (Krajewska, 2004; Laranjeira e Valfredo, 2009).

2.2.4 Argila montmorilonita

A bentonita é uma rocha constituída essencialmente por um argilomineral esmectítico (montmorilonita), formado pela desvitrificação e subsequente alteração química de um material vítreo, de origem ígnea, usualmente um tufo ou cinza vulcânica em ambientes alcalinos de circulação restrita de água (Souza Santos, 1992). As bentonitas podem apresentar outros componentes tais como: outros argilominerais (caulinita, ilita), feldspatos, anfibólios, cristobalita, quartzo, sendo que o total dos componentes não argilosos dificilmente supera os 10%. Podem apresentar cores variadas, tais como: branco, cinza, amarelo, marrom, verde e azul (Grim e Guven, 1978).

A montmorilonita (MMT) pertence à família das esmectitas, é um hidrossilicato de alumínio e apresenta a seguinte fórmula química geral $M_x(Al_{4-x}Mg_x)Si_8O_{20}(OH)_4$ (Silva e Ferreira, 2008). Este mineral caracteriza-se por apresentar partículas muito finas, que variam entre 0,1-2 μm, com tamanho médio de 0,5 μm e formato de lâminas. A montmorilonita é hidrofílica com uma elevada área superficial e porosidade (Ray e Okamoto, 2003; Tjong, 2006). Outras propriedades interessantes da montmorilonita são alta capacidade de troca catiônica expressa em meq/100 g, que varia de 80 a 150 meq/100 g de esmectita, propriedades de intercalação de outros componentes entre as camadas e resistência à temperatura e a solventes (Luckham e Rossi, 1999). Quando as lamelas individuais da argila são expostas à água, as moléculas de água são adsorvidas na superfície das folhas de sílica, que são então separadas umas das outras. Este comportamento é chamado de inchamento interlamelar e é controlado pelo cátion associado à estrutura da argila. A espessura da camada de água interlamelar, varia com a natureza do cátion adsorvido e quantidade de água disponível. A diferença no inchamento das montmorilonitas sódicas e cálcicas deve-se à força de atração entre as camadas, que é acrescida pela presença do cálcio, reduzindo a quantidade de água que poderá ser adsorvida, enquanto que o cátion sódio provoca uma menor força atrativa, permitindo que uma maior quantidade de água penetre entre as camadas, e seja então adsorvida.

2.2.4.1 Estrutura

A MMT pertence ao grupo dos filossilicatos 2:1, cujas lâminas são caracterizadas por estruturas constituídas por duas folhas tetraédricas de sílica com uma folha central octaédrica de alumina, que são unidas entre si por átomos de oxigênio que são comuns a ambas as folhas (**Figura 10**). Estas folhas possuem orientação aproximadamente paralela nos planos (001) dos cristais, o que confere a estrutura laminada (Murray, 1999).

As lâminas da montmorilonita apresentam perfil irregular, são muito finas, tem tendência a se agregarem no processo de secagem, e apresentam boa capacidade de delaminação quando colocadas em contato com a água. O diâmetro é de aproximadamente 100 nm, a espessura pode chegar até 1 nm e as dimensões laterais podem variar de 30 nm a vários micrômetros (Silva e Ferreira, 2008). Forças polares relativamente fracas e forças de Van der Waals são as responsáveis pelo empilhamento dessas lâminas, e entre elas existe um espaço interlamelar no qual residem cátions trocáveis como Na^+, Ca^{2+}, Li^+, K^+ fixos eletrostaticamente e com a função de compensar as cargas negativas geradas pelas substituições isomórficas que ocorrem no reticulado, como por exemplo, Al^{3+} por Mg^{2+} ou Fe^{2+}, ou Mg^{2+} por Li^+. Cerca de 80% dos cátions trocáveis na montmorilonita estão presentes no espaço interlamelar e 20% se encontram nas superfícies laterais (Murray, 1999).

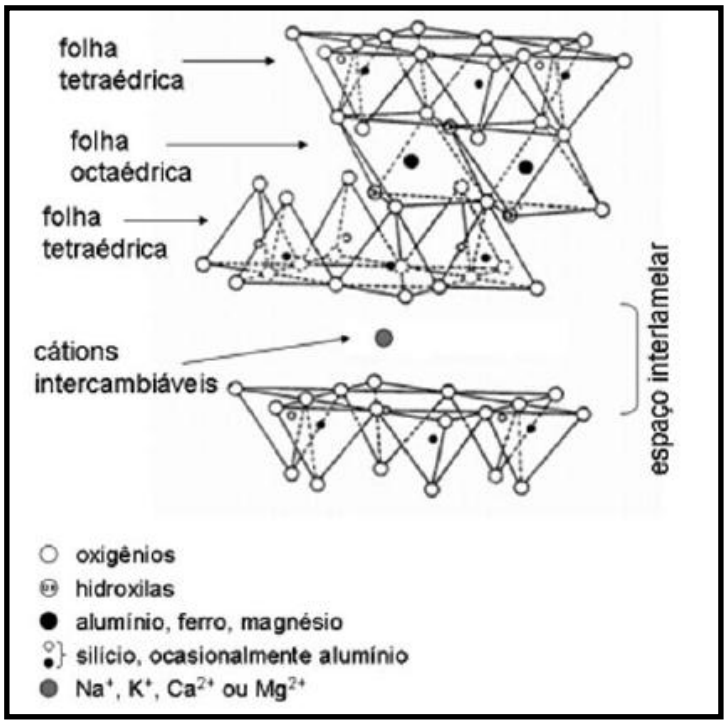

Figura 10. Estrutura da argila montmorilonita. *Fonte: Teixeira-Neto e Teixeira Neto, 2009.*

2.2.4.2 Aplicações

As argilas têm sido usadas desde a antiguidade para a fabricação de objetos cerâmicos, como tijolos e telhas e, mais recentemente, em diversas aplicações tecnológicas, como adsorção em processos de clareamento na indústria têxtil e de alimentos, recuperação de óleos isolantes e automotivos, remoção de fenol e de corantes em efluentes.

As argilas montmoriloníticas têm sido utilizadas na geologia, processos industriais, agricultura, remediação ambiental e construção (Murray, 1999). As principais aplicações industriais da montmorilonita são: componente de fluidos utilizados para a perfuração de poços de petróleo; aglomerantes de areias de moldagem usadas em fundição; pelotização de minério de ferro; descoramento de óleos e clarificação de bebidas; impermeabilizante de solo; absorvente sanitário para animais de estimação; carreadora de moléculas orgânicas em produtos farmacêuticos, rações de animais, produtos cosméticos; agente plastificante para produtos cerâmicos; composição de cimento; catalisadores e como suportes catalíticos (Murray, 2000; Stackhouse et al., 2004).

As argilas como carreadoras de tensoativos para produtos detergentes de lavanderia permitem que os produtos agridam menos a pele do usuário e que produzam melhor efeito visual em peças de vestuário. Além disso, as argilas podem atuar como carreadoras de perfume ou de substâncias antioxidantes para produtos de lavanderia. Os tensoativos, perfumes e antioxidantes são adsorvidos entre as lamelas de argilas e são liberados na sua aplicação (Teixeira-Neto e Teixeira-

Neto, 2009). A argila é um ligante para rações de animais e de aves, tem a propriedade de aumentar a capacidade de extrair mais nutrientes dos alimentos contidos nas rações. No caso das aves, a argila auxilia também a aumentar tanto o tamanho, quanto a dureza da casca dos ovos (O'Driscoll, 1988).

As argilas esmectíticas, bentoníticas ou montmoriloníticas possuem mais usos industriais que todos os outros tipos de argilas industriais reunidas, sendo um material extremamente versátil e de perfil adequado para obtenção de produtos ou insumos de elevado valor agregado (Silva e Ferreira, 2008).

2.2.5 Terra de diatomáceas

Terra de diatomáceas ou diatomito é uma rocha sedimentar originada a partir de frústulas ou carapaças silicosas de diatomáceas (**Figura 11**). As diatomáceas são algas marinhas microscópicas, de composição unicelular, de formas e tamanhos variados, e com aproximadamente 12.000 espécies. Todas elas estão compostas por uma parede celular transparente, com uma capa externa translúcida de sílica não cristalina. Quando a célula morre, todo o conteúdo orgânico se destrói, com exceção do seu esqueleto de sílica, o qual geralmente irá se depositar no fundo das águas, para formar ao longo dos séculos grandes depósitos de algas fossilizadas (diatomito). Estes organismos fotossintetizadores vivem numa grande variedade de ambientes aquáticos, desde o de águas doces ou salobras até os de regiões francamente marinhas (Campos e Santos, 1984).

Este material apresenta tamanho de partícula que varia de 4 a 500 μm e com coloração que varia do branco ao cinza escuro. A composição química geral da terra de diatomáceas é de 58-91% de sílica opalina e incluem também pequenas quantidades de substâncias inorgânicas como alumina, ferro e metais alcalinos, quantidades variáveis de matéria orgânica e componentes comuns de litologias sedimentares como, por exemplo, areia, silte e argila (Montanheiro et al., 2002).

O diatomito ($SiO_2.nH_2O$) é um material inerte, não tóxico, de baixa massa específica aparente, com uma estrutura altamente porosa e elevada área superficial, com carga elétrica negativa, que contêm grupos silanóis (-Si-OH), os quais podem reagir com grupos funcionais polares (Bakr, 2010). Estas características tornam a terra de diatomáceas um suporte ideal para imobilização de biomoléculas.

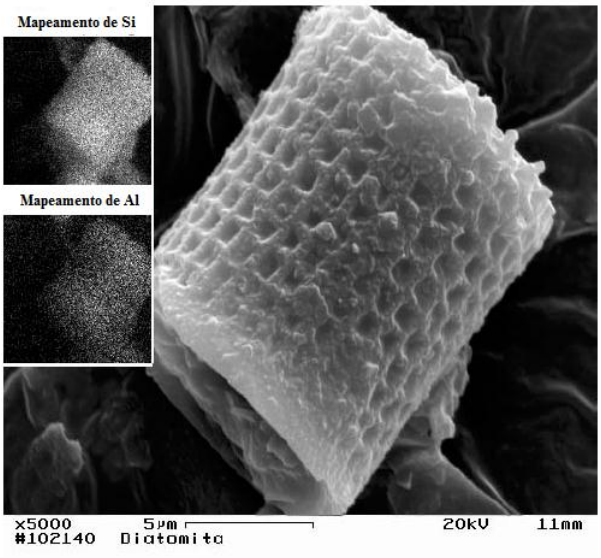

Figura 11. Frústula íntegra de diatomácea. Mapeamento de Al e Si por EDS. *Fonte: Souza et al., 2003.*

2.2.5.1 Estrutura

A reatividade da terra de diatomáceas é semelhante à sílica amorfa sintética, apresentando sítios reativos na superfície. A **Figura 12** mostra a estrutura da terra de diatomáceas contendo os grupos silanóis (-Si-OH), siloxanos (-Si-O-Si) e formação de ponte hidrogênio entre hidroxilas próximas. Nos sítios reativos, é onde acontecem as reações de enxerto e ligações químicas, além disso, são os responsáveis pela carga, acidez, solubilidade e hidrofilicidade da superfície do diatomito (Yuan et al., 2004a).

Geralmente, os grupos hidroxilas são os sítios reativos primários na superfície da sílica amorfa. Existem dois tipos de grupos silanóis na superfície do diatomito, os silanóis isolados e os silanóis ligados a um hidrogênio. À temperatura ambiente, os dois tipos de silanóis estão ligados ao hidrogênio da molécula de água. Com o aumento da temperatura de 200-1000ºC se inicia o processo de desidratação, mostrado no esquema de I a V na **Figura 13**.

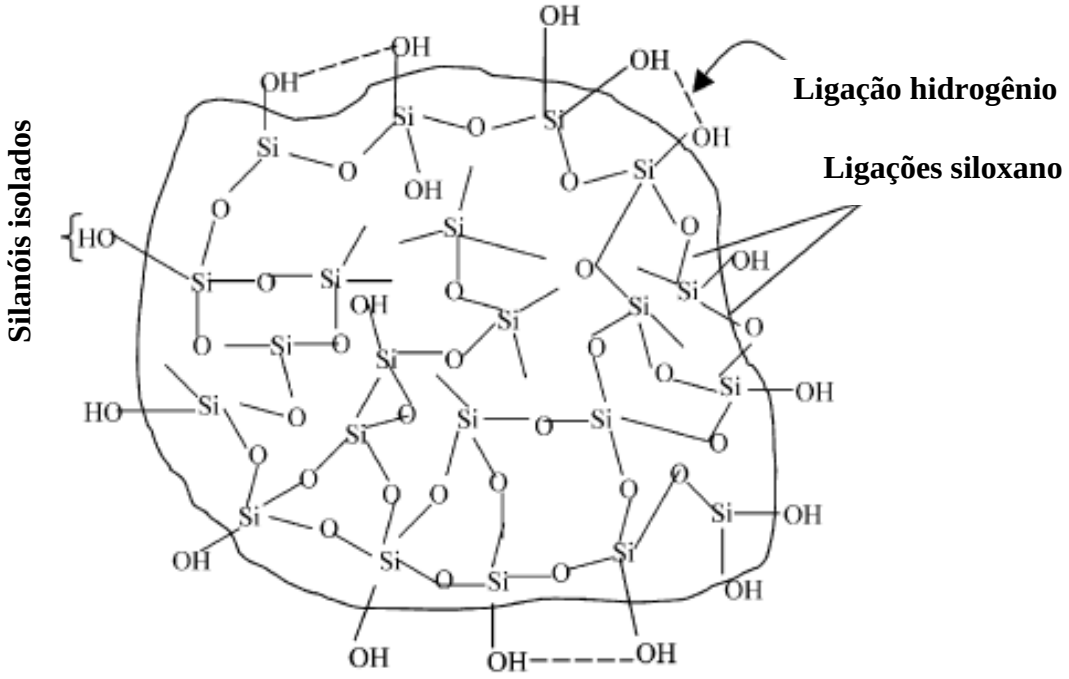

Figura 12. Estrutura da terra de diatomáceas representando os tipos de ligações e os grupos silanóis presentes. *Fonte: Al-Ghouti et al., 2003.*

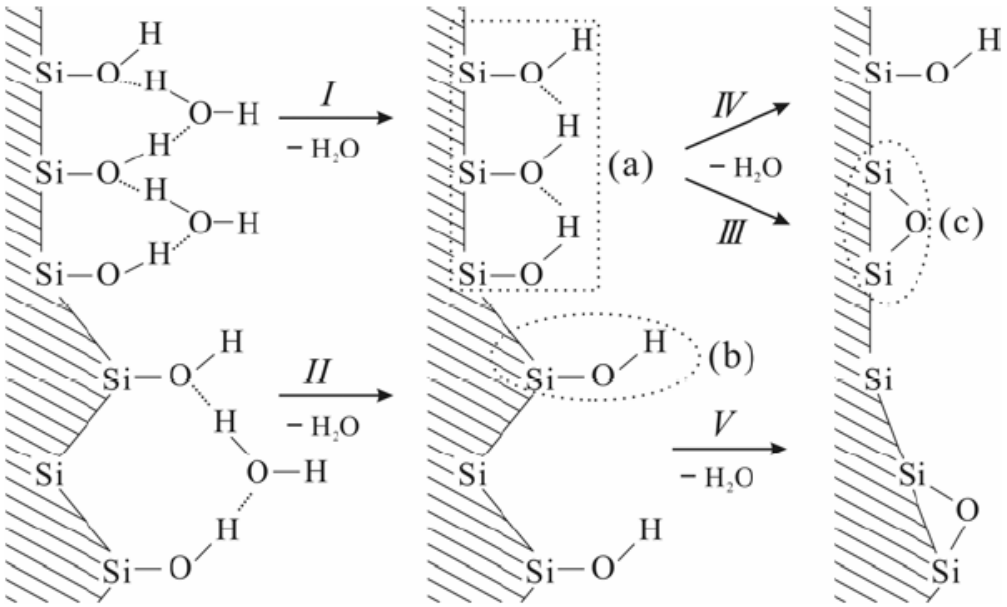

Figura 13. Estrutura hidroxila e processo de desidratação da sílica diatomácea. Grupo silanol = SiOH (a); Silanol isolado (b); Grupo siloxano= Si-O-Si (c). *Fonte: Yuan et al., 2004a.*

O processo de desidratação da terra de diatomáceas se inicia com a dessorção de água que resulta na aparição dos grupos silanóis isolados e na formação da ligação O–H entre os átomos dos grupos silanóis vizinhos (**Figura 13**, I e II). Com a dessorção contínua da água os grupos silanóis ficam expostos e os silanóis ligados ao hidrogênio começam a se condensar para formar pontes siloxano (**Figura 13**, III e IV) enquanto que a maioria dos silanóis isolados não são condensados.

Isto mostra que a ligação entre o grupo silanol e hidrogênio é fraca na sílica diatomácea e, nos grupos silanóis isolados, é mais difícil de ocorrer a condensação. A 1100ºC, a maioria dos grupos silanóis condensa como mostrado no esquema V (Yuan et al., 2004a).

2.2.5.2 Aplicações

As propriedades deste material permitem sua aplicação como auxiliar de filtração, isolante térmico e acústico, inseticida mecânico, ativador da coagulação sanguínea, catalisadores, absorventes, indicadores estratigráfico, materiais de construção, como carga ou enchimento, na fabricação de capacitor cerâmico e tijolos cerâmicos artesanais, na confecção de cosméticos e creme dental (Campos e Santos, 1984; Yuan et al., 2004b).

Recentemente, novas aplicações das terras de diatomáceas como suporte catalítico e biológico, transportador de fármacos e suporte para cromatografia têm atraído muito a atenção (Yuan et al., 2004a). Terras de diatomáceas comerciais (Celite® R-545, Celite® R-632, Celite® R-633 e Celite® R-647) foram utilizadas como suportes para a imobilização de enzimas por ligação covalente, adsorção e aprisionamento usando diferentes estratégias (Mansour and Dawoud, 2003; Chang et al., 2007; Cabana et al., 2009; Koszelewski et al., 2010; Meunier and Legge, 2010; Tomin et al., 2011). Aw et al., 2012 utilizaram também micropartículas deste mineral como biocarregadores para a liberação controlada de droga (indometacina). O diatomito natural mostrou potencial para substituir os materiais sintéticos à base de sílica. Além disso, a terra de diatomáceas foi utilizada como suporte de catalisadores à base de paládio (Pd), incluindo Pd-Cu/diatomito, Pd-Ni/diatomito e Pd-Co/diatomito para a hidrogenação seletiva de ésteres de cadeia longa (Huang et al., 2012).

A combinação única das propriedades físicas e químicas do diatomito o tornou aplicável como substrato para a adsorção de poluentes orgânicos e/ou inorgânicos e como meio de filtragem em um grande número de usos industriais (Al-Ghouti et al., 2003). Nas últimas décadas, os fenômenos de poluição da água têm se tornando mais frequentes e agudos. Poluentes inorgânicos, em particular íons de metais pesados representam uma das categorias mais comuns de poluentes, tornando as águas superficiais e/ou subterrâneas impróprias para muitos usos (incluindo potável), devido à sua toxicidade e/ou propriedades cancerígenas (Bakr, 2010). O cádmio (Cd), cromo (Cr), chumbo (Pb) e mercúrio (Hg) não são metais biodegradáveis e tendem a se acumular em organismos vivos provocando doenças e distúrbios (Al-Ghouti et al., 2004). Al-Degs et al. (2001) reportaram o uso de diatomito e diatomito modificado com óxido de manganês como adsorventes efetivos na remoção de íons chumbo (Pb^{+2}). A capacidade de sorção do diatomito e diatomito

modificado foram 24 e 99 mg/g de íons chumbo, respectivamente. Yuan et al., 2010 utilizaram nanopartículas de diatomito e diatomito magnético para a remoção de cromo (VI) através de um processo físico-químico (atração eletrostática) seguido por um processo redox em que os íons cromo (VI) foram reduzido para íons cromo (III), os quais são menos tóxicos.

Por outro lado, as terras diatomáceas destacam-se como uma das principais substâncias naturais pozolânicas, isto é, a sílica opalina reage com hidróxido de cálcio à temperatura ambiente para formar compostos com propriedades cimentícias. Este tipo de material pozolânico possibilita a produção de cimentos especiais com menor consumo de energia e, portanto, menor custo de fabricação (Montanheiro et al., 2002).

2.3 Partículas magnéticas

Há séculos observou-se que determinadas pedras tinham propriedades de atrair pedaços de ferro ou interagir entre si. Essas pedras foram chamadas de "ímãs naturais", e os fenômenos de atração e repulsão de "fenômenos magnéticos". Essas pedras correspondem a um óxido de ferro denominado magnetita ($FeO.Fe_2O_3$ ou Fe_3O_4). Outros óxidos/hidróxidos de ferro são também conhecidos, a **Figura 14** mostra uma classificação destes óxidos/hidróxidos em função da cor.

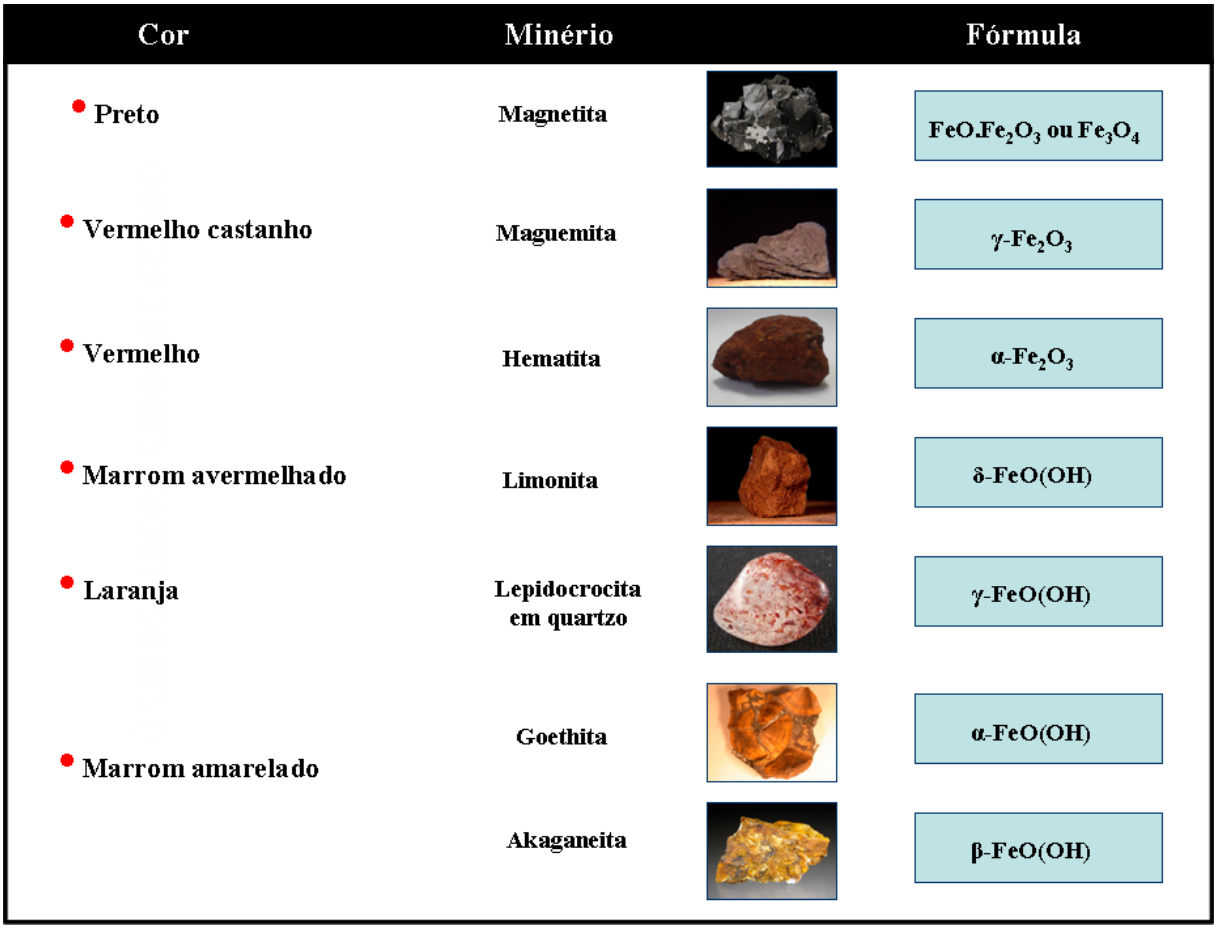

Figura 14. Cores características dos diferentes óxidos/hidróxidos de ferro. *Fonte: Bruce et al., 2004.*

O magnetismo é uma propriedade cuja natureza é de origem elétrica e está relacionada com uma carga em movimento. As propriedades magnéticas dos materiais têm sua origem no movimento de cargas elétricas ou em uma propriedade intrínseca de partículas elementares, como elétrons e prótons, denominado spin dos átomos (Hannickel, 2011). O magnetismo pode aparecer de diversas formas, e os materiais são classificados pela forma como respondem a um campo magnético aplicado, de acordo com sua susceptibilidade relativa (χ), que pode variar entre 10^{-5} até 10^{6}. Materiais diamagnéticos apresentam $\chi < 1$, paramagnéticos $\chi \geq 1$, antiferromagnéticos, ferromagnéticos e ferrimagnéticos $\chi >> 1$ (Sinnecker, 2000).

2.3.1 Estrutura

A magnetita é um dos óxidos de ferro mais interessante devido a suas propriedades magnéticas, e geralmente é preparada pelo método de co-precipitação de sais de cloreto ferroso (Fe^{+2}) e cloreto férrico (Fe^{+3}) em meio alcalino. Quando a magnetita é mantida sob atmosfera normal (presença de oxigênio), o produto chamado como de costume magnetita é na verdade "bertholide", ou seja, um óxido de ferro cuja composição está entre a magnetita (Fe_3O_4) e a maguemita (γ-Fe_2O_3). Ambos os óxidos de ferro são materiais ferrimagnéticos (Ngomsik et al., 2005).

As estruturas cristalinas das partículas magnéticas são fundamentadas na existência de muitos domínios magnéticos. A magnetita apresenta uma estrutura cristalina do tipo espinélio inverso, formada por uma rede cúbica unitária de face centrada (fcc) de ânions de oxigênio, com sítios preenchidos por cátions (**Figura 15**). Existem dois tipos de sítios, diferindo na coordenação: tetraédrica (A) e octaédrico (B). Considerando-se um cubo elementar, com aresta de 8 Å, que contém 32 íons de oxigênio, os cátions ocupam somente 8 sítios tetraédricos (sítios A) e 16 sítios octaédricos (sítio B). Na estrutura do tipo espinélio inverso, a metade dos íons Fe^{+3} encontra-se nos sítios tetraédricos, e o restante juntamente com os íons Fe^{+2} são distribuídos pelos sítios octaédricos. A maguemita é obtida por meio de um processo de oxidação da magnetita, processo que pode ser natural ou induzido, e preserva a estrutura de espinélio inverso. Além disso, se houver ainda uma variação da temperatura do meio, a magnetita pode sofrer uma transição de fase, originando uma fase mais estável, a hematita (α-Fe_2O_3) (Mendoza et al., 2005).

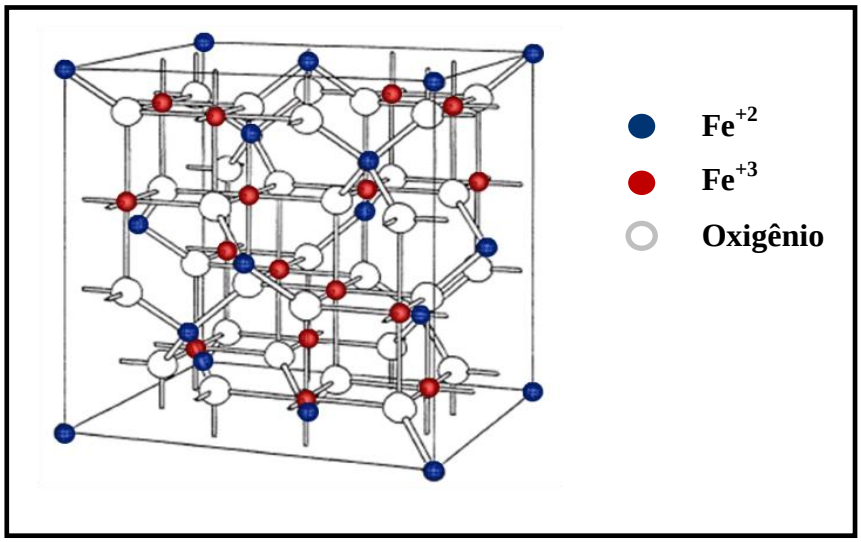

Figura 15. Estrutura cristalina da magnetita. *Fonte: http://www.ruf.rice.edu/~natelson/magnetite_str_lg.png*

2.3.2 Estabilidade: revestimento com polianilina (PANI)

O mecanismo de atuação das partículas magnéticas está relacionado com a área de superfície, uma aglomeração das mesmas inibe este mecanismo. As aglomerações acontecem devido à presença de forças de Van der Waals e da energia de superfície. Estas forças existentes no aglomerado podem ser rompidas através de processos físicos como o cisalhamento, ou químicos, que envolvem a adição de surfactantes ou funcionalização da superfície (Maity e Agrawal, 2007).

A polianilina (PANI) é um dos polímeros condutores mais estudados, devido às suas únicas propriedades eletrônicas e ópticas, excelente estabilidade ambiental, facilidade de preparação e baixo custo do monômero (anilina). Na última década, este polímero tem sido amplamente investigado como resultado do excelente desempenho em aplicações tais como polímeros condutores em sensores, membranas condutoras de separação, dispositivos anti-estáticos, controle de corrosão e como matriz para a imobilização de enzimas (Zhang et al., 2006; Jaramillo-Tabares et al., 2012; Neri et al., 2009; Neri et al., 2011). A síntese da PANI pode ser realizada quimicamente, através do uso de agentes oxidantes, ou eletroquicamente, utilizando eletrodos para aplicação de uma corrente elétrica, que dará início à síntese. A sua estrutura é constituída por dois segmentos: uma estrutura plana de dois grupos imina e um anel quinóide, e segmentos tetraédricos de dois grupos amina que separam três anéis benzênicos (Jaramillo-Tabares et al., 2012). A PANI é um dos únicos polímeros orgânicos condutores cuja estrutura e propriedades elétricas, óticas e eletroquímicas podem ser reversivelmente controladas por reações ácido/base ou eletroquímicas (**Figura 16**). Assim, este polímero apresenta diferentes estados de oxidação, dos quais a forma mais

estável é o sal de esmeraldina, 50% oxidada. A forma base de esmeraldina (isolante) do polímero pode reagir com ácidos resultando na forma sal de esmeraldina (condutora).

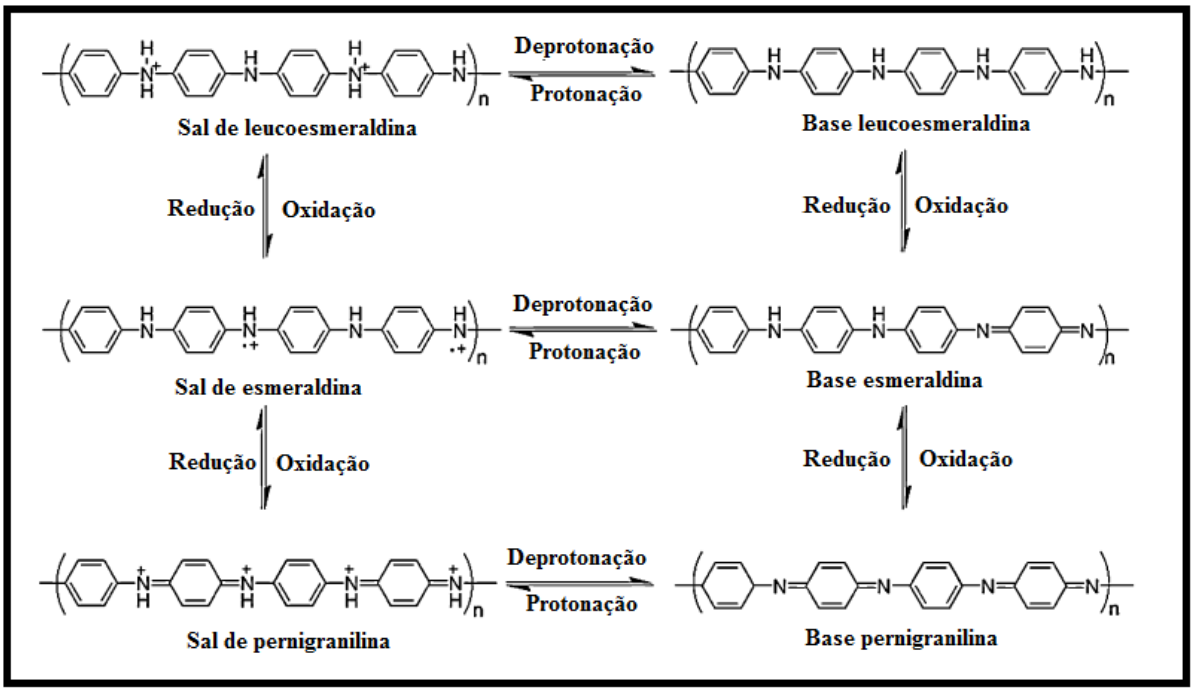

Figura 16. Estruturas químicas da polianilina em diferentes estados redox. *Fonte: Zhang, 2007.*

A PANI é utilizada como revestimento de partículas magnéticas com o intuito de funcionalizar a superfície das partículas magnéticas, também como de unir as propriedades elétricas do polímero e a propriedade magnética da partícula magnética (Maciel, 2012). A interação elétrica/eletrônica da PANI/magnetita, que é responsável pelo desempenho do compósito polimérico magnético ainda não foi elucidado. Embora, Montoya et al. (2010) reportaram uma clara indicação de interações redox entre as partículas de magnetita e o polipirrol. Os autores observaram que a presença de magnetita na matriz polimérica reduziu o processo de oxidação do polímero, estabilizando sua condutividade.

2.3.3 Aplicações

Robinson et al. (1973) utilizaram a separação magnética pela primeira vez no contexto da biotecnologia. Os autores trabalharam com sílica e celulose ambas revestidas com óxido de ferro (magnetita) como matriz para imobilizar duas enzimas: α-quimotripsina e β-galactosidase para aplicações em biorreatores. Desde então, a separação magnética tem se tornado uma ferramenta cada vez mais popular para o processo de separação de moléculas biológicas e células.

Algumas aplicações industriais da magnetita são: gravação magnética de mídia, catalisadores, pigmentos, sensores de gás, dispositivos ópticos e eletromagnéticos (Xuan et al.,

2008). Em biotecnologia, essas partículas magnéticas têm encontrado aplicações em diagnósticos médicos como pesquisa genética (Scherer et al., 20002) e tecnologias baseadas em separação magnética de células, proteínas, DNA/RNA, bactérias, vírus e outras biomoléculas (Neuberger et al., 2005); como adsorventes de bioafinidade e tratamento de água poluída via adsorção eletrostática (Ngomsik et al., 2005; Hsing et al., 2007).

A magnetita e compósitos magnéticos têm sido utilizados também como suportes para a imobilização de enzimas. (Neri et al., 2009; Neri et al., 2011; Soria et al., 2012; Maciel et al., 2012). Na biotecnologia, as enzimas são essenciais para aplicação em processos industriais. Um grande número de enzimas foi imobilizado com sucesso, tendo altos rendimentos de atividade no suporte adequado. É importante que a escolha do material e o método de imobilização sejam bem justificados. A utilização de materiais magnéticos como matrizes para a imobilização de enzimas reúne as vantagens de fácil remoção da mistura de reação por aplicação de um campo magnético externo, e a preparação é relativamente simples. No entanto, os materiais magnéticos não possuem grupos químicos funcionais. Por isto, eles devem ser funcionalizados quando forem utilizados como matriz para a imobilização covalente de biomoléculas. A PANI é utilizada como revestimento de partículas magnéticas devido às suas propriedades físico-químicas e a presença de grupos químicos funcionalizáveis. Os óxidos de ferro oferecem uma série de características magnéticas e eletrônicas que combinadas com a PANI produzem compósitos poliméricos magnéticos, uma nova classe de materiais funcionais com elevado potencial para a aplicação na separação de células, imunoensaio enzimático, liberação de droga, dispositivos eletromagnéticos e supressão de interferência eletromagnética (Zhang e Wan, 2003; Jaramillo-Tabares et al., 2012).

2.4 Enzimas para a imobilização

2.4.1 α-Ramnosidase

As α-L-ramnosidases (RASE, E.C. 3.2.1.40), são enzimas glicosídicas que podem ser produzidas por fungos (Gallego et al., 2001) mais comumente pelo *Penicillium* sp., *Penicillium decubens* e pelo *Aspergillus niger* ou por bactérias *Bacillus* sp. GL1 (Hashimoto et al., 1999).

As preparações comerciais das ramnosidases a partir dos gêneros *Aspergillus* e *Penicillium* são geralmente contaminadas com atividade β-D-glicosidase (E.C. 3.2.1.21). A **Figura 17** mostra esquematicamente a ação da α-L-ramnosidase e β-D-glicosidase. Este complexo enzimático, quando é utilizado para a hidrólise das duas ligações glicosídicas dos flavonóides ramnoglicosídeos

naringina ou hesperidina, é chamado de naringinase ou hesperidinase, respectivamente (Soria et al., 2011).

2.4.1.1 Modo de ação

Essa enzima catalisa a conversão da naringina em naringenina em um processo de duas etapas que envolvem a hidrólise de duas ligações glicosídicas (**Figura 17**). O substrato naringina (4´-5,7´-trihidroxiflavonona-7-ramnoglicosídeo) é hidrolisado pela porção α-L-ramnosidase para produzir ramnose e prunina (4´-5,7´-trihidroxiflavonona-7-glicosídeo), que é então convertida pela porção β-D-glicosidase em glicose e naringenina (4´-5,7´-trihidroxiflavonona) (Norouzian et al., 2000).

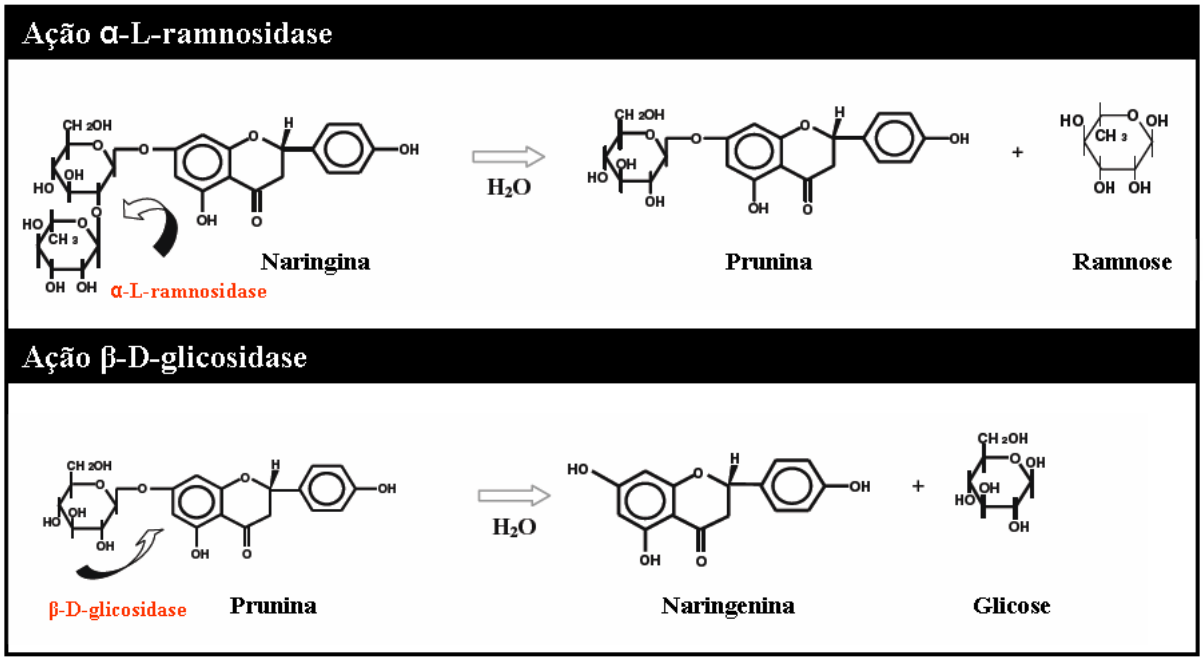

Figura 17. Hidrólise enzimática de naringina por ação da naringinase. *Fonte: Soria et al., 2012.*

2.4.1.2 Aplicações

A α-L-ramnosidase apresenta potencial de aplicação na produção de ramnose, que pode ser utilizada como componente de fármacos, e de prunina que tem ação antiinflamatória e pode ser utilizada também como adoçante para diabéticos. A naringenina tem ação antioxidante, antiúlcera e antiinflamatória, induz a apoptose através da ativação da cascata da caspase-3 e prevê doenças neurodegenerativas, como o Alzheimer (Vila-Real et al., 2010).Os produtos da ação da naringinase podem, ainda, ser utilizados no realce do aroma de vinhos e na indústria de sucos cítricos por degradar a naringina, formando compostos menos amargos (Soria et al., 2004).

As α-L-ramnosidases de *Aspergillus niger* e *Penicillium sp*. foram imobilizadas em diversos suportes, tais como fibras ocas (Olson et al., 1979), DEAE Sephadex A-25 (Ono et al., 1977), alginato de cálcio (Ellenrieder et al., 1998; Norouzian et al., 1999), triacetato de celulose (Tsen e Yu, 1991), quitina (Tsen, 1984), vidro de poro controlado (Roitner et al., 1984).

2.4.2 Invertase

A invertase (β-D-frutofuranosidase, E.C.3.2.1.26) também conhecida como sacarase, sucrase, pertence à família das hidrolases glicosídicas e está amplamente distribuída nos organismos vivos (bactérias, fungos, plantas, insetos). Esta enzima é principalmente biosintetizada por cepas de leveduras tais como a *Saccharomyces cerevisiae*.

Invertase de levedura é uma glicoproteína contendo 50% de manana e 2-3% de glucosamina (Neumann e Lampen, 1967). Uma diferença no teor de carboidratos distingue duas formas da enzima. A invertase externa (forma predominante) contém carboidrato, é homodimérica, com peso molecular de 270 kDa, localizada fora da membrana celular. O conteúdo de carboidratos na invertase externa não é essencial para a atividade da enzima, mas assegura uma proteção contra a degradação proteolítica (Chu et al., 1978; Chu e Maley, 1980), aumenta a estabilidade térmica (Neumann e Lampen, 1967), solubilidade (Gascon et al., 1968), e faz à enzima extremadamente estável a temperatura ambiente (Chu et al., 1978). As invertases externas contêm grupos fosfatos covalentemente unidos a manose (Trimble et al., 1983). Os dímeros desta enzima podem estar associados a tetrâmeros, hexâmeros e octâmeros (Esmon et al., 1987). A invertase interna não contém carboidratos, tem um peso molecular de 135 kDa e encontra-se dentro do citoplasma (Goldstein e Lampen, 1975). As duas enzimas diferem também na composição de aminoácidos, em particular, a invertase interna não contém cisteína (Gascon et al., 1968).

2.4.2.1 Modo de ação

A invertase catalisa a hidrólise da sacarose para produzir glicose e frutose (açúcar invertido). A sacarose é um dissacarídeo não redutor, composto por dois açucares redutores: α-D-glicose e β-D-frutose, unidos por um enlace glicosídico β-1,2 (**Figura 18**). A denominação "invertase" deve-se ao fato que esta enzima quando hidrolisa a sacarose ocorre uma inversão no sinal da rotação óptica de positivo para negativo. A sacarose gira o plano de luz polarizada para a direita (+66,5º) e os produtos de hidrólise para a esquerda (-39,7º), o sinal negativo surge porque a glicose tem um desvio de +52,5 º e a frutose de -92º.

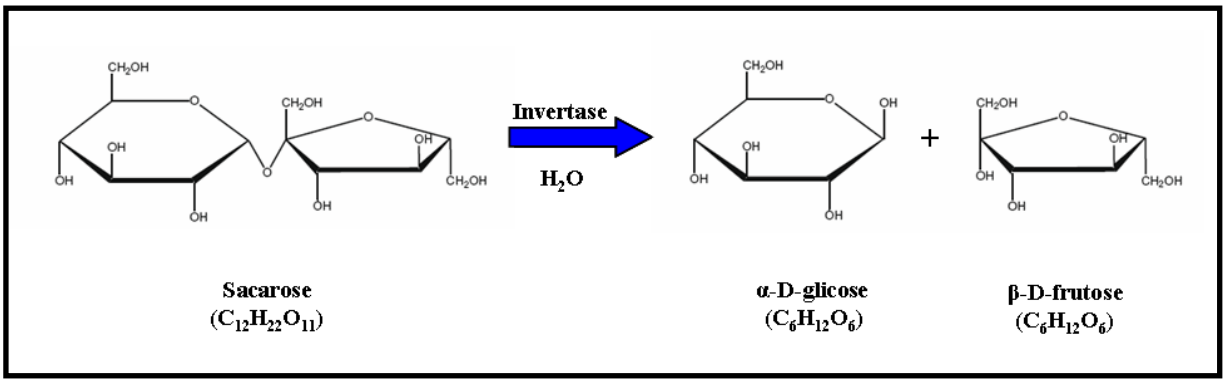

Figura 18. Modo de ação da invertase.

2.4.2.2 Aplicações

A invertase é principalmente usada para a produção de açúcar invertido, e tem sido amplamente utilizada nas indústrias de alimentos e cerveja, pois possui poder adoçante superior ao da sacarose. Esta enzima também é usada para a fabricação de mel artificial, agentes plastificantes usados em cosméticos e nas indústrias farmacêuticas e de papel, bem como eletrodos de enzima para a detecção de sacarose (Kotwal e Shankar, 2009). Outra aplicação da invertase é na hidrólise de xarope de cana de açúcar para obter frutose. Este monossacarídeo redutor possui alta capacidade de adoçante e algumas aplicações dele são: substituinte da sacarose para os diabéticos e potencialização da absorção de ferro em crianças (Gill et al., 2006), matérias-primas para produtos farmacêuticos, indústria de alimentos (pão, chocolate, sumos de fruta), também na indústria de cosméticos, entre outros.

A hidrólise enzimática da sacarose é preferível à hidrólise ácida, pois não produz agentes aromatizantes indesejáveis também como impurezas coloridas. No entanto, o uso de invertase imobilizada para a obtenção de frutose é limitado devido à utilização da glicose isomerase imobilizada, utilizada também para produzir frutose a partir de glicose de forma mais econômica (Kotwal e Shankar, 2009).

A invertase tem sido imobilizada por diferentes métodos e em uma ampla variedade de suportes orgânicos, inorgânicos e resinas de poliestireno, alguns deles são citados: DEAE-celulose, quitosana, flanela de algodão revestida com polietilenimina, poliacrilamida, contas de vidro poroso, bentonita, montmorilonita-K10, sílica gel, álcool polivinílico, grãos de milho, CM-celulose, Dacron magnético, poliuretano, fibras de poliacilonitrila revestida com polianilina, álcool polivinílico-alginato, celite, microesferas de álcool polivinílico magnético, casca de arroz, quitina modificada com ácido hialurônico (Kotwal e Shankar, 2009).

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4 Objetivos

4.1 Objetivo Geral

Propor suportes magnéticos (Dacron, POS/PVA, quitosana, argila montmorilonita e terra de diatomáceas) de fácil síntese, baixo custo e resistentes para a imobilização de enzimas.

4.2 Objetivos Específicos

- Imobilizar α-L-ramnosidase por ligação covalente nos suportes magnéticos: Dacron, POS/PVA e quitosana;
- Escolher o melhor derivado enzimático da α-L-ramnosidase para a hidrólise e hidrólise inversa;
- Sintetizar novos compósitos magnéticos a partir de minerais de baixo custo (argila montmorilonita e terra de diatomáceas) e investigar metodologias de funcionalização dos suportes;
- Caracterizar por diferentes técnicas físico-químicas os novos materiais propostos, através da determinação do tamanho de partícula, difração de raios X (DRX), infravermelho por transformada de Fourier (FTIR), microscopia eletrônica de varredura (MEV), área de superfície e porosimetria, medidas de magnetização e espectroscopia de Mössbauer (MS);
- Imobilizar a invertase por ligação covalente em argila montmorilonita magnética (mMMT), terra de diatomáceas magnética (mTD) e terra de diatomáceas magnética revestida com polianilina (mTD-PANI);
- Estudar os processos de imobilização e caracterização dos novos derivados imobilizados, tais como: pH ótimo, temperatura de máxima atividade, estabilidade térmica, reuso.

Capítulo 2

Artigos

5.1 Artigo publicado no periódico *Applied Microbiology and Biotechnology*

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

α-L-Rhamnosidase of *Aspergillus terreus* immobilized on ferromagnetic supports

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Abstract α-L-Rhamnosidase from *Aspergillus terreus* was covalently immobilized on the following ferromagnetic supports: polyethylene terephthalate (Dacron-hydrazide), polysiloxane/polyvinyl alcohol (POS/PVA), and chitosan. The powdered supports were magnetized by thermal coprecipitation method using ferric and ferrous chlorides, and the immobilization was carried out via glutaraldehyde. The activity of the Dacron-hydrazide (0.53 nkat/μg of protein) and POS/PVA (0.59 nkat/μg of protein) immobilized enzyme was significantly higher than that found for the chitosan derivative (0.06 nkat/μg of protein). The activity–pH and activity–temperature profiles for all immobilized enzymes did not show difference compared to the free enzyme, except the chitosan derivative that presented higher maximum temperature at 65 °C. The Dacron-hydrazide derivative thermal stability showed a similar behavior of the free enzyme in the temperature range of 40–70 °C. The POS/PVA and chitosan derivatives were stable up to 60 °C, but were completely inactivated at 70 °C. The activity of the preparations did not appreciably decrease after ten successive reuses. Apparent K_m of α-L-rhamnosidase immobilized on magnetized Dacron-hydrazide (1.05±0.22 mM), POS/PVA (0.57±0.09 mM), and chitosan (1.78±0.24 mM) were higher than that estimated for the soluble enzyme (0.30±0.03 mM). The Dacron-hydrazide enzyme derivative showed better performance than the free enzyme to hydrolyze 0.3% narigin (91% and 73% after 1 h, respectively) and synthesize rhamnosides (0.116 and 0.014 mg narirutin after 1 h, respectively).

Keywords α-L-Rhamnosidase · *Aspergillus terreus* · Dacron · POS/PVA · Chitosan · Immobilization

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Introduction

The enzyme α-L-rhamnosidase (RASE, E.C. 3.2.1.40) releases rhamnose from glycosides, glycolipids, and other natural products. RASEs are not among the most studied glycosidases, but they can be used in the following applications: debittering of grapefruit and other citrus juices by hydrolysis of the bitter rhamnoside naringina (Soares and Hotchkiss 1998), aroma enhancement of wines and juices by hydrolysis of terpenyl glycosides precursors (Caldini et al. 1994), production of the rhamnose (raw material for the synthesis of several compounds of interest in the food industry) by hydrolysis of natural glycosides (Ellenrieder et al. 1998), enzymatic synthesis of rhamnosides (Martearena et al. 2003), and agent for structural study of glycosides, which hydrolyzes specifically L-rhamnosides (Monti et al. 2004). The commercial preparations of RASEs obtained from the genera *Aspergillus* and *Penicillium* are usually contaminated with β-D-glucosidase (βG, EC 3.2.1.21) activity. Figure 1 depicts schematically α-L-rhamnosidase and β-D-glucosidase actions. This enzyme mixture when used for the hydrolysis

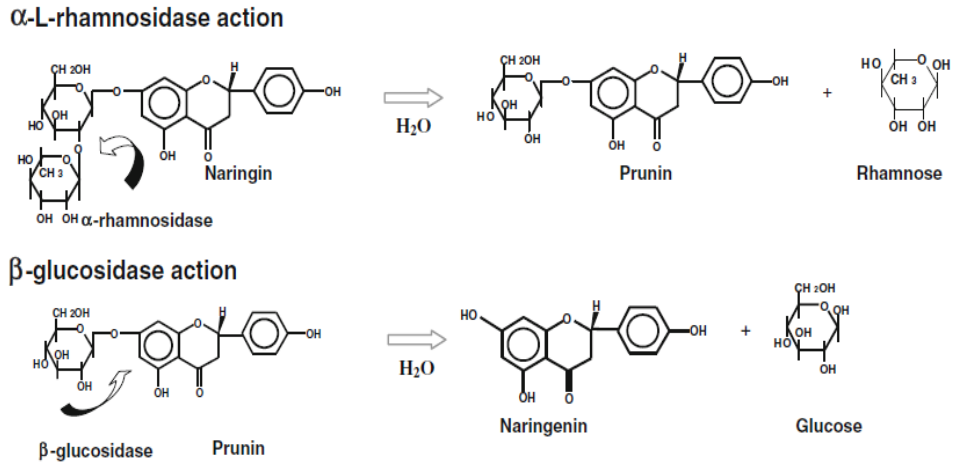

Fig. 1 Enzymatic hydrolysis of naringin by action of naringinase

of the two glycosidic bonds of the flavonoid rhamnoglucosides naringin or hesperidin, it is called naringinase or hesperidinase, respectively. *Aspergillus niger* and *Penicillium* sp. naringinases have been immobilized on several supports such as hollow fibers (Olson et al. 1979), DEAE Sephadex A-25 (Ono et al. 1977), calcium alginate (Ellenrieder et al. 1998; Norouzian et al. 1999), cellulose triacetate (Tsen and Yu 1991), and chitin (Tsen 1984) for their applications in the debittering of juices. For studies on thermal stability, naringinase of *Penicillium decumbens* has been immobilized on protein-rich supports such as silk fibers, bovine horn keratin, sheep wool, and collagen (Ellenrieder and Daz 1996).

The advantages of the use of immobilized enzymes are well known: they facilitate the product and substrates surpluses recovery and the reusability of the catalyst. The ease of separation of the enzyme of products and other components in the reaction mixture can be remarkably increased using magnetic supports, mainly in the cases where there exist insoluble substances at the end of the reaction (Demirel et al. 2004).

In the present study, α-L-rhamnosidase of *Aspergillus terreus* was covalently immobilized on three magnetic supports (Dacron-hydrazide, polysiloxane/polyvinyl alcohol (POS/PVA), and chitosan), and the following properties have been studied: retention of activity, optima pH and temperature, kinetics constants, and thermostability. Also, the L-rhamnose production by the flavonoid glycoside naringin hydrolysis in supersaturated solutions and rhamnosides synthesis under the immobilized enzymatic derivatives were studied.

Materials and methods

Hydrazine hydrate, *p*-nitrophenyl α-L-rhamnoside (pNPR), and chitosan were purchased from Sigma (St. Louis, Mo, USA). Polyethylene terephthalate (Dacron) was produced by Terphane S.A (Cabo, Brazil), polyvinyl alcohol (PVA; MW 72000) was from Reagen (Quimibras Indústrias Químicas S.A., Rio de Janeiro, RJ, Brazil), and tetraethyl orthosilicate and glutaraldehyde (25%) were from Merck S. A. The standards for naringin, prunin, and narirutin high-performance liquid chromatography (HPLC) were of Extrasynthese (France). All other reagents were of analytical grade.

Supports synthesis and magnetization

The Dacron-hydrazide and POS/PVA particle syntheses were obtained according to Amaral et al. (2006) and Coêlho et al. (2002), respectively. The magnetization of Dacron-hydrazide, POS/PVA, and chitosan (particles size ≤250 μm) was performed according to Amaral above cited. From now on, the magnetic particles derivatives were recovered by a magnetic field (Ciba Corning; 0.6 T). The formed magnetic particles (about 2.7 g) were then washed with deionized water until pH 7 and dried at 50 °C overnight.

Activation of Dacron-hydrazide, POS/PVA, and chitosan magnetic particles with glutaraldehyde

The magnetized Dacron-hydrazide and chitosan particles (10 mg) were incubated with 1 ml of 5% *v*/*v* glutaraldehyde in the buffer under mild stirring for 2 h at 25 °C. Afterwards, they were washed ten times with 1 ml of the buffer. The POS/PVA magnetized particles (10 mg) were incubated with 1 ml 2.5% (*v*/*v*) glutaraldehyde in 0.1 M H_2SO_4 with mild stirring for 2 h at 25 °C, washed ten times with 1 ml deionized water and finally with the buffer. In all the cases, the treated particles were recovered using magnetic field.

Enzyme preparation

Production of the *A. terreus* CECT 2663 kindly gifted by the Colección Española de Cultivos Tipo (Valencia, Spain) naringinase complex was carried out in submerged cultures using naringin as inducer and carbon source, following the procedure previously described (Soria et al. 1999). The β-glucosidase was partially removed from the enzymatic complex by an inactivating treatment at pH 11 prior to the purification procedure. The partial purification procedures of the enzyme α-L-rhamnosidase included ammonium sulfate precipitation and ion exchange chromatography on DEAE Sepharose CL-6B according to the method previously described (Soria et al. 1999). This preparation was 135-fold purified yielding 29% of the initial activity and presented a specific activity of 1.46 nkat/μg of protein. Furthermore, this preparation contained about 8% of the original β-glucosidase activity.

α-L-Rhamnosidase immobilization on magnetic Dacron-hydrazide, POS/PVA, and chitosan

Glutaraldehyde-activated magnetic particles (10 mg) were incubated with 1 ml of α-rhamnosidase solutions in the buffer in concentrations ranging from 20 to 500 μg protein per milliliter for 19 h at 4 °C with mild stirring. The α-rhamnosidase-magnetized derivatives were recovered by action of a magnetic field, and the supernatants were collected for protein determination according to Sedmak and Grossberg (1977). The immobilized enzyme preparations were washed ten times with 1 ml of the buffer and stored at 4 °C for their later use. The supernatant of these washings was also collected for protein determination. The fixed protein was calculated by the difference between the content of the offered enzyme preparation and those determined in the supernatants.

Enzyme assays

The activity of soluble α-rhamnosidase was determined as described by Romero et al. (1985) using pNPR (1.75 mM) as substrate prepared in 0.02 M succinate buffer, pH 5.5 (from now on abbreviated as the buffer). The unit of catalytic activity katal (kat) was taken as the amount of enzyme that liberates 1 mol of *p*-nitrophenol per second.

The activity of immobilized enzymes was determined by incubating 1.2 ml of pNPR (1.75 mM) with the water-insoluble derivative (10 mg), under shaking at 50 °C. Samples of 50 μl were withdrawn at time intervals of 2 min after removing the magnetic enzymatic derivatives by a magnetic field and added to 1.5 ml of 0.5 M NaOH and their absorbencies measured at 400 nm. In these experiments, the solid catalyst quantity was measured by pipetting a given volume of homogenized suspension (10 mg/ml). To validate this procedure, it was previously observed that there was a linear relationship between the volume of suspension and mass of recovered magnetic immobilized enzyme derivatives. The standard deviation of replicate determinations was lesser than 2.6%. Furthermore, a linear relation was also observed between the measured enzyme activity (from 0 to 8.7 nkat) and the volume of original suspension.

Effect of pH and temperature on enzymatic activity

Optimal pH and temperature of the immobilized RASEs were established by measuring their activities at several pH values from 3 to 8, using Na_2HPO_4 citric acid (McIlvaine buffer) at 50 °C and at different temperatures (in the buffer) in the range from 40 °C to 70 °C, respectively.

Thermostability

The thermostability of the immobilized derivatives was determined by incubating the appropriate amount of the immobilized derivatives suspended in the buffer at temperatures from 40 °C to 70 °C for 20 min. After 30 min at 25 °C (temperature equilibration), their activities were established as above described.

Reusability of the immobilized derivatives

The activity of the immobilized derivative was measured ten times consecutively. After each derivative activity determination, they were recovered by a magnetic field, washed with the buffer, and reused.

Kinetic studies

Michaelis constants for the immobilized derivatives were determined using pNPR as the substrate in the concentration ranges from 0.4 to 3.5 mM. The reaction was carried out by using 5.9, 4.7, and 0.5 nkat/mg of the magnetic Dacron-hydrazide, POS/PVA, and chitosan derivatives of RASE, respectively, in 2 ml of the substrate prepared in the buffer at 50 °C, under stirring. The initial reaction rate was recorded measuring the absorbance of the *p*-nitrophenol produced. The graphics, nonlinear regressions, and statistic calculations were done using the Prism software of GraphPad, USA.

Hydrolysis of supersaturated naringin solutions

The immobilized RASEs were those obtained by using the supports Dacron-hydrazide and POS/PVA. Naringin was dissolved in the buffer at 80–90 °C to achieve a concentration of 0.30%. After cooling the temperature to 60 °C, the

substrate (1 ml) was mixed to the Dacron-hydrazide (10 mg; 32 nkat) and POS/PVA (5 mg; 26.7 nkat) derivatives suspensions and free enzyme (50 µl; 116.9 nkat). Aliquots of 50 µl were taken at different times, and the residual substrate and the prunin were determined by HPLC in equipment Shimadzu LC-4 with a C_{18} column and a detector UV (280 nm). The movable phase was a mixture 32:68 of acetonitrile/water at a speed of flow of 0.8 ml min^{-1}.

Synthesis of rhamnosides

The immobilized RASEs were also those obtained by using the supports Dacron-hydrazide and POS/PVA. For the glycosylation of prunin with L-rhamnose like donor, by inverse hydrolysis, a solution (0.5 ml) containing L-rhamnose (1,220 mM) and prunin (96 mM) prepared in the buffer was added by 32 nkat (0.5 ml) of the free and immobilized enzyme. The reaction mixture was incubated into a closed tube at 60 °C with rotary agitation. Aliquots of 25 µl were taken at different times and analyzed by HPLC in equipment Shimadzu LC-4 with a C18 column and a detector UV (280 nm). A mixture 70:15:10:5 of water/acetonitrile/methanol/acetic acid was used as fluent at a speed of flow of 1 ml min^{-1}.

Results

Characterization of the magnetic supports

The relationship between the amounts of protein fixed on the magnetized supports and that offered in the immobilization process is shown in Fig. 2a. An increase of the fixed protein is observed for all of these materials in the tested range (0 to 50 µg of protein per milligram of support). POS/PVA and chitosan showed a higher immobilization capacity than Dacron-hydrazide, achieving amounts of 22, 19, and 11 µg per milligram of support, respectively, at the highest amount of offered protein (50 µg per milligram of support). Figure 2b and c displays the retained and specific activities for all enzymatic derivatives, respectively. The retained activity for all enzymatic derivatives showed hyperbolic curves because as the offered protein increases, the fixed ones also increase but limited by the supported load capacity. Regarding the specific activity (co), the highest value was found to be 5 µg/mg of support after which these values decreased. This decline can be attributed to enzyme overloading. Table 1 resumes the properties of the magnetic RASE derivatives obtained using the proportion of 15 µg offered protein per milligram of support that it is lower than the highest and far from the zone of saturation (50 µg offered per milligram of support in Fig. 2a). This

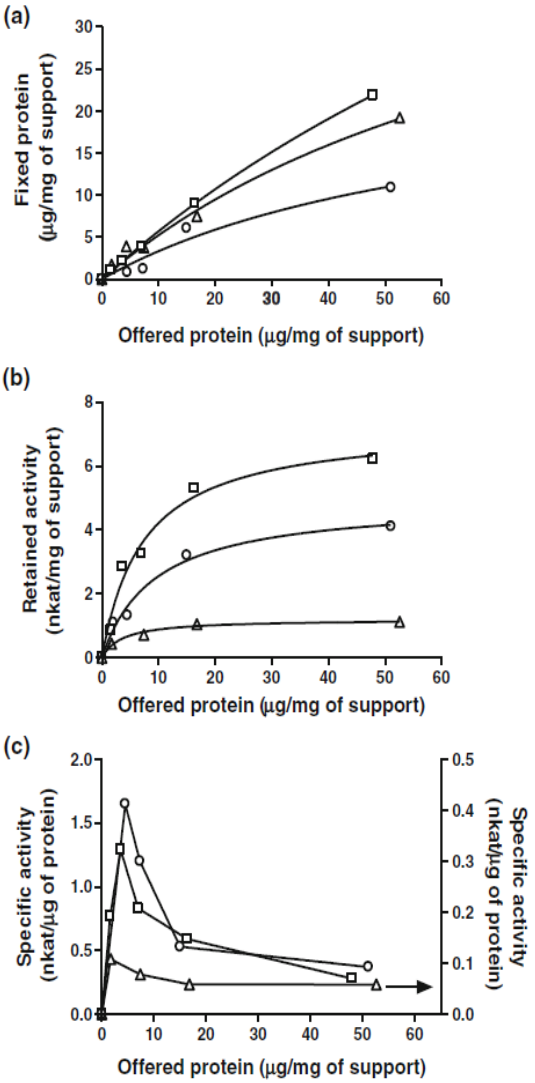

Fig. 2 Relationship between the fixed α-L-rhamnosidase (a) retained (b) and specific activities (c) on magnetized Dacron (*white circle*), POS/PVA (*white square*), and chitosan (*white triangle*) and the amount of offered enzyme. Magnetic supports' glutaraldehyde particles (10 mg) were incubated with 1 ml of α-L-rhamnosidase solutions prepared in the buffer for 19 h at 4 °C

would avoid the possibility of steric hindrance due to overload of immobilized enzyme. Magnetic POS/PVA, chitosan, and Dacron-hydrazide RASE derivatives yielded preparations containing, respectively, 9.0, 7.5, and 6.0 µg of protein/milligram of support. However, the retained activities were, respectively, 5.3, 0.4, and 3.2 nkat/mg of support. These results mean that the specific activity of the magnetic POS/PVA and Dacron-hydrazide are similar (0.53 and 0.59 nkat/µg of protein, respectively) and higher than that estimated for the magnetic chitosan

Table 1 Properties of free and immobilized *A. terreus* α-L-rhamnosidase on magnetic Dacron-hydrazide, POS/PVA, and chitosan

Preparation	Retained activity (nkat/mg)[a]	Fixed protein (μg/mg)[a]	Specific activity (nkat/μg)[b]	Activity retention (%)	K_m (mM) Mean±SD
Free enzyme	–	–	1.46	100.0	0.30±0.03
Dacron-hydrazide	3.2	6.0	0.53	36.3	1.05±0.22
POS/PVA	5.3	9.0	0.59	40.4	0.57±0.09
Chitosan	0.4	7.5	0.06	4.0	1.78±0.24

The magnetic RASE derivatives were synthesized using the proportion of 15 μg enzyme offered per milligram of support

[a] Per milligram of support

[b] Per microgram of protein

(0.06 nkat/μg of protein). The retention of activity for the magnetic POS/PVA and Dacron-hydrazide derivatives was of the same magnitude (40.4% and 36.3%, respectively, of the soluble enzyme activity), whereas the magnetic chitosan retained only 4% of activity. Although the amount of fixed protein was higher on magnetized chitosan than on magnetized Dacron-hydrazide, its activity was smaller. Table 1 also shows the apparent K_m for the α-L-rhamnosidase immobilized on magnetized Dacron-hydrazide (1.05±0.22 mM), POS/PVA (0.57±0.09 mM), and chitosan (1.78±0.24 mM) were higher than that estimated for the soluble enzyme (0.30±0.03 mM).

Effect of pH and the temperature on the enzymatic activity

The influence of the pH on the activity of free and immobilized RASE on the different supports is shown in Fig. 3a. The pH profiles of all immobilized enzymes were not significantly different with respect to the free enzyme. The activity of the free and immobilized enzyme as a function of temperature is shown in Fig. 3b. The maximum temperature for the immobilized enzyme on magnetized Dacron-hydrazide and POS/PVA was 60 °C, in coincidence with that of the free enzyme, but this value was 65 °C for the enzyme immobilized in magnetized chitosan.

Thermostability and reusability of the immobilized derivative

The thermostability of immobilized RASEs was studied between 40 °C and 70 °C and compared with the free enzyme (Fig. 4). The Dacron-hydrazide derivative showed a similar performance of the free enzyme. POS/PVA and chitosan derivatives were stable up to 60 °C but lost all activities at 70 °C, whereas the Dacron-hydrazide derivative and the free enzyme retained 40% of the activity. The possible reuse of the immobilized derivatives was carried out, and all of them were stable after ten consecutive reuses. The Dacron-hydrazide, POS/PVA, and chitosan enzymatic derivatives showed at the tenth reuse 98.4±0.4%, 99.1±0.3%, and 96.4±1.1% of activity retentions, respectively.

Hydrolysis of naringin solutions and synthesis of rhamnosides

The immobilized enzyme on Dacron-hydrazide was capable to hydrolyze 99% of 0.3% naringin in a lapse of 3 h (Table 2). Furthermore, higher percent of naringin hydrolysis compared to the fee enzyme was observed in all investigated time. For instance, 80% of the naringin was hydrolyzed by the immobilized enzyme in half hour whereas 63% for the free enzyme. The yields of narirutin production (Table 3) were higher for both immobilized enzyme on Dacron-hydrazide and on POS/PVA than to the free enzyme. After 7 h of reaction, the amount of narirutin produced were, respectively, about seven- and fourfold higher than for the free enzyme.

Discussion

The use of magnetic supports allows the rapid isolation of the catalyst from reaction mixtures with suspended solid components and provides high-performance separation of even finely dispersed particles (Demirel et al. 2004). Compared to conventional separation, the advantages of magnetic separation are attributed to its speed, accuracy, and simplicity. The RASE immobilization on magnetic supports can render convenient catalysts for use in most of the applications of this enzyme because the presence of undissolved solids is very frequent in these system due to low solubility of its components. The immobilizations by covalent bond of an enzyme to a support present the advantage of higher stability and the consequent lack of enzyme leakages. Dacron (polyethylene terephthalate), POS/PVA, and chitosan have been used as supports to immobilize proteins (Coêlho et al. 2002; Amaral et al. 2006; Krajewska 2004)

A. terreus RASE was covalently bound, via glutaraldehyde, on Dacron-hydrazide; POS/PVA, and chitosan. These supports were previously magnetized by coprecipitating Fe^{2+} and Fe^{3+} yielding Fe_3O_4 particles. Dacron was used after a partial hydrazinolysis. The hybrid inorganic–organic composite POS/PVA network was obtained by a sol–gel technique. The alkoxide sol–gel process is an efficient method to prepare silica glass by the hydrolysis of alkoxysilane precursors and by subsequent condensation of the remaining silanols, followed by aging and drying under ambient atmospheres and sintering.

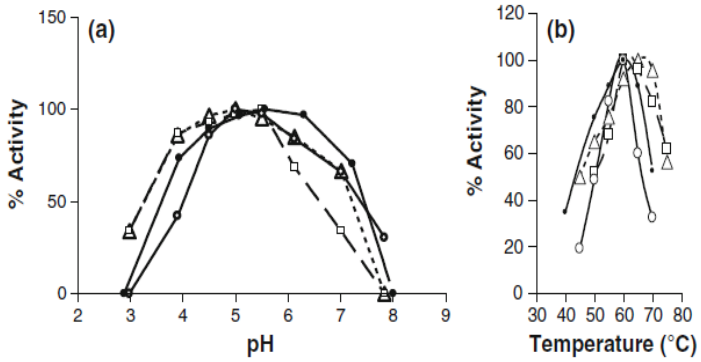

Fig. 3 Effect of pH (a) and temperature (b) on the enzymatic activity of free (*black circle*) and immobilized α-L-rhamnosidase on magnetized Dacron (*white circle*), POS/PVA (*white square*), and chitosan (*white triangle*). Values of pH were obtained by using Na_2HPO_4 citric acid (Mcilvaine buffer) and activities determined at 50 °C, whereas the temperature activities were established in the buffer

Chitosan is easily obtained by *N*-deacetylation of chitin. Therefore, as linear polyglucosamine chains of high molecular weight, it presents reactive amino and hydroxyl groups amenable to chemical modifications. Commercially, chitosan is obtained at a relatively low cost from shells of shellfish (mainly crabs, shrimps, lobsters, and krills) and wastes of the seafood processing industry (Krajewska 2004).

The K_m (0.30 mM) of the free *A. terreus* enzyme (Soria et al. 1999) is one of the lowest reported for fungal α-rhamnosidases, which ranges from 0.29 to 2.9 mM (Caldini et al. 1994; Romero et al. 1985; Manzanares et al. 1997, 2000, 2001), indicating a high affinity for the chromogenic substrate. Conformational/steric, partitioning, diffusional/mass transfer, and microenviromental effects are well known to influence the immobilized enzyme kinetics. Kinetics parameters particularly, the apparent K_m, can provide the degree of these interferences when compared to that calculated for the soluble enzyme. Hydrolysis kinetic of pNPR with RASE immobilized on the magnetized supports showed that the initial velocity versus substrate concentration

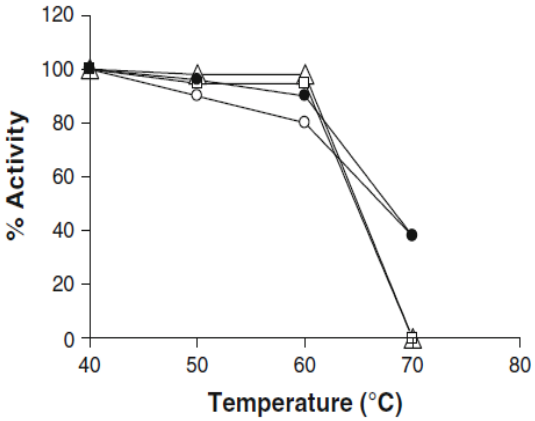

Fig. 4 Thermostability of free (*black circle*) and immobilized α-L-rhamnosidase on magnetized Dacron (*white circle*), POS/PVA (*white square*), and chitosan (*white triangle*). The different immobilized derivatives were incubated at temperatures varying from 40 °C to 70 °C for 20 min in the buffer, and after standing for 30 min at 25 °C, their activities were measured

curve was hyperbolic, as expected for a Michaelis–Menten mechanism. The statistical analysis showed that the deviation of the model was not significant. The apparent K_m values for all magnetic RASE derivatives were higher than that estimated for the soluble enzyme, probably, as the result of impairment of the enzyme action under those effects. Nevertheless, this increase showed the following pattern: magnetic chitosan > magnetic Dacron-hydrazide > magnetic POS/PVA. This behavior is according to the retained and specific activities of the magnetic derivatives. The values of apparent K_m reported for the other immobilized enzymes are also higher than those of *A. terreus*, when the substrate used was the pNPR (Puri et al. 1996; Tsen et al. 1989). It is worthwhile to notice that the K_m for the *A. terreus* RASE immobilized on bagasse particles was found to be 0.24 mM (Yadav and Yadav 2001). However, all magnetic RASE derivatives synthesized in the present work are appreciably more active than this using bagasse as support (5.8×10^{-4} nkat/mg of support). They are also higher than those reported for the *P. decumbens* enzyme immobilized derivatives: 0.3 nkat/mg of glass (Turecek and Pittner 1987) and 8.83×10^{-3} nkat/mg of silk fiber (Ellenrieder and Daz 1996); and than that from *A. niger*, 5.67×10^{-2} nkat/mg of calcium alginate (Ellenrieder et al. 1998). A high value of retained

Table 2 Hydrolysis of supersaturated naringin (0.30%) by free and immobilized α-L-rhamnosidase on magnetic Dacron-hydrazide and POS/PVA

	Free enzyme	Immobilized enzyme	
		Dacron-hydrazide	POS/PVA
Time (h)	% Hydrolysis		
0.5	63	80	58.0
1.0	73	91	73.0
2.0	84	97	85.0
3.0	92	99	92.0

In all cases, 32 nkat was used per milliliter of mixture of reaction at 60 °C

Table 3 Synthesis of narirutin by free and immobilized α-L-rhamnosidase on magnetic Dacron-hydrazide and POS/PVA

	Free enzyme	Immobilized enzyme Dacron-hydrazide	Immobilized enzyme POS/PVA
Time (h)	Narirutin (mg)		
1.0	0.014	0.116	0.044
2.0	0.023	0.172	0.089
5.0	0.037	0.263	0.170
6.0	0.040	0.279	0.189
7.0	0.043	0.292	0.206

The glycosylation was carried out using solutions of 610 mM L rhamnose and 48 mM prunin and 32 nkat for all enzyme preparations (final volume of 1 ml)

activity could be very convenient for certain applications such as the production of rhamnose and prunin.

The pH profiles of the immobilized enzyme derivatives were not significantly different from that observed for the free enzyme (5.0–6.5). These values are higher than those reported in the literature for other immobilized RASEs: pH 4.5 (Norouzian et al. 1999), pH 3.7 (Tsen et al. 1989), and pH 4.0 (Puri et al. 1996) for the *Penicillium* sp. enzyme and pH 4.0 for that from *A. terreus* (Yadav and Yadav 2001).

The maxima temperatures were not discrepant from those immobilized RASE reported: 65 °C (Norouzian et al. 1999), 55 °C (Tsen et al. 1989), and 60 °C (Puri et al. 1996) for the *Penicillium* sp. and 55 °C for the *A .terreus* (Yadav and Yadav 2001).

The thermal stability of Dacron-hydrazide-immobilized enzyme showed a behavior similar to that of the free enzyme. The magnetized POS/PVA and magnetized chitosan-immobilized enzymes were stable up to 60 °C, but were completely inactivated at 70 °C. An increase of thermostability of immobilized RASEs was also observed in previous reports (Ellenrieder and Daz 1996; Tsen et al. 1989).

An important characteristic of the immobilized enzymes is the possibility of its reusability. This is a very important factor as far as cost evaluation is concerned when compared to the use of a soluble enzyme. All immobilized derivatives were stable after ten consecutive reuses.

The immobilized enzyme on Dacron-hydrazide presented a better naringin hydrolysis performance compared to the free enzyme. Additionally, this magnetic derivative can be easily removed from the reaction mixture and reused. Therefore, this water-insoluble enzymatic derivative can be proposed to remove the naringin responsible for the bitter taste of citrus fruits.

Glycosydases catalyze the hydrolysis of polysaccharides and glycosides, and are frequently commercialized as industrial enzymes, particularly as hydrolases in the food industry. However lately, they are also becoming important catalysts in glycosides and oligosaccharides synthesis (Martearena et al. 2003). In the present work, the glycosylation of the prunin flavonoid with L-rhamnose like donor by inverse hydrolysis took place to give like narirutin product. The yields of narirutin synthesis were higher for both immobilized enzyme on Dacron-hydrazide and POS/PVA compared to the free enzyme. The best yield of narirutin production with the immobilized enzyme depends on the nature of the support. The Dacron-hydrazide enzymatic derivative showed to produce about 30% more narirutin than the POS/PVA derivative at the seventh hour.

α-L-Rhamnosidase of *A. terreus* immobilized onto magnetic supports can be of interest for some of the proposed applications of the enzyme. That fixed on Dacron-hydrazide presented advantages compared with the other two matrices: (1) it has similar properties of POS/PVA derivative regarding specific activity and activity retention, (2) the best performance as far as hydrolysis of naringin and rhamnosides synthesis are concerned, (3) it was stable up to 65 °C where the solubility, reaction rate, and stability of supersaturated solutions of substrates and products are elevated, (4) showed good stability during its reuse, and (5) the magnetic property increases the separation facility of the catalyst from other insoluble components present in the reaction mixture.

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Capítulo 3

5.5. Artigo submetido ao periódico *Hyperfine Interactions*

Título: Magnetic composites from minerals: study of the iron phases in clay and diatomite using Mössbauer spectroscopy, magnetic measurements and XRD

Autores: M. Cabrera, J. C. Maciel, J. Quispe-Marcatoma, B. Pandey, D. F. M. Neri, F. Soria, E. Baggio-Saitovitch, L. B.Carvalho Jr.

Magnetic composites from minerals: study of the iron phases in clay and diatomite using Mössbauer spectroscopy, magnetic measurements and XRD

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Abstract

Magnetic particles as matrix for enzyme immobilization have been used due to the enzymatic derivative can be easily removed from the reaction mixture by a magnetic field. This work presents a study about the synthesis and characterization of iron phases into magnetic montmorillonite clay (mMMT) and magnetic diatomaceous earth (mDE) by ^{57}Fe Mössbauer spectroscopy (MS), magnetic measurements and X-ray diffraction (XRD). Also these magnetic materials were assessed as matrices for the immobilization of invertase via covalent binding. Mössbauer spectra of the magnetic composites performed at 4.2 K showed a mixture of magnetite and maghemite about equal proportion in the mMMT, and a pure magnetite phase in the sample mDE. These results were verified using XRD. The residual specific activity of the immobilized invertase on mMMT and mDE were 83% and 92.5%, respectively. Thus, both magnetic composites showed to be promising matrices for covalent immobilization of invertase.

Keywords: magnetic particles, montmorillonite, diatomite, immobilization, invertase

1. Introduction

Inorganic materials have been widely used as carriers for enzyme immobilization. Their advantages are rigid structure, durability, high mechanical strength and relatively low cost [1]. Montmorillonite belongs to the smectite clays and its crystal structure consists of two tetrahedral silicate layers with an edge-shared octahedral layer of either alumina or magnesia [2]. On the other hand, diatomaceous earths or diatomite are mineral deposits of diatomaceous algae and are the major silica source on earth [3]. These minerals have many properties that make them interesting matrices for immobilization of proteins. Some of them are chemical inertness, large surface area, high porosity and mechanical strength, besides being readily available mineral in nature. It is advantageous to use magnetic particles as matrix for enzyme immobilization because the enzymatic derivatives are insoluble in water and can be easily removed from the reaction mixture by a magnetic field. Our group has reported several works related to biomolecules immobilized on magnetite and different magnetic composites [4-10]. The objective of the present work is to study the different iron phases in the magnetic montmorillonite clay (mMMT) and magnetic diatomaceous earth (mDE) by the ^{57}Fe Mössbauer spectroscopy (MS), magnetic measurements and X-ray diffraction (XRD). Also as to propose and assess the mMMT and mDE as matrices for the immobilization of invertase via covalent binding.

2. Materials and methods

2.1. Magnetization of clays

Montmorillonite (MMT) and diatomaceous earth (DE) were kindly supplied by Minarmco S.A. (Neuquén, Argentina) and TAMER S.A. (Salta, Argentina), respectively. The synthesis of magnetic composites was performed according to Amaral et al.[11]. The magnetic particles obtained were washed with distilled water and recovered by a magnetic field (Ciba Corning; 0.6 T). The mMMT and mDE were dried at 50 ºC overnight.

2.2. Characterization of magnetic composites

The phases of iron presents in the resulting mMMT and mDE were investigated by X-ray diffraction and Mössbauer spectroscopy. X-ray diffraction patterns were measured at room temperature in a Siemens D5000 X-ray diffractometer, using CuKα radiation (λ= 1.5406 Å). Mössbauer spectra were recorded at 4.2 K in a transmission geometry using a conventional ^{57}Fe Mössbauer spectrometer employing a 50 mCi ^{57}Co/Rh source. The spectra were analyzed using least squares method assuming Lorenzian line shapes and hyperfine field distribution. The isomer shift (δ) values are relative to α-Fe at room temperature. Magnetization measurements were

performed at 298 K in magnetic fields varying from 0 to 50 kOe (5.0 T) using a SQUID magnetometer (Quantum Design Model MPMS-5S).

2.3. Immobilization process

The magnetic composites were silanized with aminopropyltriethoxysilane (APTES, 2.5% v/v) stirring at 25ºC. The activation of the silanized mMMT and mDE with glutaraldehyde (10% v/v) also was carried out stirring at 25ºC. Functionalized materials were washed several times with distilled water. The invertase from Baker's yeast (1 mL, prepared in 0.2 M sodium acetate buffer, pH 5.0) was incubated with mMMT and mDE (0.01 g) 4 ºC under mild stirring. Afterwards the material was washed five times with 0.2 M sodium acetate buffer, pH 5.0. The invertase immobilized on mMMT (mMMT-invertase) and mDE (mDE-invertase) were collected by the magnetic field and the supernatants including the first two washings were used for protein determination according to Lowry et al.[12] using bovine serum albumin as the standard protein. The derivatives immobilized were stored in sodium acetate buffer at 4 ºC for further use. Invertase activity was determined by using 0.15 M sucrose (10 mL) prepared in sodium acetate buffer (0.2 M, pH 5.0). After exactly 15 min of incubation at 25ºC, 20 µl the sample was withdrawn and added to 2.0 mL of working solution in order to measure released glucose using a glucose oxidase-peroxidase (GOD/POD) enzymatic kit (Doles, Goiás, Brazil). The enzyme activity unit (U) was defined as the amount of enzyme releasing 1 µmol of glucose per minute under the assay conditions.

3. Results and discussion

The XRD patterns of the magnetic particles are presented in Fig.1. The 2θ peaks at 18.44°, 30.32°, 35.75°, 43.32°, 53.89°, 57.34° and 62.96° are attributed to the crystal planes of magnetite at (111), (220), (311), (400), (422), (511) and (440) respectively [13]. The characteristic peaks of magnetite and quartz were observed in all magnetic composites. By analyzing the XRD patterns it is observed that the magnetite is the most predominant crystalline phase in mDE, while that in mMMT the aluminosilicates are the predominant crystalline phase. The mMMT and mDE exhibited broad and low intensity peaks in the base line (Fig.1b and Fig.1c). This broad X-ray structure suggests an amorphous component in the prepared composite [14]. The grain size estimated from the main reflections of each diffractogram, by using the Scherrer formula is shown in Table 1. In this calculus we are not considering possible contributions of crystal stress. The XRD patterns of magnetite and maghemite are very similar. The main difference consists of a few low-intensity diffractions (<5%) which are only present for the maghemite structure [15]. These diffraction lines

are found in the patterns presented in mMMT and mDE (Fig.1b and Fig.1c), but this does not confirm or exclude the presence of maghemite in the composites produced. Furthermore, the fact that the low intensity peaks become visible in the XDR pattern does not prove that the transition from magnetite to maghemite took place; it could simply be due to an increase of the particle size [15]. However, in a more recent report [16], the differentiation between magnetite and maghemite were made on the basis of high angle peaks corresponding to plane (511) and (440) peak-heights and its resolution through the deconvolution.

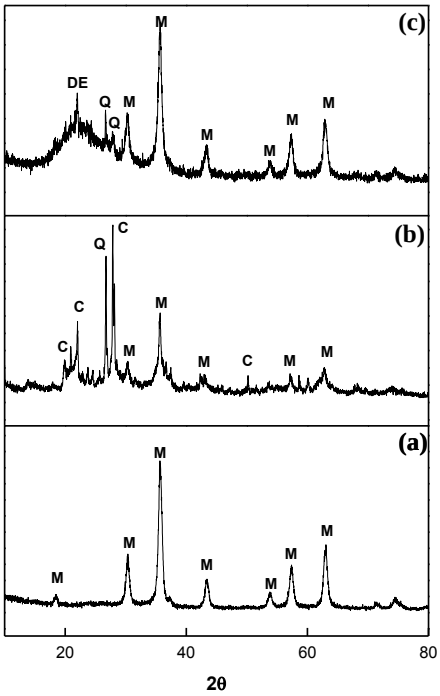

Figure 1. XRD patterns of (a) magnetite, (b) mMMT and (c) mDE.M=magnetite; C=montmorillonite clay; Q=quartz; DE=diatomaceous earth

The hyperfine parameters of the Mössbauer Spectroscopy (MS) at an appropriate temperature can be used to identify the magnetic signal of the iron oxide and to obtain information about the Fe^{3+} linking the components of the material [17]. The measurements were carried out at 4.2 K to check any superparamagnetic state present in the samples. Only sample of pure magnetite may be analyzed at room temperature (300 K). ^{57}Fe Mössbauer spectrum of pure magnetite shows two sextets (Fig. 2a). The first one (A sites) has a hyperfine magnetic field, B= 47.5 T, and an isomer shift, δ= 0.31 mm/s; assigned to Fe^{3+} ions; the second sextet (B sites) has a, B= 43.2 T, and δ= 0.33 mm/s; this sextet corresponds to the mixed Fe^{2+} –Fe^{3+} ions [18]. The line width (Γ) of the second sextet corresponding to B-site is quite high. This could be because of defect in the sample, particle size distribution and presence of different iron environment which leads to intermediate iron oxidation states. Due to high line width the two components in the spectrum are not fully resolved.

As it is also clear from broad hyperfine field distribution (Fig. 2b) that there may be more than two sextets exist in the sample which corresponds to different iron oxidation states or different iron minerals. These values are similar to the bulk material (sextet 1: B= 49.0 T and δ= 0.26 mm/s and sextet 2: B= 46.0 T and δ= 0.67 mm/s) [19], but the δ for second component is significantly lower, this indicate presence of some other iron mineral such as maghemite. The deviation in the ideal area ratio (1:2) of the iron in tetrahedral and octahedral position obtained from the subspectra area is due to the smaller particle size compared to their bulk counterpart [20].

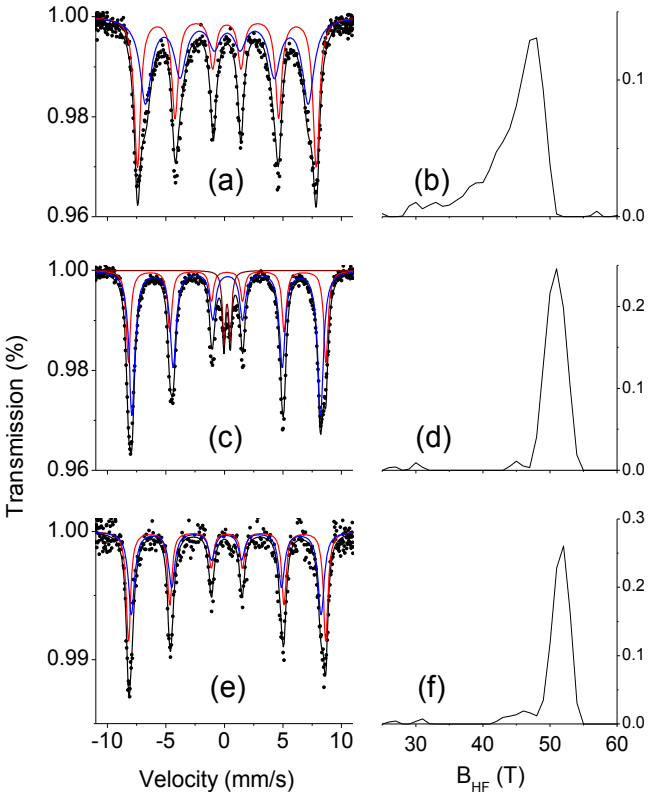

Fig.2. Mössbauer spectra and their corresponding p-B distribution (a) and (b) magnetite at room temperature (c) and (d) mMMT at 4.2 K and (e) and (f) mDE at 4.2 K. Scattered points are data point and the fitted spectrum is shown in black line. The subspectra shown in red and blue lines are the component subspectra corresponding to A-site and B-site iron respectively, whereas in (c) the subspectrum shown in dark line is showing doublet.

The hyperfine magnetic fields for mMMT (sextet 1 equal to 52.3 T and sextet 2 equal to 50.0 T) and mDE (sextet 1 equal to 52.3 T and sextet 2 equal to 50.3 T) showed slightly higher values than pure magnetite (Fig.2c, Fig.2e and Table 1). Addition to the signals relating to magnetite, the Fig.2c (mMMT) also shows a doublet having an isomer shift equal to 0.33 mm/s and an area equal to 7.5%. This doublet emanates from ferric iron in a non-spherical local surrounding, maybe coming from the rim of the iron oxide core, i.e., the magnetic relaxation effect which is attributed to the presence of superparamagnetism as well as the ferromagnetic nanoparticles [20]. The absence of doublet in the Mössbauer spectrum of the mDE suggests that there is no non-magnetic and non-

spherical iron surrounding present in mDE sample. The mMMT and mDE spectra (Fig.2c and Fig.2e) show an increase in the B sites compared to the spectrum for pure magnetite, whereas the intensity of A sites (Fe^{3+}) decreases. Table 1 shows the hyperfine parameters obtained from fitting of Mössbauer spectra. The percentage area reported under the curve of the lines of best fit is related to the composition of the obtained materials. According to these spectra and the hyperfine parameters, it is evident that the mMMT showed a higher increase in the B sites than mDE as compared to the pure magnetite, i.e., the presence of the clay caused more modifications in the magnetite produced relative to diatomaceous earth.

Table 1. Mössbauer parameters. The isomer shift (δ); Quadrupole splitting (Δ) and Line width (Γ) is 0.02 mm/s while that in hyperfine field (B) is 0.5 T; Areas are accurate within 2%.

Sample	Grain size XRD (nm)[a]	Component	δ (mm/s)	Δ (mm/s)	Γ (mm/s)	B (T)	Area %
Magnetite (RT=300K)	11	Sextet 1	0.31	-0.01	0.65	47.5	49.0
		Sextet 2	0.33	-0.03	1.20	43.2	51.0
mMMT (4.2K)	25	Sextet 1	0.33	0.01	0.45	52.3	30.0
		Sextet 2	0.38	-0.12	0.60	50.0	62.5
		Doublet	0.33	0.53	0.30	-------	7.5
mDE (4.2K)	12	Sextet 1	0.33	0.01	0.47	52.3	49.5
		Sextet 2	0.28	-0.03	0.64	50.3	50.5

[a]Uncertainty in particle size is 0.5 nm.

The magnetic properties of magnetite, mMMT and mDE particles were measured by applying an external magnetic field at 298 K. The saturation magnetization for magnetite, mMMT and mDE was determined by the magnetization curve at maximum magnetic field. As shown in Fig. 3, the saturation magnetization of mMMT and mDE was around 10 emu g^{-1} lower than the value of 60 emu g^{-1} found for the magnetite particles at 298 K. The decreased saturation magnetization can be attributed to surface effects, such as magnetically inactive layer producing disordered surface [21]. In addition, the magnetite, mMMT and mDE particles exhibited superparamagnetic behavior.

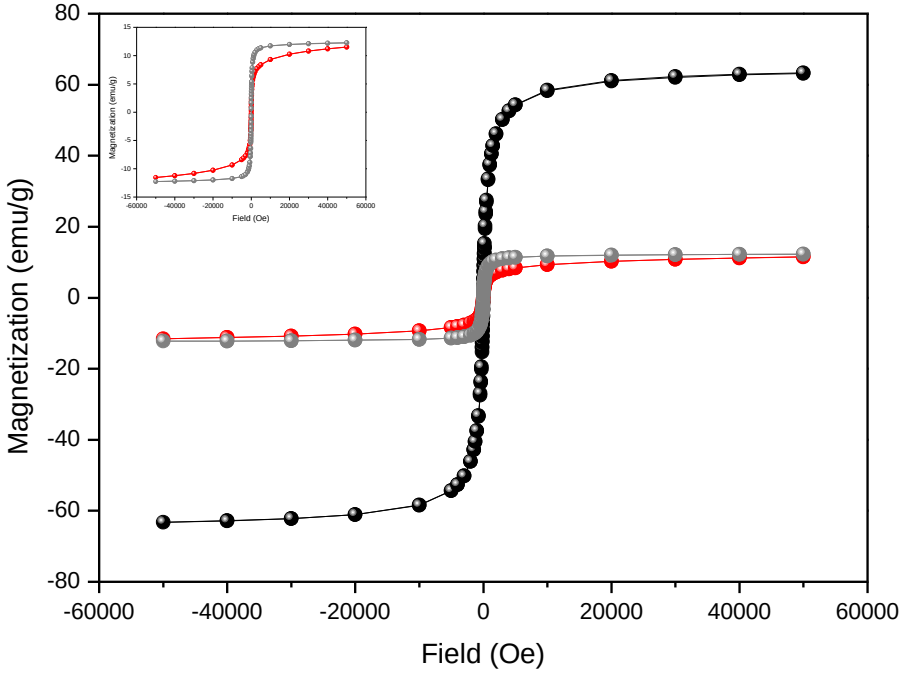

Fig.3. Magnetization measurements for the magnetite (black), mMMT (red) and mDE (gray). The inset shows a magnified view of the magnetization curves of the mMMT and mDE.

Important parameters in the immobilization process such as reaction conditions of enzyme, solid support and linker determine the biochemical, mechanical and kinetic properties of the immobilized enzyme. The magnetic composites proposed as matrices for the immobilization of invertase via covalent binding showed excellent results. Thus, the residual specific activity of mMMT-invertase and mDE-invertase was 83% and 92.5%, respectively. No decrease in specific activity was observed, suggesting the potential application of these magnetic composites from minerals of low cost as matrices for the immobilization of invertase or other enzymes.

4. Conclusion

The synthesis of magnetic composites from two mineral of low cost as well as the study of the iron phases in mMMT and mDE was successfully performed. X-ray diffraction measurement of the mMMT and mDE exhibited similar peak compared to those of magnetite and showed that the montmorillonite clay and diatomaceous earth minerals does not significantly interfere with the structure of synthesized magnetite. Through Mössbauer spectra we observed that the nanoparticles of mMMT are composed by a mixture of magnetite and maghemite whereas the mDE showed a pure magnetite phase. All magnetic particles displayed a superparamagnetic behavior in agreement with particles size distribution at the nano scale. The residual specific activity of the mMMT-invertase and mDE-invertase were 83% and 92.5%, respectively. Finally, these results suggest that

the mMMT and mDE will be a promising matrices for covalent immobilization of invertase and could be used for the immobilization or purification the other enzymes of industrial interest for biotechnological applications.

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Capítulo 4

5.2. Artigo a ser submetido ao periódico *Journal of Magnetism and Magnetic Materials*

Título: Magnetic raw montmorillonite as matrix for protein immobilization

Autores: Mariana Cabrera, Eduardo H.L. Falcão, Fernando Soria, David F.M. Neri, Luiz B. Carvalho Jr.

Magnetic raw montmorillonite as matrix for protein immobilization

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Abstract

A magnetic composite was synthetized from the raw montmorillonite clay and magnetite. The montmorillonite-magnetite composite was characterized by particle size analysis, XRD, FTIR, surface area measurements, SEM and magnetization measurements. The results indicate that the magnetite particles were deposited on the flat surface of clay. The montmorillonite-magnetite composite showed superparamagnetic behavior and mesoporous. The functionalized material with aminopropyltriethoxysilane and glutaraldehyde was used as matrix for the covalent protein immobilization using invertase as model. The optimal pH for both free and immobilized invertase was 5.0. The optimum temperature for free and immobilized enzymes was 45ºC and 55ºC, respectively. The K_m value was 4-fold higher than that found for the free enzyme. The effectiveness factor was equal to 0.83 whereas the catalytic efficiency decreased around 4.8-fold upon immobilization. The immobilized derivative presented higher thermal stability (70% residual activity at 60°C) and was reused for seven continuous cycles keeping around 91 % of activity. The success of immobilizing invertase on magnetic composite allows proposing this matrix and immobilization procedure to be used for other enzymes or biomolecules.

Keywords: magnetic particle; raw clay; immobilization; invertase; sucrose hydrolysis.

1. Introduction

In recent years, the production of magnetic materials have had an increased attention for several uses in many biological fields, including bioseparation [1], tumor hyperthermia [2], magnetic resonance imaging (MRI) diagnostic contrast agents [3], magnetic drug delivery [4]and biomolecules immobilization [5-11]. Insoluble supports for the immobilization of biomolecules are an important tool for the fabrication of a diverse range of functional materials and these will have a particular interest if presents magnetic property. The magnetite ($FeO.Fe_2O_3$ or Fe_3O_4) is one of the most important iron oxides used as matrix for the study of enzyme immobilization. These magnetic particles offers the desired magnetic properties to the ensembles formed with the specie to be separated, and they can have surface properties which enable a selective separation [12]. Their main advantage is easy removal of the reaction mixture by an external magnetic field. Furthermore, the use of them can reduce the capital and operational costs [13].

On the other hand, clays minerals have several applications due to their interesting properties [14]. The surface modification of clays is an area that has received considerable attention from researchers because by means of various types of modification is possible to prepare new materials with interesting applications. Montmorillonite belongs to the smectite clays, which possess a sandwich structure of tetrahedral–octahedral–tetrahedral aluminosilicate lamellas formed by condensation of an octahedral Al_2O_3 (or MgO) between two tetrahedral SiO_2 layers [15]. Several enzymes have been immobilized on montmorillonite K-10, clay commercially available in the acid activated form [16-19]. However, to the best of our knowledge invertase has not been immobilized on montmorillonite-magnetite composite yet. The rigid structure, high mechanical strength, hydrophilic character, great number of reactive hydroxyl groups, appreciable surface area and low cost of the raw montmorillonite together with the magnetic property of magnetite allow proposing a montmorillonite-magnetite composite as matrix for the covalent protein immobilization using invertase as model. This enzyme is mainly used to hydrolyze sucrose in the production of glucose and fructose (invert syrup) and these latter monosaccharides have lower crystallinity than sucrose at higher concentrations [16].

In the present study, montmorillonite-magnetite composite (mMMT)) was prepared and characterized by size distribution, X-ray diffraction (XRD), scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, surface area and porosity, and magnetization measurements. For the immobilization process, the mMMT was functionalized with aminopropyltriethoxysilane and glutaraldehyde. Some physicochemical properties of the immobilized derivative were investigated and thermal stability and reuse were also performed. Up

to now, magnetic raw montmorillonite (mMMT) as matrix for invertase immobilization was never reported in the literature previously.

2. Experimental

2.1. Materials

Montmorillonite (MMT) sample was a gift from Minarmco S.A. (Neuquén, Argentina). Invertase from Baker's yeast was obtained from NOVO-Nordisk (Denmark). The aminopropyltriethoxysilane (APTES), glutaraldehyde, 3,5-dinitro salicylic acid (DNS) and bovine serum albumin were purchased from Sigma Aldrich Chemicals (St. Louis, USA). All other chemicals were of high purity available commercially.

2.2. Synthesis and functionalization of mMMT

Firstly, clay mineral was concentrated by a simple process of sedimentation. This way, a process of water washing and repeated sedimentation was applied to eliminate bigger particles than the clay mineral. The magnetization process for the montmorillonite (MMT) was performed according to Amaral et al. [20]. The magnetic clay (mMMT) obtained was washed with distilled water until pH 7.0 and recovered by a magnetic field (Ciba Corning; 0.6 T). The mMMT was dried at 50 ºC overnight and then sieved. The functionalization of mMMT with aminopropyltriethoxysilane (APTES) and glutaraldehyde was performed according to Sanjay and Sugunan [16]. Functionalized mMMT was washed several times with distilled water and 0.2 M sodium acetate buffer, pH 5.0 until the washings became colorless. The treated particles were recovered using magnetic field (0.6 T).

2.3. Characterization

The size and size distribution of both MMT and mMMT were determined with a Microtrac S3500 particle size analyzer. The qualitative mineralogical analysis of MMT and mMMT was performed using a Siemens D5000 X-ray diffractometer. Representative powder samples were analyzed at a range of $2^o<2\theta<50^o$ by using CuKα radiation (λ= 1.5406 Å) in steps of 0.02º and with a counting time of 1.0 s per step. The morphological characterization of MMT, mMMT and invertase immobilized on mMMT (mMMT-invertase) was carried out by using a scanning electron microscope (SEM, FEI Model QUANTA 200 FEG) equipped with energy dispersive spectroscopy (EDS). The samples were coated with gold prior to analysis. The identification of the chemical elements present in the material was performed by EDS. Surface area and porosity were determined

for all materials with a Micromeritics ASAP 2420 porosimeter. The isotherms were obtained at 77 K using N_2 as an adsorbate. The specific surface area was calculated using the Brunauer-Emmett-Teller (BET) model. Pore size distribution and pore volume were determined from the desorption branch of the isotherms using the Barrett-Joyner-Halenda (BJH)-plot method. FTIR spectra of magnetite and mMMT in the range of 4000-400 cm^{-1} were recorded in a BRUKER instrument model IFS 66. The samples were pressed into pellets with KBr. Magnetization measurements were performed at 298 K in magnetic fields from 0 to 50 KOe (5.0 T) using a SQUID magnetometer (Quantum Design Model MPMS-5S).

2.4. Immobilization process

Invertase (1 mL containing 0.15 mg protein prepared in 0.2 M sodium acetate buffer, pH 5.0) was incubated with mMMT (0.01 g) for 12 h at 4ºC under mild stirring. Afterwards the material was washed five times with 0.2 M sodium acetate buffer, pH 5.0. The magnetic particles (mMMT-invertase) were collected by the magnetic field and the supernatants including the first two washings were used for protein determination according to Lowry et al. [21] using bovine serum albumin as standard. The amount of immobilized protein was calculated by the difference between the amount of offered protein and that one found in the supernatants and washings. The immobilized enzyme was stored in sodium acetate buffer at 4 ºC for further use. The pH and time of immobilization were also investigated in order to study what happens in the immobilization process to prevent enzyme inactivation at inappropriate pH and longer reaction time. For this, invertase solutions were prepared in different buffers (pH 4.0 - 5.5, 0.2 M, sodium acetate buffer and pH 5.7 - 7.0, 0.2M, sodium phosphate buffer). Immobilization time was set up according to the time variation in the procedure of the immobilization (0.5 to 12 h). The activity of invertase was determined by using 4 mL of 0.2 M sucrose solution prepared in sodium acetate buffer (0.2 M, pH 5.0). The reducing sugars produced by sucrose hydrolysis were measured by the DNS method according to Miller [22]. One unit of enzyme (U) was defined as the amount of enzyme that hydrolyzed one µmol of sucrose per minute at pH 5.0 and 55ºC.

2.5. Characterization of free and immobilized enzyme

The kinetic parameters, Michaelis-Menten constant and the maximum velocity values for the free and immobilized invertase were determined by measuring initial rates of the reaction with sucrose (3.5-200 mM) prepared in 0.2 M sodium acetate buffer, pH 5.0. K_m and V_{max} values were determined by non-linear regressions, using the PRISM software of GraphPad, USA.

The optimum pH and reaction temperature of free and immobilized invertase were determined using 0.2 M sucrose under a variety of pH (0.2 M sodium acetate buffer for pH 4.0-5.5 and 0.2 M sodium phosphate buffer for pH 5.7-7.0) and temperature (25 to 65ºC).

2.6. Thermal stability and reusability

The thermal stability of the free and immobilized invertase was carried out by measuring the residual activity of the enzyme pre-incubated at different temperatures (25 to 100 ºC) in sodium acetate buffer (0.2 M, pH 5.0) for 30 minutes. After cooling (25^{o}C) the enzymatic activity was determined as above described.

In order to investigate the reusability of immobilized invertase, the activity of the same preparation under batch operation mode was carried out in 0.2 M sucrose at 55 ºC seven times at 30 minutes intervals. After each activity cycle the immobilized derivative was magnetically collected and washed several times with 0.2 M sodium acetate buffer, pH 5.0.

2.7. Statistical analyses

All experiments were performed in triplicate and the standard errors of the mean were evaluated. The level of significance was set at $p<0.05$.

3. Results and discussion

3.1. Characterization of MMT and mMMT particles

The MMT particle size has been reported in the range from 0.1 to 2 μm with an average particle diameter around 0.5 μm [23] whereas this value for the mMMT was found to be 5.6 μm. This increase of the magnetic particles size can be attributed to the formation of heteroaggregates of clay and magnetite particles [24].

XRD patterns of montmorillonite (MMT) and montmorillonite-magnetite composite (mMMT) particles are shown in Fig. 1. The XRD analysis showed the characteristic peaks of MMT as well as quartz as a minor phase (Fig.1A). Similar peaks were observed for the mMMT (Fig. 1B) except that peaks corresponding to magnetite were also visible.

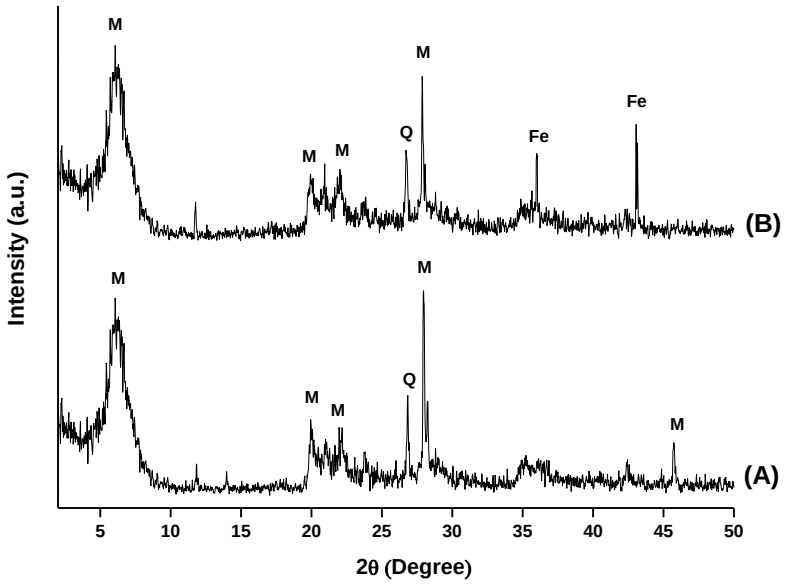

Figure. 1. X-ray diffraction of MMT (A) and mMMT (B). M=MMT; Q=quartz and Fe= magnetite.

Figure 2 shows SEM micrographs and corresponding EDS analyses of the MMT, mMMT and mMMT-invertase. The morphology of the MMT particles was irregular and showed a typical sheet like structure. The EDS spectrum for the MMT (Fig. 2A) showed the expected peaks of Si, Al and O, and other elements (Na, K, Mg, Ca, Fe). The mMMT showed a different texture in which the layers seemed more irregular (Fig. 2B). Its EDS spectrum displayed an increase in the signal corresponding to Fe supporting the presence of magnetite on the mMMT. The addition of invertase on mMMT surface changed the morphology of this material (Fig. 2C). Probably, this surface alteration can be attributed to the highly polymeric material of the enzyme covering the magnetic particles. This finding has been reported by Sanjay and Sugunan [19] that worked with immobilized enzymes on montmorillonite K-10. It is worthwhile to register the presence of S in the EDS of mMMT-invertase (Fig. 2C).

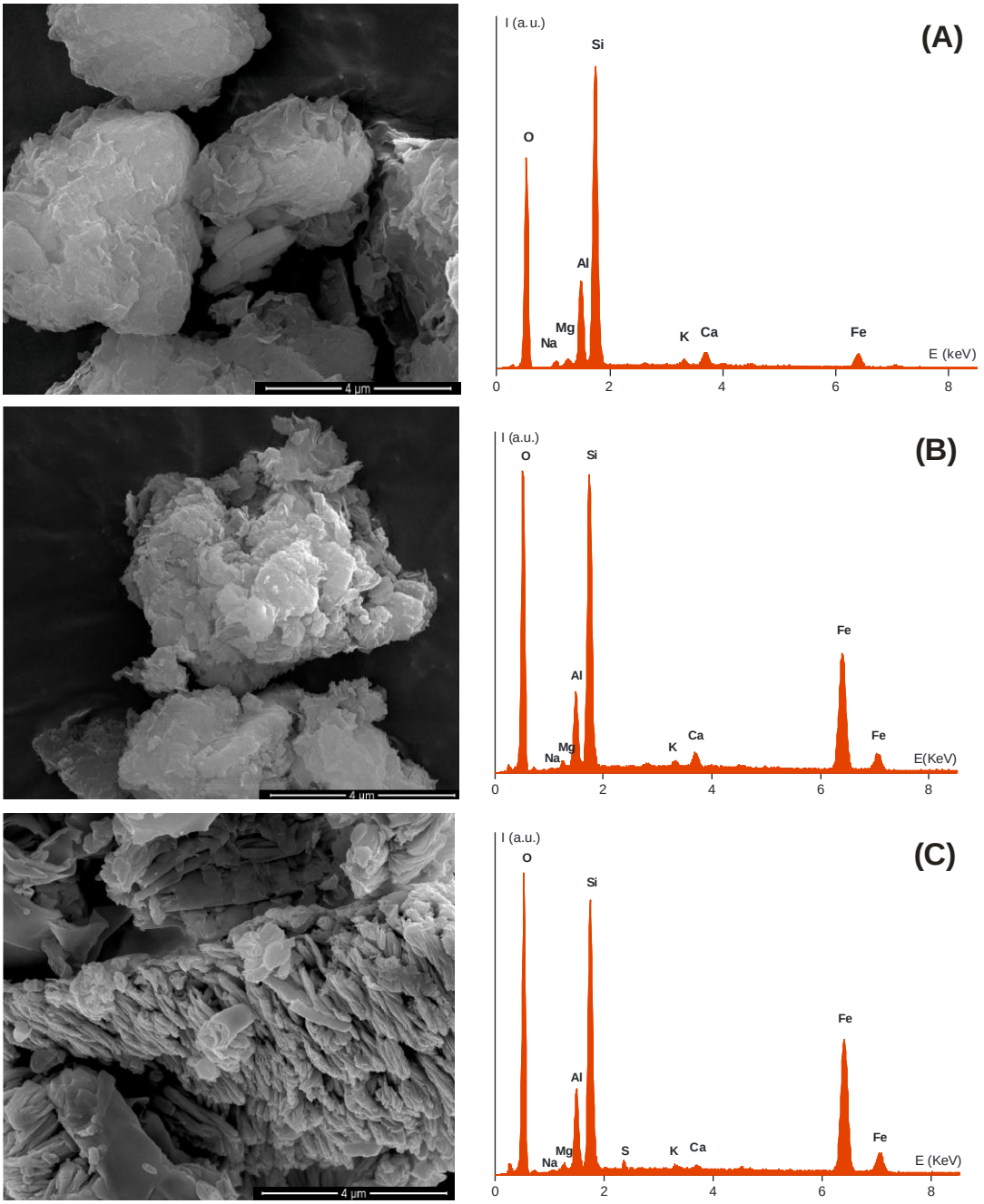

Figure 2. Scanning electron micrographs and corresponding EDS analyses of MMT (A), mMMT (B) and mMMT-invertase (C).

The FTIR spectra of magnetite and mMMT are shown in Fig.3. The magnetite spectrum exhibited absorption bands at around 630 and 583 cm^{-1}characteristic of the Fe-O bond [25]. The bands near 3421 and 1638 cm^{-1} are ascribed to the hydroxyl characteristic peaks of water adsorbed on the surface or the OH-streching bands and its bending vibration peak [26]. The mMMT spectrum presents an absorption band at around 1041 cm^{-1} for the Si-O-Si stretching vibrations. The band around 3631 cm^{-1} of Mg-OH-Al bond is typical of MMT with high content of Al on octahedral

positions [27]. After magnetization the absorption bands of Fe-O bond around 523 and 467 cm^{-1} were also observed, which supported the presence of magnetite particles.

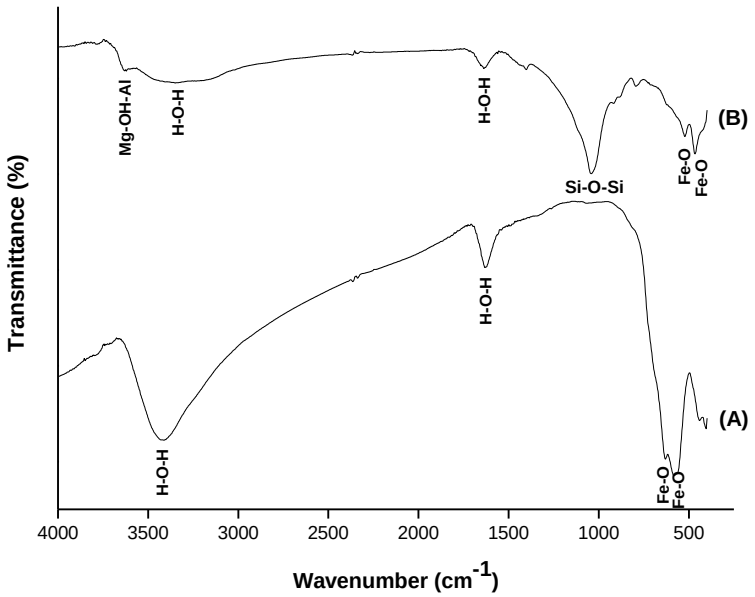

Figure 3. FTIR spectra of magnetite (A) and mMMT (B).

The magnetic properties of magnetite and mMMT particles were measured by applying an external magnetic field at 298 K. The saturation magnetization for magnetite and mMMT was determined by the magnetization curve at maximum magnetic field. As shown in Fig. 4, the saturation magnetization of mMMT was around 10 emu g^{-1} lower than the value of 60 emu g^{-1} found for the magnetite particles at 298 K. The decreased saturation magnetization can be attributed to surface effects, such as magnetically inactive layer producing disordered surface [28]. In addition, there is no hysteresis in the magnetization with both remanence and coercivity being zero, suggesting that the mMMT particles exhibited superparamagnetic behavior.

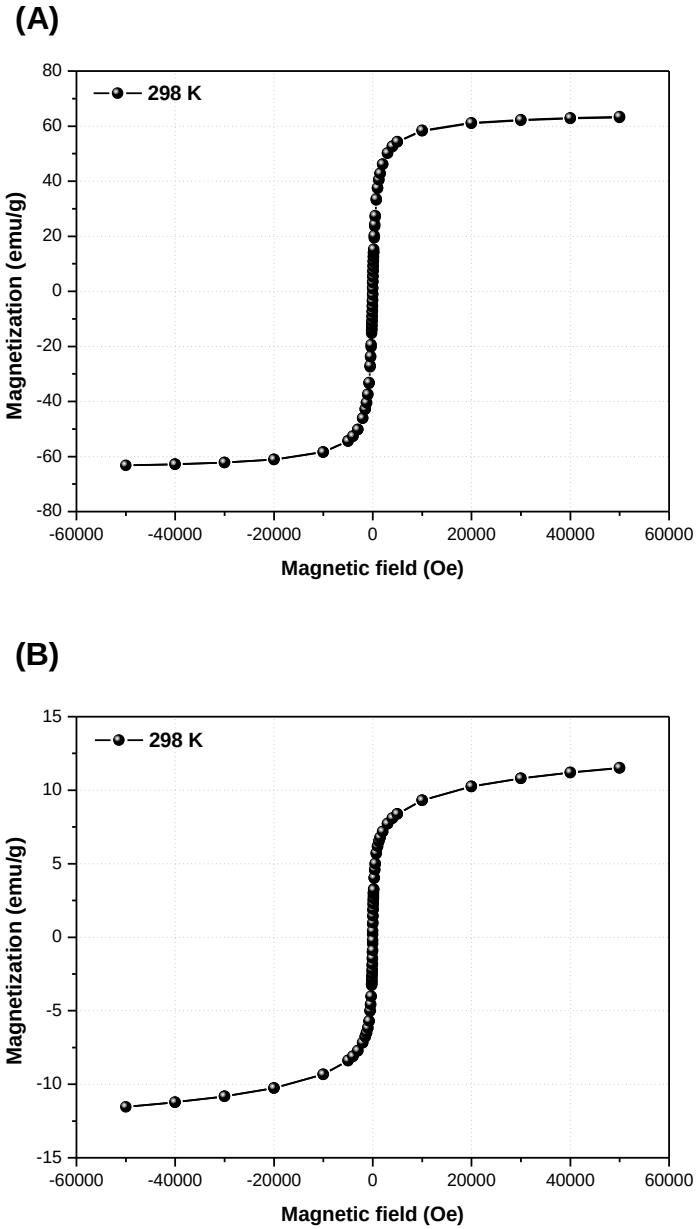

Figure 4. Magnetization curves of magnetite (A) and mMMT (B) at 298 K.

The surface area, pore volume and pore size of MMT, mMMT and mMMT-invertase are presented in Table 1. As a result of the magnetization process, the mMMT presented a higher surface area and pore volume compared to MMT. This increase can be explained because the magnetite particles were deposited on the flat surface of clay. A maximum value of S_{BET} of 116.3 m^2 g^{-1} was found after the immobilization process indicating that enzyme molecules are located around the sheets of clay and not between them. Sanjay and Sugunan [16] showed that the functionalization with APTES and glutaraldehyde takes place within the clay layers but the whole enzyme molecules are not intercalated. They reported that the polypeptide backbone is situated at the periphery of the clay and does not enter the interlayer space. The values of pore size for MMT, mMMT and mMMT-invertase are in the range of mesoporous solid (between 2-50 nm) according to IUPAC. The pores

in mMMT were slit shaped openings as depicted by the type of the adsorption-desorption isotherms and the characteristic hysteresis loops [29]. The decrease in pore size with the magnetization and immobilization process may be due to the elimination of bigger pores.

Table 1. Surface area, pore volume and pore size of MMT, mMMT and mMMT-invertase.

Sample	S_{BET} ($m^2\ g^{-1}$)	Pore Volume ($cm^3\ g^{-1}$)	Pore Size (nm)
MMT	65.1	0.10	7.9
mMMT	106.6	0.18	6.2
mMMT-invertase	116.3	0.19	5.2

3.2. Immobilization of enzyme

The invertase is one of the most studied enzymes and has been immobilized for different methods and supports as reported by Kotwal and Shankar [30]. In this work, we have proposed a magnetic composite from the raw montmorillonite clay as matrix for the immobilization of invertase or other biomolecules as well as general study of immobilization process and characterization of immobilized derivative. The effect of pH on immobilization process can be observed in Fig. 5A that shows the maximum immobilization efficiency at pH 4.0 achieving a value of 5.0 mg protein g^{-1} mMMT. However, the retained activity was 10 times lower than that observed at pH 5.0. At this value was observed a maximum activity and therefore this pH was chosen for the immobilization process. The immobilization time course (Fig 5B) showed that either retained protein or enzyme activity firstly increases and decreases afterwards. Therefore, the time of 1h is more advantageous than 12 h due to the decrease of immobilization efficiency (from 4.7 to 2.6 mg protein g^{-1} mMMT) and the recovered activity (from 100 to 48.4 %). The decrease in the immobilized enzyme activity can be due to the multipoint attachment of the enzyme molecules on mMMT particles and/or overloading of immobilized enzyme. Kumar et al. [31] reported that 45 minutes was enough for the invertase covalently immobilization on PVC. Immobilization time for immobilized invertase has been usually reported to be 12-24 h [32, 33].

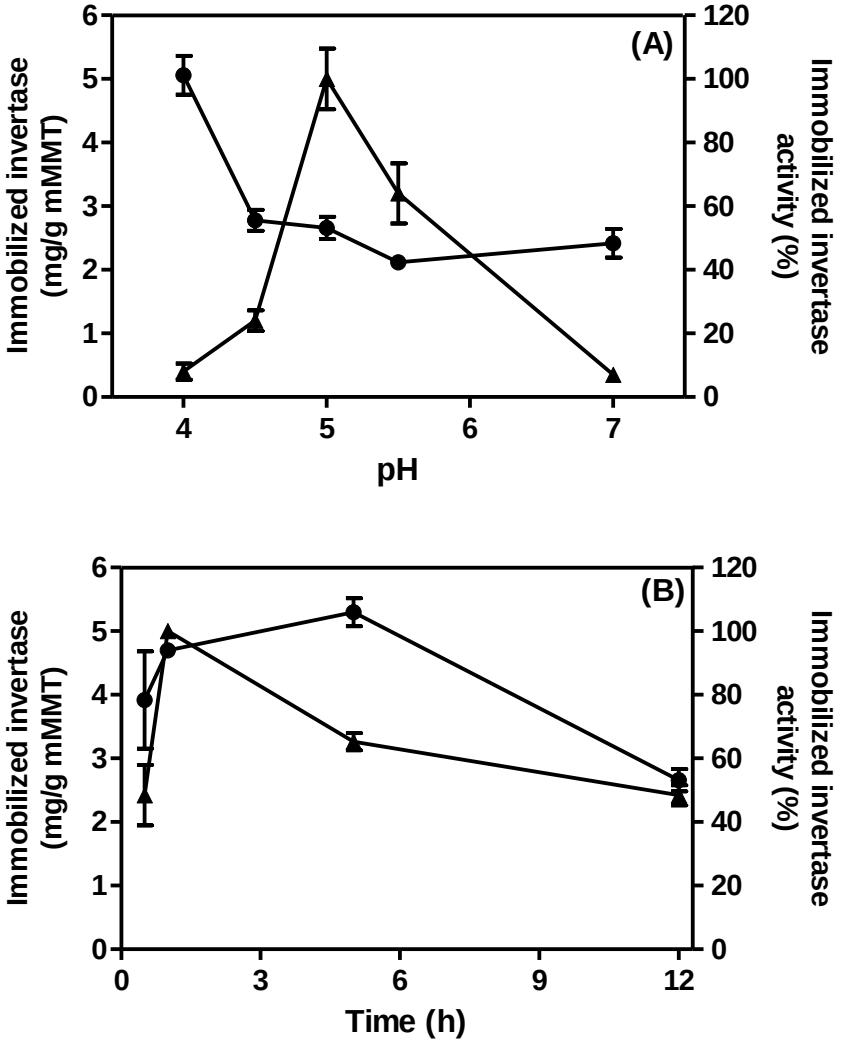

Figure 5. Effect of pH (A) and time (B) on the efficiency (●) and recovered activity (▲) of the immobilized invertase on mMMT. The experimental immobilization temperature and invertase concentration were 4ºC and of 0.15 mg/mL, respectively.

3.3. Effect of pH and temperature on activity

The influence of the pH on the activity of the free and immobilized invertase for sucrose hydrolysis was examined in the pH range from 4.0 to 7.0 at 55ºC (Table 2). The optimum pH-activity was found to be 5.0 for both enzymes. The mMMT presents isoeletric point at this pH [34]. Therefore, this matrix does not present charge to affect the microenvironmental pH. The same pH was already observed for the free and immobilized invertase on montmorrilonite K-10 [18]. Effect of temperature on the activity of free and immobilized invertase was performed varying the temperature from 25 to 65ºC at pH 5.0 (Table 2). The immobilized enzyme showed the maximum temperature at 55ºC whereas the free enzyme was 45ºC. An increase at 10°C in the optimum temperature for the immobilized invertase was also observed [35-38].

3.4. Kinetic parameters

The K_m values were calculated as 40.0 and 160.7 mM for free and immobilized enzyme, respectively (Table 2). The higher K_m value for the immobilized derivative (4-fold higher) suggests that the substrate has restricted access to the enzyme active site either to diffusional and mass transfer limitations or to steric hindrance. Sanjay and Sugunan [16] reported for immobilized invertase on montmorillonite K-10 the K_m value near 7-fold higher than that one obtained for free enzyme. Others increases in the K_m values for immobilized invertase were also reported [39, 40]. The effectiveness factor (η) calculated from the maximum reaction rates of the immobilized enzyme over that of free enzyme was equal to 0.83. The catalytic efficiency (V_{max}/K_m) of invertase decreased about 4.8-fold upon immobilization (Table 2). Effectiveness factor and catalytic efficiency for the immobilized invertase on montmorillonite K-10 were, respectively, 0.36 and 22.1 [18].

Table 2. Properties and kinetic parameters of free and immobilized enzyme on mMMT.

	pH	T (ºC)	K_m (mM)	V_{max} (U mg^{-1}enzyme)	Effectiveness factor (η)	Catalytic efficiency (V_{max}/K_m)
Free enzyme	5.0	45	40.0	150.4	-	3.76
Immobilized enzyme	5.0	55	160.7	124.5	0.83	0.77

3.5. Thermal stability of the enzyme

The thermal stability of the immobilized invertase is one of the most important criteria with respect to applications. For this reason the thermal stability of free and immobilized invertase was determined by incubating them in buffer at different temperatures (25 to 100 ºC) during 30 minutes. Residual activity of invertase was calculated with respect to its initial activity. For both preparations of enzymes was observed a slight decrease on residual activity until 50°C (Fig. 6). However, immobilized invertase due to restricted conformational mobility showed better thermal stability than the free enzyme. The free and immobilized enzyme at 50 ºC retained around of 94% of its initial activity, but at 60 ºC lost 70% and 40% of initial activity, respectively. The mMMT showed to be a successful matrix in reducing the rate of thermal inactivation of invertase. Results similar were presented by others authors: the activity of immobilized invertase on rice husk dropped to 69% after 30 minutes at 60 ºC [32]; immobilized invertase on nylon-6 microbeads at 50 ºC preserved 80% of their activity, but at higher temperatures, their activity decayed more than 60% in 10 minutes [41].

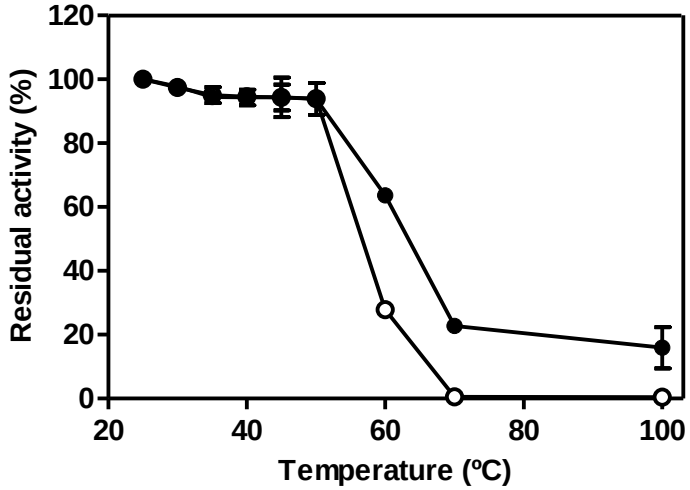

Figure 6. Thermal stability of the free (○) and immobilized invertase (●) on mMMT.

3.6. Reuse of the enzymatic derivative

The main advantage of immobilization is the repeated use of the enzyme. The catalyst reusability was carried out to determine the operational stability of the immobilized invertase on mMMT particles. The invertase immobilized showed high stability when it was repeatedly used for sucrose hydrolysis under batch operation mode (Fig. 7). The data shows that the immobilized derivative retained around 91 % of its initial activity after seven cycles of reuse. Similar results were observed by Emregul et al[40], Cirpan et al [42] and Bagal and Karve [43]. A high operational stability could significantly reduce the costs in practical applications.

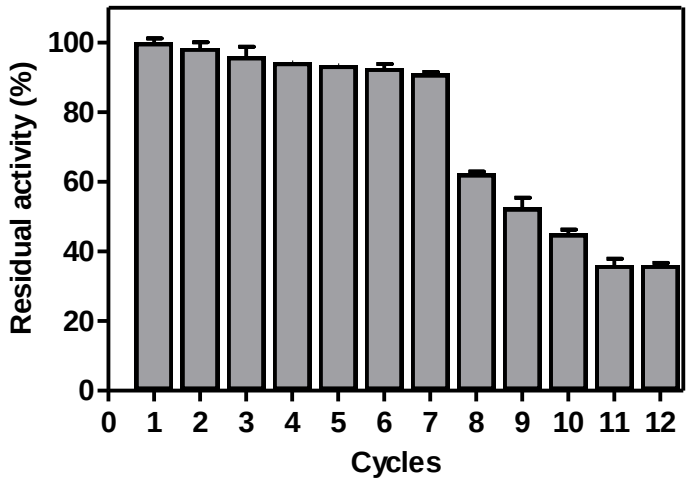

Figure 7. Reusability of immobilized invertase on mMMT.

4. Conclusion

In this study, magnetic composite from the raw montmorillonite clay was prepared, characterized and used as matrix for the covalent protein immobilization using invertase as model. The presence of magnetite added on the MMT particles was supported by XRD, MEV-EDS and FTIR analysis. The mMMT particles showed superparamagnetic behavior and mesoporous. The MMT particles after magnetization showed a different texture in which the layers seemed more irregular. Furthermore, the addition of invertase on mMMT surface changed the morphology of this material. The experimental conditions, such as pH (5.0) and time (1 h), for invertase immobilization were optimized. The immobilized invertase on mMMT presented the same optimum pH and higher maximum temperature and thermal stability compared to the free enzyme. The K_m value was 4-fold higher than that found for the free enzyme. The effectiveness factor was equal 0.83 whereas the catalytic efficiency decreased about 4.8-fold upon immobilization. In addition, the immobilized derivative retained around 91 % of its initial activity after seven cycles of reuse. It can be concluded that the possibility of an efficient reuse of the invertase makes this system attractive from the view point of practical application. Finally, these results suggest that the mMMT particles showed to be a promising matrix for covalent immobilization of invertase or other biomolecules. Thereby, we propose a simple immobilization protocol based in the low time for immobilization and mainly cheap due to using of raw montmorillonite clay, a mineral highly available in nature.

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Capítulo 5

5.3. Artigo a ser submetido ao periódico *Journal of Molecular Catalysis B: Enzymatic*

Título: Preparation and characterization of magnetite-modified diatomite as support for protein imobilization

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Preparation and characterization of magnetite-modified diatomite as support for protein immobilization

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Abstract

A raw diatomite was magnetized by co-precipitation method and this material was proposed as matrix for the covalent protein immobilization using invertase as model. The characterization of support and study of immobilization process were carried out. Magnetic diatomite showed a superparamagnetic behavior and characteristic of a mesoporous solid. A 2^{7-2}_{IV} fractional factorial design was employed to evaluate the effects of the most important variables on the immobilized protein (%) and enzymatic activity in the immobilization process. As result of the analysis of both responses, the operational conditions chosen for the immobilization process were: aminopropyltriethoxysilane concentration (2.5%), contact time aminopropyltriethoxysilane (2 h), glutaraldehyde concentration (10%), contact time glutaraldehyde (1 h), immobilization time (12 h), immobilization pH (5.5) and invertase concentration (0.15 mg/mL). In these conditions, the immobilized derivative presented the highest residual specific activity of 92.5%. Since the synthesis of magnetic diatomite and immobilization process are simple, this material proved to be an attractive and efficient matrix for invertase immobilization and could be used for other biomolecules of industrial interest.

Keywords: Diatomite; Magnetic particle; Immobilization; Invertase; Invert syrup

1. Introduction

Heterogeneous biocatalysts prepared by immobilization of enzymatic active substances on water-insoluble supports form the basis for development of novel modern bioprocesses. The immobilization process is one way to extend the operational stability, and thus decrease the effective cost of the enzyme. There are many supports for enzyme immobilization but the great interest in inorganic supports is due to good operational characteristics and relatively low cost, further if the support is natural. Diatomaceous earth (DE) or diatomite is naturally occurring clay from geological deposits composed predominantly of the fossilized skeletons of diatoms. The diatoms in turn are one type of algae which adsorb silica from water, metabolize and deposit it as an external skeleton. Therefore, these plants are an extremely abundant and inexpensive source of silica [1] . DE typically consists of 87-91% silicon dioxide (SiO_2), with significant quantities of alumina (Al_2O_3) and ferric oxide (Fe_2O_3) [2]. Due to its specific properties such as porous structure, high silica content, low density, high specific surface area, low conductivity coefficient, chemically inert in most liquids and gases, and a high fusion point, the diatomite are of great industrial importance. DE has numerous applications as filter aid, adsorbent, catalyst support or carrier, natural insecticide or grain protectant, to removing of dye in the effluent [2, 3].

The magnetic particles have drawn increasing interest in biotechnology and are used as matrix for the study of enzyme immobilization. These particles as support fulfill two functions in that they contain a magnetic material which confers the desired magnetic properties to the ensembles formed with the species to be separated and they can have surface properties which enable a selective separation [4]. Magnetic bio-separation technology is a promising strategy for recovering the immobilized enzyme on magnetic particles using an external magnetic field for recycled use. So, in our research group, recently have been published papers that used magnetic particles as matrix for the enzyme immobilization [5-7].

The invertase (EC 3.2.1.26) plays a catalytic role in the conversion of sucrose into glucose and fructose (invert syrup). This syrup is used to a great extent in the food industry. Invertase is one of the most studied enzymes and has been immobilized for different methods and supports as reported by Kotwal and Shankar [8]. On the other hand, few papers were published using silica particles as support for invertase immobilization or other enzymes. Bergamasco [9] and Mansour [10] reported immobilized invertase on silica particles by covalent binding and absorption, respectively. Meunier and Legge [11, 12], Tomin [13] and Koszelewski [14] reported immobilized lipase on diatomaceous earth (Celite) by sol-gel entrapment, and Cabana [15] immobilized laccase on Celite by covalent binding. However, a systematic investigation of the using magnetic diatomaceous earth particles for the invertase immobilization or other biomolecules by covalent binding is not available in literature

yet. Therefore, the objective of the present work was to propose and assess the magnetic diatomaceous earth (mDE), from raw DE as matrix for the enzyme immobilization using the invertase as enzyme model. Invertase immobilized on magnetic diatomaceous earth particles (mDE-invertase) can be used in the analytical field for the construction of the enzyme reactors for hydrolysis of sucrose. The mDE was characterized by particle size analysis, X-ray diffraction (XRD), Fourier transform infrared (FTIR), surface area measurements, scanning electron microscopy (SEM) and magnetization measurements. The immobilization process of the invertase on mDE particles was studied using a 2^{7-2}_{IV} fractional factorial design to screening of the most important variables, and to better understand the relationships between the immobilization variables and the responses (immobilized protein and enzymatic activity). All the results obtained in this study would provide a sound basis for further exploration.

2. Materials and methods

2.1. Materials

Diatomaceous earth (DE) was kindly supplied by TAMER S.A. (Salta, Argentina). A process of water washing and repeated sedimentation was applied to purify the raw DE. Invertase from Baker's yeast, aminopropyltriethoxysilane (APTES), glutaraldehyde and bovine serum albumin were purchased from Sigma Aldrich Chemicals (St. Louis, USA). All other chemicals were of high purity available commercially.

2.2. Diatomaceous earth magnetization

The synthesis of magnetic DE was performed according to Amaral et al. [16] with the next modifications: (a) incubation temperature of DE with $FeCl_3.6H_2O/FeCl_2.4H_2O$ by 30 minutes was extended from 80ºC to 100ºC; (b) final pH magnetization was 11.0 adjusted with ammonium hydroxide (7.6 M). The magnetic diatomaceous earth (mDE) obtained was washed with distilled water until pH 7.0 and recovered by a magnetic field (Ciba Corning; 0.6 T). The mDE was dried at 50 ºC overnight and then sieved (< 250 μm).

2.3. Diatomaceous earth functionalization and immobilization process

The functionalization of mDE with APTES and glutaraldehyde, and immobilization process were performed by a study design of experiments (DOE). For the silanization process the mDE particles (0.10 g) were submerged in APTES (2 mL, prepared in acetone) stirring at 25ºC. The activation of the silanized mDE (0.01 g) with glutaraldehyde (2 mL, prepared in 0.2 M sodium acetate buffer, pH 5.5) also was carried out stirring at 25ºC. Functionalized mDE was washed several times with

distilled water and 0.2 M sodium acetate buffer, pH 5.0 until the washings became colorless. The treated particles were recovered using magnetic field (0.6 T). For the immobilization process, invertase (1 mL, prepared in 0.2 M sodium acetate buffer, pH 5.0) was incubated with mDE (0.01 g) 4ºC under mild stirring. Afterwards the material was washed five times with 0.2 M sodium acetate buffer, pH 5.0. The invertase immobilized on mDE (mDE-invertase) was collected by the magnetic field and the supernatants including the first two washings were used for protein determination. The amount of immobilized protein was calculated by the difference between the offered protein amount and that found in the supernatants and washings. The mDE-invertase was stored in sodium acetate buffer at 4 ºC for further use. The pH and immobilization time were also investigated in order to study what happens in the immobilization process. Thus invertase solutions were prepared in different pH (0.2 M, sodium acetate buffer). Immobilization time was set up according to the time variation in the procedure of immobilization.

2.3.1. Design of experiments: screening analysis using a fractional factorial design

The statistical design of experiments (DOE) is a structured and systematized method of experimentation in which all factors are varied simultaneously over a set of experimental runs [17]. DOE was used to study the effects of variables on the DE functionalization, and immobilization process of the invertase. A 2^{7-2}_{IV} fractional factorial design (resolution IV) with seven variables where each with two levels namely low (-1) and high (+1) were employed. The variables studied were: APTES concentration, APTES contact time, glutaraldehyde concentration, glutaraldehyde contact time, immobilization time, immobilization pH and invertase concentration. Table 1 shows the range of process variables studied. Altogether 32 experiments were required and the experimental sequence was randomized in order to minimize the effects of the uncontrolled factors (Table 2). The data were statistically analyzed by variance analysis (ANOVA) using the software Statistica 8.0 (Stat Soft, Inc., 2008, USA). The level of significance was set at $p<0.05$.

Table 1. Experimental independent variables

Variables	Factor code	Unit	Level and range (coded)	
			-1	+1
[APTES]	A	%	2.5	10.0
APTES contact time	B	H	1	2
[Glutaraldehyde]	C	%	2.5	10.0
Glutaraldehyde contact time	D	h	1	2
Immobilization time	E	h	2	12
Immobilization pH	F	-	4.0	5.5
[Invertase]	G	mg/mL	0.05	0.15

Table 2. Experiment runs and responses for the diatomaceous earth functionalization and immobilization process of the invertase.

Run	Factors							Response 1	Response 2
	A	B	C	D	E	F	G	Immobilized protein (%)	Enzimatic activity (U/mg mDE)
1	-1	-1	-1	-1	-1	+1	+1	36	0.330
2	+1	-1	-1	-1	-1	-1	-1	100	0.057
3	-1	+1	-1	-1	-1	-1	-1	100	0.093
4	+1	+1	-1	-1	-1	+1	+1	47	0.616
5	-1	-1	+1	-1	-1	-1	+1	88	0.182
6	+1	-1	+1	-1	-1	+1	-1	80	0.199
7	-1	+1	+1	-1	-1	+1	-1	83	0.278
8	+1	+1	+1	-1	-1	-1	+1	87	0.085
9	-1	-1	-1	+1	-1	-1	-1	100	0.094
10	+1	-1	-1	+1	-1	+1	+1	27	0.354
11	-1	+1	-1	+1	-1	+1	+1	34	0.324
12	+1	+1	-1	+1	-1	-1	-1	100	0.057
13	-1	-1	+1	+1	-1	+1	-1	92	0.532
14	+1	-1	+1	+1	-1	-1	+1	94	0.185
15	-1	+1	+1	+1	-1	-1	+1	98	0.183
16	+1	+1	+1	+1	-1	+1	-1	91	0.102
17	-1	-1	-1	-1	+1	+1	-1	100	0.100
18	+1	-1	-1	-1	+1	-1	+1	79	0.209
19	-1	+1	-1	-1	+1	-1	+1	79	1.043
20	+1	+1	-1	-1	+1	+1	-1	66	0.420
21	-1	-1	+1	-1	+1	-1	-1	100	0.735
22	+1	-1	+1	-1	+1	+1	+1	27	0.626
23	-1	+1	+1	-1	+1	+1	+1	36	0.886
24	+1	+1	+1	-1	+1	-1	-1	100	0.481
25	-1	-1	-1	+1	+1	-1	+1	75	0.251
26	+1	-1	-1	+1	+1	+1	-1	72	0.701
27	-1	+1	-1	+1	+1	+1	-1	73	0.765
28	+1	+1	-1	+1	+1	-1	+1	78	0.330
29	-1	-1	+1	+1	+1	+1	+1	27	0.620
30	+1	-1	+1	+1	+1	-1	-1	100	0.694
31	-1	+1	+1	+1	+1	-1	-1	100	0.566
32	+1	+1	+1	+1	+1	+1	+1	28	0.493

2.4. Enzyme assay

Invertase activity was determined by using 0.15 M sucrose (10 mL) prepared in sodium acetate buffer (0.2 M, pH 5.0). After exactly 15 min of incubation at 25ºC, 20 µl the sample was withdrawn and added to 2.0 mL of working solution in order to measure released glucose using a glucose

oxidase-peroxidase (GOD/POD) enzymatic kit (Doles, Goiás, Brazil). The enzyme activity unit (U) was defined as the amount of enzyme releasing 1 μmol of glucose per minute under the assay conditions.

Protein determination was according to Lowry et al. [10] using bovine serum albumin as the standard protein.

2.5. Characterization

The size and size distribution of mDE were determined with a Microtrac S3500 particle size analyzer. Mineralogy of raw DE and mDE was characterized by powder X-ray diffraction (XRD) analysis using a Siemens D5000 X-ray diffractometer. Representative powder samples were analyzed in the range $10^o<2\theta<90^o$ by using CuKα radiation (λ= 1.5406 Å) in steps of 0.02º and with a counting time of 1.0 s per step. The morphological characterization of DE, mDE and mDE-invertase was carried out with a scanning electron microscope (SEM, FEI Model QUANTA 200 FEG) equipped with energy dispersive spectroscopy (EDS). The samples were coated with gold prior to analysis. The identification of the chemical elements present in the material was performed by EDS. Surface area and porosity were determined for all materials with a Micromeritics ASAP 2420 porosimeter. The isotherms were obtained at 77 K using N_2 as an adsorbate. The specific surface area was calculated using the Brunauer-Emmett-Teller (BET) model. Pore size distribution and pore volume were determined from the desorption branch of the isotherms using the Barrett-Joyner-Halenda (BJH)-plot method. FTIR spectra in the 4000-400 cm^{-1} range of magnetite, DE and mDE were recorded in a BRUKER instrument model IFS 66. The samples were pressed into pellets with KBr. Magnetization properties of the samples were performed at 298 K in magnetic fields from 0 to 50 KOe (5.0 T) using a SQUID magnetometer (Quantum Design Model MPMS-5S). The magnetic properties of the particles were expressed in electron mass units (emu).

3. Results and discussion

3.1. Characterization of mDE

The DE particle size has been reported in the range from 10 to 200 μm [18]. We have found this value for DE and mDE at 13.4 μm and 16.1 μm, respectively. Galindo-González [19] reported for magnetic clay particles that after magnetization process there is an increase of size of composite particles and this can be attributed to the formation of heteroaggregates of clay and magnetite particles. Thus, the increase of size (around 20%) of the mDE particles also can be attributed to the formation of heteroaggregates of DE and magnetite particles.

The XRD analysis showed the characteristic peaks of DE (amorphous silica), quartz (crystalline silica) as well as kaolinite as a minor phase (Fig.1A) [3, 20]. Similar peaks were observed for the mDE (Fig. 1B) except that peaks corresponding to magnetite were also visible. The kaolinite peaks non-appeared after the magnetization process, this can be due to kaolinite particles are more heavy than diatomaceous earth and thus during the magnetization these particles were removed.

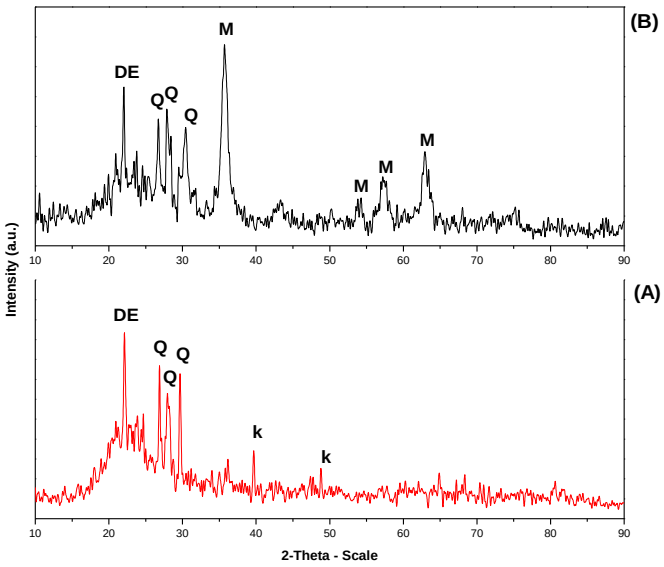

Figure 1. X-ray diffraction of DE (A) and mDE (B). DE=diatomaceous earth; K=kaolinite; M=magnetite and Q= quartz.

Fig. 2 shows SEM micrographs and corresponding EDS analyses of the DE, mDE and mDE-invertase. The morphology of the particles was irregular, and showed a typical sheet like structure. The EDS spectrum for the DE (Fig. 2A) showed the expected peaks of Si and O, and other elements (Al, K, Ca, Fe). The magnetite composite (mDE) showed a different texture in which the layers seemed more irregular and a high surface roughness (Fig. 2B). This roughness can be attributed at aggregates of magnetite on DE surface. Its EDS spectrum displayed an increase in the signal corresponding to Fe supporting the presence of magnetite on the mDE. The addition of invertase on mDE further changed the morphology of the material. The Fig. 2C shows the layer formation and agglomeration that occurred between magnetic particles and enzyme. Probably, this surface alteration can be attributed to the highly polymeric material of the enzyme covering the magnetic particles. This finding has been reported by Gopinath and Sugunan [21] working with immobilized enzymes including invertase. It is worthwhile to register the presence of S in the EDS of mDE-invertase (Fig. 2C).

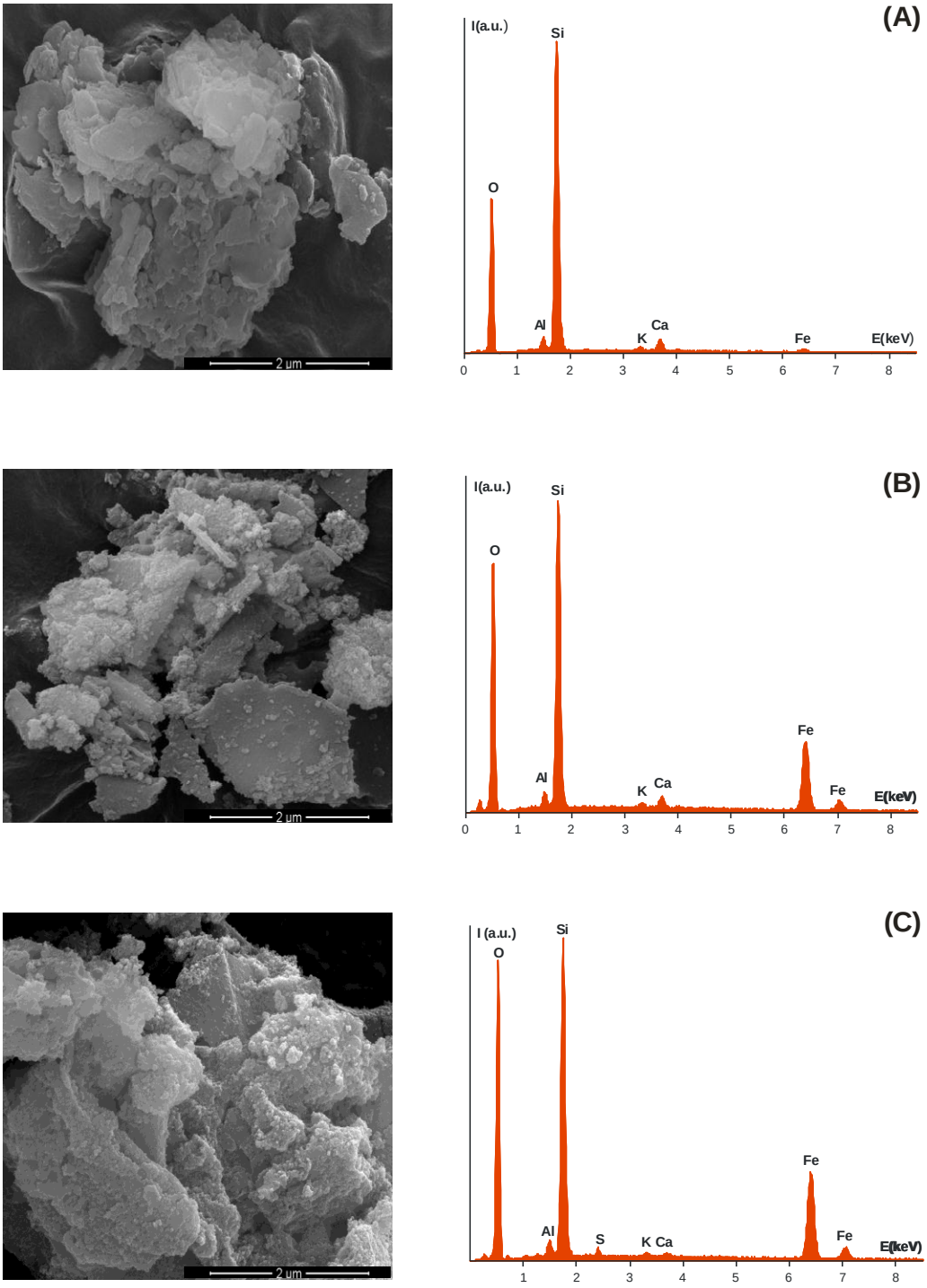

Figure 2. Scanning electron micrographs and corresponding EDS analyses of DE (A), mDE (B) and mDE-invertase (C).

The FTIR spectra of magnetite, DE and mDE are shown in Fig. 3. The magnetite spectrum exhibited absorption bands at around 630 and 583 cm^{-1}characteristic of the Fe-O bond [22]. The bands near 3421 and 1638 cm^{-1} are ascribed to the hydroxyl characteristic peaks of water adsorbed in the surface or the OH-streching bands and its bending vibration peak [23]. The spectral band

intensities of DE were at 3648, 1630, 1200, 1097, 800 and 470 cm^{-1}. The band at 3648 (weak) is due to free surface silanol group (Si-OH), the bands at 1200 and 1097 cm^{-1} (strong and broad) are mainly due to siloxane (Si-O-Si) stretching, while the bands at 800 and 470 (strong and narrow) cm^{-1} are due to (Si-O) stretching of silanol group and (Si-O-Si) bending vibration, respectively [3, 18]. After magnetization process, the mDE spectrum showed the same absorption bands of Fe-O bond at around 633 and 583 cm^{-1}, which supported the presence of magnetite particles.

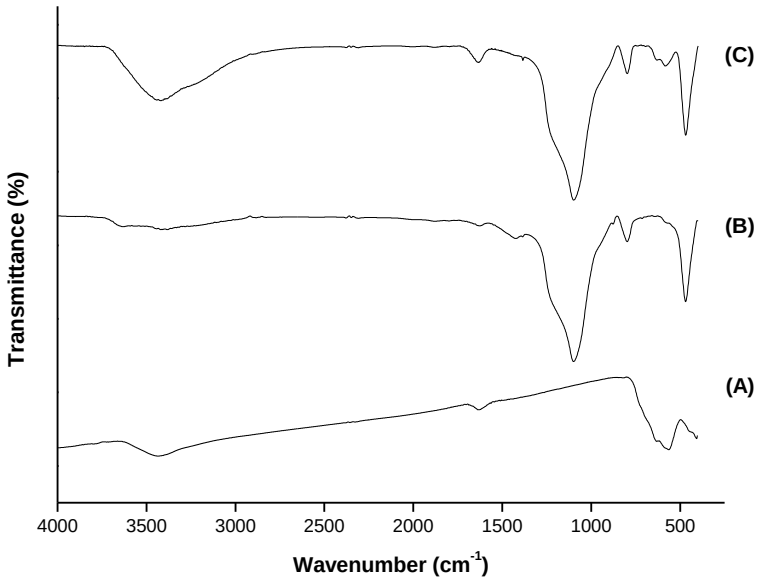

Figure 3. FTIR spectra of magnetite (A), DE (B) and mDE (C).

The magnetic properties of magnetite and mDE particles were measured by applying an external magnetic field at 298 K. The saturation magnetization for magnetite and mDE was determined by the magnetization curve at maximum magnetic field. As shown in Fig. 4, the saturation magnetization of the mDE was above 10 emu g^{-1} lower than the value of 60 emu g^{-1} found for the magnetite particles at 298 K. Thus, the saturation magnetization of the magnetic composite was affected nevertheless the mDE particles had a good response against the external magnetic field applied in the immobilization process. This decrease can be attributed to surface effects, such as magnetically inactive layer producing disordered surface [24]. In addition, the magnetite and mDE particles exhibited ferromagnetic and superparamagnetic character.

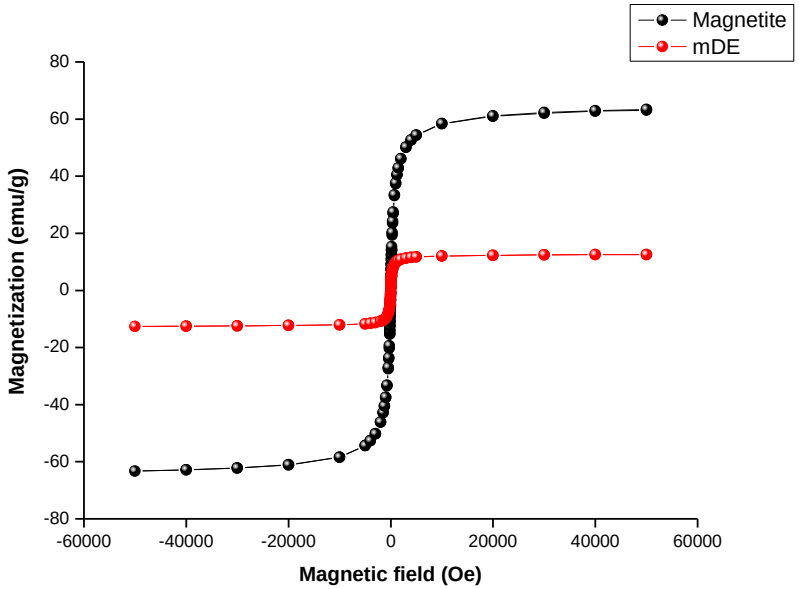

Figure 4. Magnetization curves of magnetite and mDE at 298 K.

The BET test was conducted to determine textural parameters, such as BET surface area, pore volume and pore size of raw DE, mDE and mDE-invertase (Table 3). As a result of the magnetization process of DE the magnetic composite presented a higher surface area and pore volume compared to DE. This increase can be indicative of the creation of open pores on the diatomite backbone surface, as a consequence of magnetite deposits on surface of DE. Furthermore, with this increase is possible that immobilization process is better, i.e. enzyme molecules will have more chemical groups on mDE particles so that happen to the covalent binding. It is worthwhile to show that this result is in agreement with the size particle analysis. Al-Degs et al. [18] reported that the surface area of diatomite depends mainly on the hydroxyl groups (-OH) present on the surface this material. The authors showed values at 33 and 80 m^2g^{-1} of surface area for the diatomite and manganese-diatomite, respectively. Although was not shown an increase in surface area after the immobilization process, probably indicating that enzyme molecules were deposited on the surface of mDE, which decreases the surface area of magnetic composite. The values of pore size for DE, mDE and mDE-invertase are in the range of mesoporous solid (between 2-50 nm) according to IUPAC. The pores in mDE were slit shaped openings as depicted by the type of the adsorption-desorption isotherms (type II) and the characteristic hysteresis loops (H3). Also can be observed that there are not change in pore size in the immobilization process (mDE-invertase) evidencing the confinement of enzyme out the pores of diatom structure.

Table 3. Surface area, pore volume and pore size of DE, mDE and mDE-invertase.

Sample	S_{BET} ($m^2\ g^{-1}$)	Pore Volume ($cm^3\ g^{-1}$)	Pore Size (nm)
DE	32.9	0.08	11.8
mDE	56.6	0.16	12.7
mDE-invertase	41.2	0.12	11.9

3.2. Study of the immobilization process by design of experiments

A fractional factorial design was used to screening of the most important factors that affected the immobilization process of invertase on mDE particles, so as to obtain the optimum operational conditions in this process. Therefore, to obtain the optimum conditions (i.e. APTES concentration, APTES contact time, glutaraldehyde concentration, glutaraldehyde contact time, immobilization time, immobilization pH and invertase concentration) was performed a 2^{7-2}_{IV} fractional factorial design with a fixed amount of support (0.01 g mDE). The factors were studied at two levels, low level coded (-1), and high level coded (+1) as presented in Table 1. This design of experiments and the results of both responses obtained in the experiments are given in Table 2. For the statistical analysis, the data are plotted against a theoretical normal distribution in such a way that the points should form an approximate straight line. Departures from this straight line indicate departures from normality. Therefore, the significant effect values lie off the line passing close to the origin, whereas the dummy effect values fit this line quite well.

3.2.1. Response 1: Immobilized protein (%)

Fig. 5 shows the corresponding normal probability plot for the immobilized protein (%). The most of values of these effects are represented by the points closest from a straight line passing through the origin in the normal plot. In contrast, the invertase concentration and immobilization time were the factors lie farther to this line. Thus, the results from the screening of seven different factors indicated that only invertase concentration and immobilization pH were the main factors affecting the immobilization process. That is, the effects of these factors were negative indicating that low levels favoring this response. Other factors were also significant, for example, the interaction factor of order second: glutaraldehyde concentration*immobilization time (C*E) was also important in this immobilization process but not more than the invertase concentration and immobilization pH that were farther from the straight line.

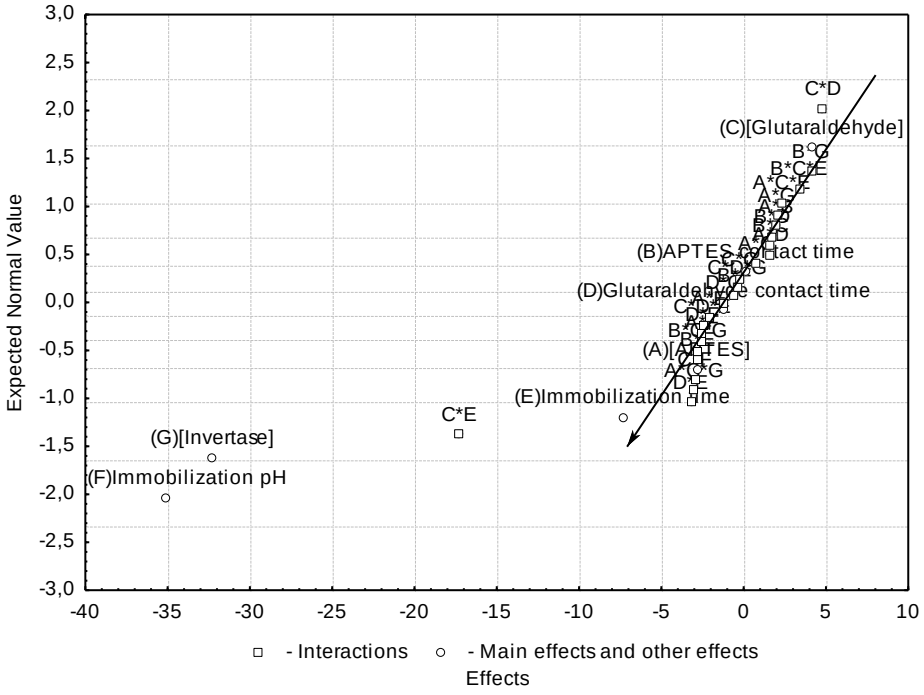

Figure 5. Normal probability plot for the response 1: Immobilized protein (%)

3.2.2. Response 2: Enzymatic activity

Fig. 6 shows the corresponding normal probability plot for the enzymatic activity. In the same way as for the response immobilized protein, the most of values of these effects are represented by the points closest from a straight line passing through the origin in the normal plot. In contrast, the immobilization time was the factor lie farther to this line. Thus, the results from the screening of seven different factors indicated that only immobilization time was the main factor affecting the enzymatic activity. That is, the effect of this factor was positive indicating that high level favoring this response. Other main and interactions factors were also significant in this immobilization process but not more than the immobilization time that was farther from the straight line.

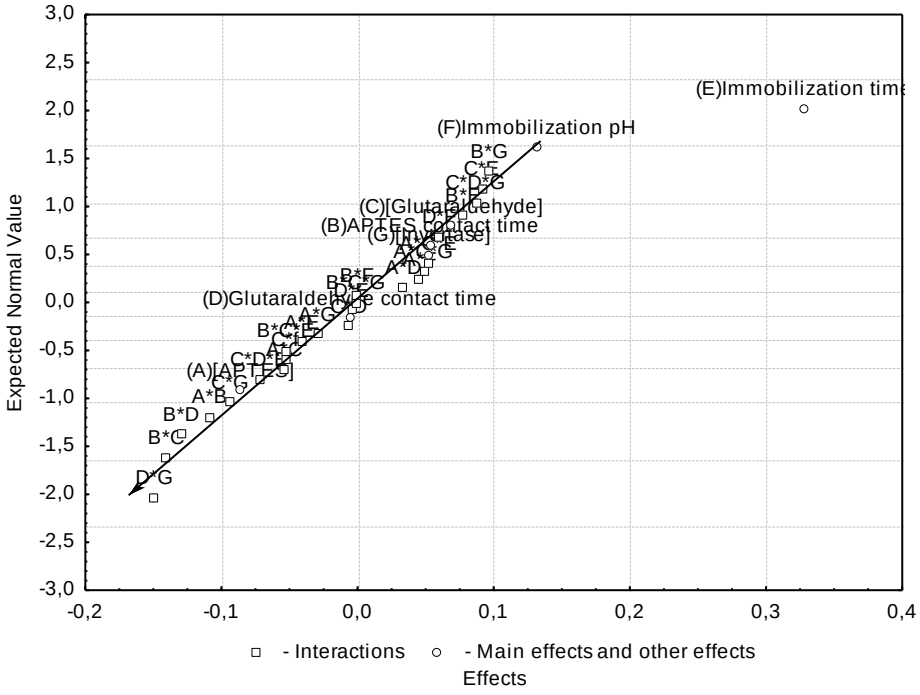

Figure 6. Normal probability plot for the response 2: Enzymatic activity (U/mg mDE)

3.2.3. Analysis of both responses

As final result of this fractional factorial design the fig. 7 shows the scatterplot of both responses: immobilized protein (%) and enzymatic activity (U/mg mDE). Thereby, analyzing the responses simultaneously were not chosen a main factor or interaction factor as a result of this DOE, but was chosen the best experimental conditions for the immobilization process of invertase using as matrix the magnetic diatomaceous earth particles. Thus, the screening of seven different factors indicated that the run 19 and 23 were that presented the best operational conditions in the immobilization process of invertase on mDE particles. The difference between these run were the glutaraldehyde concentration and immobilization pH. The run 19 had the low levels of these parameters whereas the run 23 had the high levels of the same parameters (Table 2). The immobilized derivatives for run 19 and 23 presented a residual specific activity of 48.9% and 92.5%, respectively. Therefore, the operational conditions of the run 23 were chosen for to use in the immobilization process: APTES concentration (2.5%), APTES contact time (2 h), glutaraldehyde concentration (10.0%), glutaraldehyde contact time (1 h), immobilization time (12 h), immobilization pH (5.5) and invertase concentration (0.15 mg/mL). The decrease in the immobilized enzyme activity can be due to the multipoint attachment of the enzyme molecules onto the mDE particles and/or overloading of immobilized enzyme. The level of activity decrease is mainly dependent on the properties of support, enzyme nature and immobilization conditions/activators. Bergamasco [9] reported that for the invertase immobilization on controlled pore silica (CPS) particles was used a APTES

concentration (0.5%), APTES contact time (3 h), glutaraldehyde concentration (2.5%), glutaraldehyde contact time (45 minutes), immobilization time (15 h) and immobilization pH (4.5), in these conditions the immobilized derivative showed a low activity yield around 24%. Sanjay and Sugunan [25] also reported that when the conditions for the invertase immobilization on montmorillonite K10 were: APTES concentration (10%), APTES contact time (1 h), glutaraldehyde concentration (10%), glutaldehyde contact time (1 h), immobilization time (1 h) and immobilization pH (5.0), the immobilized invertase retained 36% activity of the soluble enzyme.

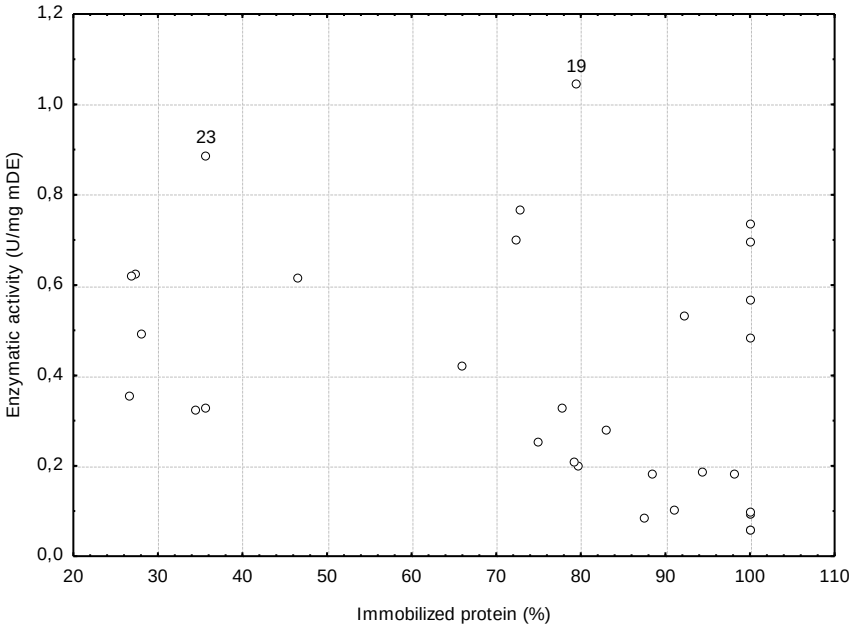

Figure 7. Scatterplot for both responses: Immobilized protein (%) and Enzymatic activity (U/mg mDE)

4. Conclusions

Fabrication of the functional magnetic diatomaceous earth (mDE) particles, from the raw diatomite as matrix for the invertase immobilization has been developed successfully in this work. These magnetic particles were characterized for different techniques and exhibit a good capacity and properties for invertase immobilization by covalent binding. Magnetic diatomite showed characteristic of a mesoporous solid with a pore size of around 13 nm, and the particles size of 16.1 µm. As result of screening carried out of the immobilization process through of fractional factorial design the run 23 presented the best operational conditions. The immobilized derivative presented 92.5% of residual specific activity when compared with the free enzyme. Finally, these results suggest that the magnetized diatomaceous earth showed to be a promising matrix for covalent immobilization of invertase and can be used for the industrial production of invert syrup. Furthermore, this magnetic composite could be used for the immobilization or purification the other enzymes of industrial interest for biotechnological applications.

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Capítulo 6

5.4. Artigo a ser submetido ao periódico *Journal of Molecular Catalysis B: Enzymatic*

Título: Optimization of the sucrose hydrolysis by invertase immobilization on magnetic mesoporous diatomite

Autores: Mariana Cabrera, Luciana Lopes Silveira, David Fernando Morais Neri, Fernando Soria, Luiz Bezerra de Carvalho Jr.

Optimization of the sucrose hydrolysis by invertase immobilization on magnetic mesoporous diatomite

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Abstract

A magnetic mesoporous diatomite was used as matrix for invertase immobilization via covalent binding. The sucrose hydrolysis process by invertase immobilized was also optimized using 2^4 factorial experimental design. Four variables were investigated and the best operational conditions for sucrose hydrolysis were pH 4.5, temperature of 45°C, 0.25M sucrose concentration and 0.05 mg mL^{-1} invertase concentration. The thermal stability of immobilized invertase showed a best performance that the free enzyme. At 35°C and 45°C for 60 minutes the immobilized derivative showed activity values around 85% and 31%, respectively, and the free enzyme around 71% and 11%, respectively. Furthermore, the immobilized derivative has also presented a good performance in the storage stability and reuse at short and long term with a retained activity 88% (2 months), 60% (10 reuses) and 50% (4 months), respectively. Finally, the mDE-invertase proved to be a potential biocatalyst for the production of inverted sugar due to its excellent stability and reuse. We are also proposing a matrix with interesting properties and low cost as well as a simple immobilization process for invertase immobilization or other biomolecules.

Keywords: invertase; covalent immobilization; magnetic particle; diatomite

1. Introduction

The immobilized enzymes have been used for several practical applications such as food and pharmaceutical industry, bioseparators or biosensors. These biocatalysts have presented excellent advantages such as reusable forms of enzymes with easy separation from products, longer half-life and convenient handling, thus leading to a process cost reduction [1]. At present, a vast number of methods of immobilization are currently available. Unfortunately, there is no a universal enzyme support, i.e. the best method of immobilization might differ from enzyme to enzyme, from application to application and from carrier to carrier. On the other hand, the screening of several variables that may affect the immobilization process are not designed and some of the industrial enzymes are working below their optimum conditions [2]. Inverted sugar is a valuable commercial product for the food industry in countries where the main sources of sugar is beet or cane. Compared with its precursor, sucrose, inverted sugar is sweeter and its products tend to retain moisture and are less prone to crystallization [3]. The hydrolysis can be induced simply by heating an aqueous solution of sucrose in acid medium or by invertase (EC 3.2.1.26). The free and immobilized invertase produces high quality inverted sugar with low concentrations of 5-hydroxymethyl-2-furfural (HMF) and without color development compared to the colored version obtained through acid hydrolysis [3, 4]. Invertase has been immobilized by different methods and on a variety of carriers in order to extend its stability, providing re-usage possibility and inverted sugar production [5].

Magnetic bio-separation technology is a promising strategy for recovering the immobilized enzyme on magnetic particles using an external magnetic field for recycled use [6-12]. It can also reduce the capital and operation costs [13]. Sadasivan and Sukhorukov [14] reported that due to low enzyme loading on the conventional magnetic beads, further attention was paid to the magnetic mesoporous support. Hybrid materials from magnetite and inorganic silica have showed a perfect combination of its properties and improvement in enzyme immobilization [15]. Diatomaceous earth (DE) or diatomite and this typically consists of 86-94% silicon dioxide (SiO_2), with a significant quantity of alumina (Al_2O_3) and ferric oxide (Fe_2O_3). This mineral has been applied as a filter aid, adsorbent, filler, packing material for gas chromatography or high-performance liquid chromatography, insulator, catalyst (support), drilling-mud thickener, extender in paints, anticaking agent, natural insecticide, or grain protectant [16]. Nowadays there are few papers that to propose silica particles as support for protein immobilization [17-24]. To the best of our knowledge the invertase has not been immobilized on magnetic mesoporous diatomite yet. Nevertheless, this enzyme has been studied by several authors, seeks to find the best experimental conditions of the immobilized invertase to be used in industrial applications. Therefore, the main point of this paper was to

propose a cheap matrix from raw diatomite and easy synthesis for the invertase immobilization. Optimization of the sucrose hydrolysis process by immobilized derivative was studied using design of experiments (DOE) as a structured and systematized method of experimentation in which all factors are varied simultaneously over a set of experimental runs. The thermal stability, storage stability, shelf life and reusability of immobilized invertase were also performed.

2. Materials and methods

2.1. Materials

Diatomaceous earth (DE) was kindly supplied by TAMER S.A. (Salta, Argentina). A process of water washing and repeated sedimentation was applied to purify the raw DE. Invertase from Baker's yeast, aminopropyltriethoxysilane (APTES), glutaraldehyde and bovine serum albumin were purchased from Sigma Aldrich Chemicals (St. Louis, USA). All other chemicals were of high purity available commercially.

2.2. Diatomaceous earth magnetization

The synthesis of magnetic DE was performed according to Amaral et al. [25] with the next modifications: (a) incubation temperature of DE with $FeCl_3.6H_2O/FeCl_2.4H_2O$ by 30 minutes was extended from 80ºC to 100ºC; (b) final pH magnetization was 11.0 adjusted with ammonium hydroxide (7.6 M). The magnetic diatomaceous earth (mDE) obtained was washed with distilled water until pH 7.0 and recovered by a magnetic field (Ciba Corning; 0.6 T). The mDE was dried at 50 ºC overnight and then sieved.

2.3. Immobilization of invertase on magnetic hybrid material

Firstly, the mDE (0.10 g) was functionalized with APTES (2.5% v/v) prepared in acetone and it was kept under stirring for 2 hours at 25°C. The activation of the silanized mDE (0.01 g) with glutaraldehyde (10% v/v, prepared in 0.2 M sodium acetate buffer, pH 5.5) also was carried out under stirring for 1 hour at 25ºC. In each step of functionalization process, the mDE was washed several times with distilled water and 0.2 M sodium acetate buffer, pH 5.0 until the washings became colorless. The treated particles were recovered using magnetic field (0.6 T).

For the immobilization process, invertase (1 mL, prepared in 0.2 M sodium acetate buffer, pH 5.5) was incubated with functionalized mDE (0.01 g) for 12 hours at 4 ºC under mild stirring. Afterwards the material was washed five times with 0.2 M sodium acetate buffer, pH 5.0. The immobilized invertase on mDE (mDE-invertase) was collected by the magnetic field and the supernatants including the first two washings were used for protein determination. The amount of

immobilized protein was calculated by the difference between the offered protein amount and that found in the supernatants and washings. The mDE-invertase was stored in sodium acetate buffer at 4 ºC for further use.

2.4. Enzyme assay

Invertase activity was determined by using sucrose (10 mL) prepared in sodium acetate buffer (0.2 M, pH 5.0). After exactly 15 min of incubation at 25ºC, 20 µl the sample was withdrawn and added to 2.0 mL of working solution in order to measure released glucose using a glucose oxidase-peroxidase (GOD/POD) enzymatic kit (Doles, Goiás, Brazil). The enzyme activity unit (U) was defined as the amount of enzyme releasing 1 µmol of glucose per minute under the assay conditions.

Protein determination was according to Lowry et al. [26] using bovine serum albumin as the standard protein.

2.5. Experimental design and statistical analysis

Based on the preliminary results, the characterization of immobilized invertase on mDE was carried out through design of experiment (DOE) for investigated the best conditions of sucrose hydrolysis by mDE-invertase. Thus was performed a 2^4 factorial experimental design to study the effect of four variables on the response: Specific activity (%).This approach enables experimental investigation of the individual factors and the interactions of the factors simultaneously as opposed to one factor at-a-time approach. The independent variables X_1, X_2, X_3 and X_4 were as follows (low/high value): pH 4.5/5.5; temperature (°C) 45/65; sucrose concentration (M) 0.15/0.25 and invertase concentration (mg mL^{-1}) 0.05/0.15. Each variable was coded at three levels: −1, 0 and +1. The coded value of these factors were obtained according to the Eq. (1),

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

where x_i is the coded value of the factor, X_i is the real value of the factor, X_0 is the real value of the factor at the center point, and $\triangle X_i$ is the step change value of the factor.

The independent variables (factors) and their levels, real values as well as coded values are presented in Table 1. Altogether 20 experiments were carried out randomized in order to minimize the effect of unexpected variability in the observed response due to extraneous factors (Table 2). The data were statistically analyzed by variance analysis (ANOVA) using the software Statistica 8.0 (Stat Soft, Inc., 2008, USA). The level of significance was set at $p<0.05$.

Table 1. Independent variables and their levels used in experimental design.

Factors	Code	Variables levels		
		-1	0	+1
pH	X_1	4.5	5.0	5.5
Temperature (ºC)	X_2	45	55	65
[Sucrose] (M)	X_3	0.15	0.20	0.25
[Invertase] (mg mL^{-1})	X_4	0.05	0.10	0.15

Table 2. Experiment runs and response value for the optimization the sucrose hydrolysis of the mDE-invertase.

Run	Coded independent variable levels				Response value
	pH	Temperature (ºC)	[Sucrose] (M)	[Invertase] (mg mL^{-1})	Specific activity (%)[a]
1	-1	-1	-1	-1	59.21
2	+1	-1	-1	-1	36.93
3	-1	+1	-1	-1	36.87
4	+1	+1	-1	-1	27.90
5	-1	-1	+1	-1	100.00
6	+1	-1	+1	-1	48.11
7	-1	+1	+1	-1	31.38
8	+1	+1	+1	-1	43.23
9	-1	-1	-1	+1	13.02
10	+1	-1	-1	+1	12.86
11	-1	+1	-1	+1	1.90
12	+1	+1	-1	+1	9.07
13	-1	-1	+1	+1	13.63
14	+1	-1	+1	+1	16.96
15	-1	+1	+1	+1	6.46
16	+1	+1	+1	+1	16.98
17	0	0	0	0	23.57
18	0	0	0	0	41.55
19	0	0	0	0	30.15
20	0	0	0	0	30.10

[a]Specific activity (%) was estimated from the most value of specific activity (U mg^{-1} protein) of the mDE-invertase and was considered as 100%.

2.6. Thermal stability

The thermal stability of the free and immobilized invertase were carried out by measuring the specific activity (%) of the enzyme pre-incubated at different temperatures (35 to 55 ºC) in sodium acetate buffer (0.2 M, pH 5.0) for 30 to 120 minutes. After cooling (25°C), the enzyme activity was determined as above described.

2.7. Storage stability

Storage stability of mDE-invertase was studied for a period of 120 days at 4°C. The specific activity (%) of immobilized derivative was checked from time to time using different samples, with sucrose (0.25 M) as substrate by the method described in section on enzyme assay.

2.8. Reusability of the mDE-invertase in short and long term

The mDE-invertase was stored in sodium acetate buffer (0.2 M, pH 5.0) at 4°C and was reused 10 times at 30 minutes interval. The residual activity (%) was measured with sucrose (0.25 M) as substrate. After assay, immobilized preparation was washed with sodium acetate buffer and magnetically collected for the next activity cycle.

The long term reuse of the mDE-invertase was performed by measure of residual activity (%) during 120 days. For this, the same mDE-invertase was measured the activity as above described, using sucrose (0.25 M) as substrate. After the immobilized derivative was washed with sodium acetate buffer (0.2 M, pH 5.0) and stored at 4 ºC.

3. Results and discussion

3.1. Optimization of sucrose hydrolysis by mDE-invertase

The effects of sucrose hydrolysis parameters such as pH, temperature, sucrose concentration and invertase concentration were investigated on specific activity (%) of the mDE-invertase. Fig. 1 shows the Pareto chart of standardized effects that was used to graphically summarize and display the relative importance of the differences between different variables studied in the sucrose hydrolysis process by mDE-invertase. The length of each bar was proportional to the value of its associated regression coefficient or estimated effect. The chart included a vertical line that corresponded to the 95% limit indicating statistical significance. A factor was, therefore, significant if its corresponding bar crossed this vertical line [27]. As indicated in Fig.1, only two main factors (X_2 and X_4) were significant statistically ($p<0.05$). It is evident that temperature (X_2) and invertase concentration (X_4) were the most significant variables in the sucrose hydrolysis process. The negative effects of X_2 and X_4 indicated that low level for the temperature and invertase concentration favoring the response variable (specific activity, %). The statistical combination of the independent variables in coded values along with the experimental response was presented in Table 2. The software Statistica 8.0 was used to calculate the effect of each factor and its interactions.

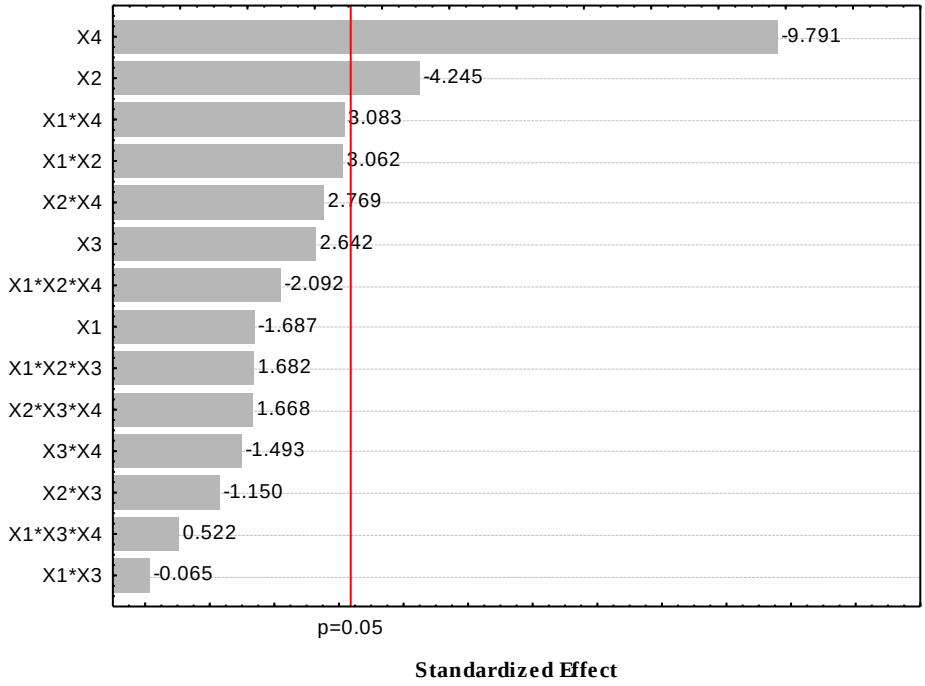

Figure 1. Pareto chart of standardized effects for the full design experiment. The line indicates the confidence level of 95%, and factors with standardized effect values to the right of this line are statistically significant.

The model expressed by Eq. (2) represents specific activity (y) as a function of temperature (X_2) and invertase concentration (X_4). The statistical significance of Eq. (2) was controlled by F-test and the analysis of variance (ANOVA). Values of probability (P) > F less than 0.05 indicate model terms are significant. According to analysis of ANOVA only temperature (X_2) and invertase concentration (X_4) variables were the most significant parameters. The coefficient of determination (R^2=0.965) indicated that the accuracy of the model was adequate.

$$y = 29.99 - 7.93\,X_2 - 18.30\,X_4 \qquad (2)$$

The relationship between predicted and experimental specific activity (%) is shown in Fig. 2. It can be seen that there is a high correlation between the predicted and experimental specific activities. The lack of fit measures the failure of the model to represent data in the experimental domain at points, which are not included in the regression. The value of lack of fit (F-value: 1.51) for regression of Eq. (2) is not significant. This way the model equation was adequate for predicting the specific activity (%) of mDE-invertase under any combination of values of the variables. Therefore, run 5 (pH 4.5; temperature 45°C; sucrose concentration, 0.25 M and invertase concentration, 0.05 mg mL^{-1}) presented the best operational conditions for sucrose hydrolysis process by mDE-invertase. Marquez et al. [3] performed a study of sucrose hydrolysis by CCD (central composite design) of immobilized invertase. The authors studied the influence of pH and temperature, who

observed that the maximum response (enzymatic activity) was found a temperature of 40°C and the pH of 4.9.

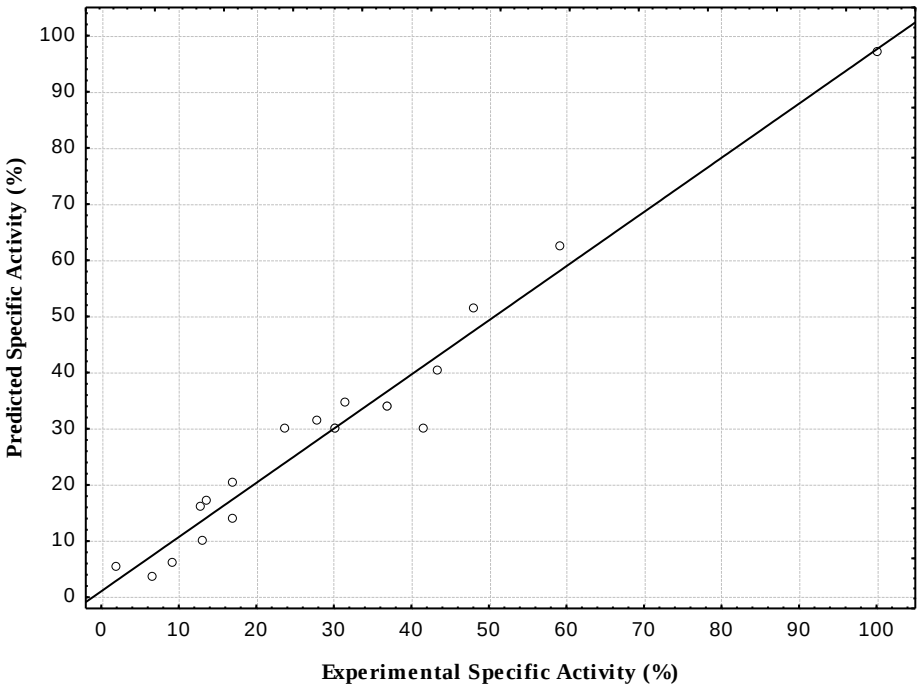

Figure 2. Predicted specific activity versus experimental specific activity.

3.2. Thermal stability

Many studies about immobilized enzymes have shown that its activity and stability can differ from those of free enzyme [28]. Therefore, the thermal stability of mDE-invertase was compared with that of the free enzyme (Fig. 3). Thus, best performance was observed at 35°C and 45°C for 60 minutes for the immobilized derivative (85% and 31%, respectively) than that for the free enzyme (71% and 11%, respectively). At 45°C the free form was inactivated at a much faster rate than the immobilized form but both enzymatic preparations at 55°C lost their initial activity after 120 minutes treatments. Thermal stability of the mDE-invertase is better than the free invertase probably due to the covalent bond formation that might reduce the conformational flexibility of the enzyme and make it more stable against temperature changes [29]. Other authors also observed that after immobilization there was an improvement in thermal stability of enzyme [30-32].

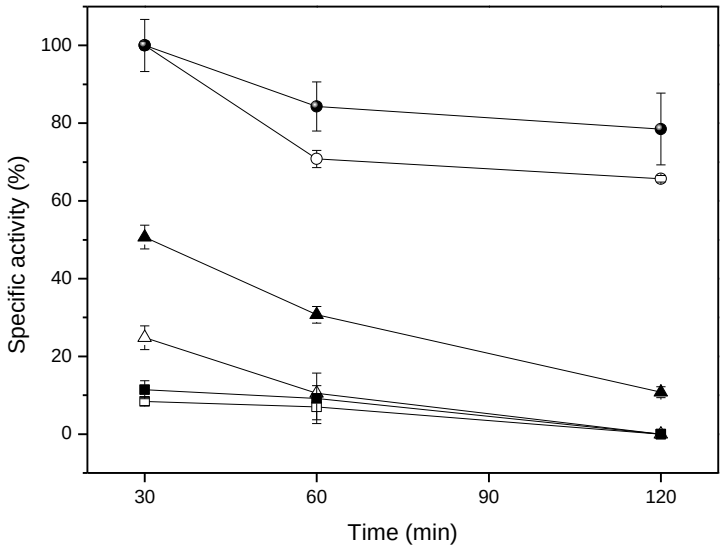

Figure 3. Thermal stability of immobilized (dark) and free (hollow) invertase at 35 ºC (●), 45°C (▲) and 55°C (■).

3.3. Storage stability

Definitely, immobilization puts the enzyme into a more stable position in comparison to free enzyme as showed in Fig. 4. After storage at 4°C for 30 days, the specific activities were found to be 28 and 88% of the initial activity values for free and immobilized enzyme, respectively. Thereafter, the mDE-invertase presented a retention activity around 83% for 120 days storage period whereas the free enzyme lost all its activity in the same period. This is an expected result since immobilization enhances the stability of the enzyme by reducing its denaturation rate [29]. Akgöl et al.[33] also observed an increase of the storage stability of the immobilized invertase onto magnetic polyvinylalcohol microspheres. In this work, the authors showed that the immobilized derivative retained above 20% of its initial activity during fifty days. On the other hand, we are proposing an immobilized derivative with a good retained activity (above 80%) during 120 days storage period.

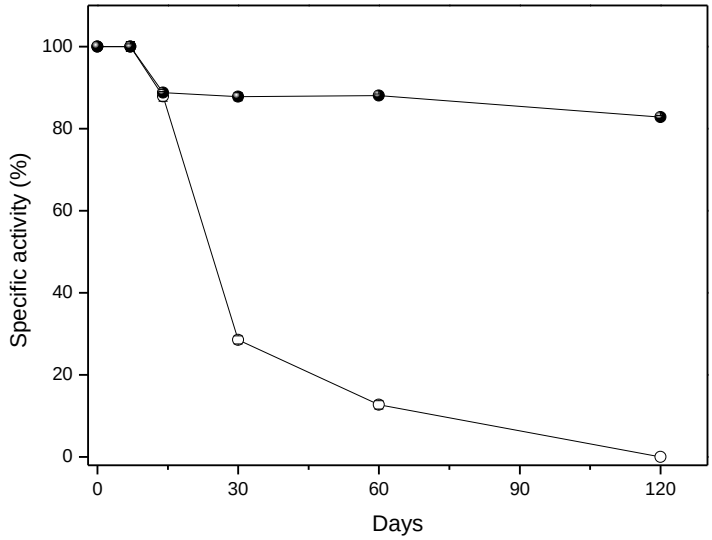

Figure 4. Effect of storage stability on the activity of free (○) and immobilized (●) invertase.

3.4. Reuse of mDE-invertase

The reusability of the mDE-invertase in short and long term is shows in the Fig. 5. The effect of repeated use capability on the activity of immobilized invertase on mDE composite is shown in the Fig.5A. The activity of mDE-invertase was stable for 10 cycles retaining more than 60% of residual activity. Raj et al.[32] reported the appreciable reusability of the immobilized invertase on nanogel-matrix up to eight cycles, but the relative activity (%) decreased to 11.03% after the 9th cycle.

The mDE-invertase stored at 4°C had a long term reuse of 120 days with about 50% of residual activity (Fig.5B). Tuncagil et al. [34] showed a retain activity of 30 and 40% in the first 10 days for the immobilized invertase onto random and block copolymers, respectively, with loss of its activities at the end of 25 days. The mDE-invertase presented 64% of residual activity during 30 days storage period. Therefore, our results show that the support and immobilization protocol are efficient for enzyme stability. The decrease of activity of immobilized invertase during the reuse may be due to loss of magnetic composite particles between each cycle.

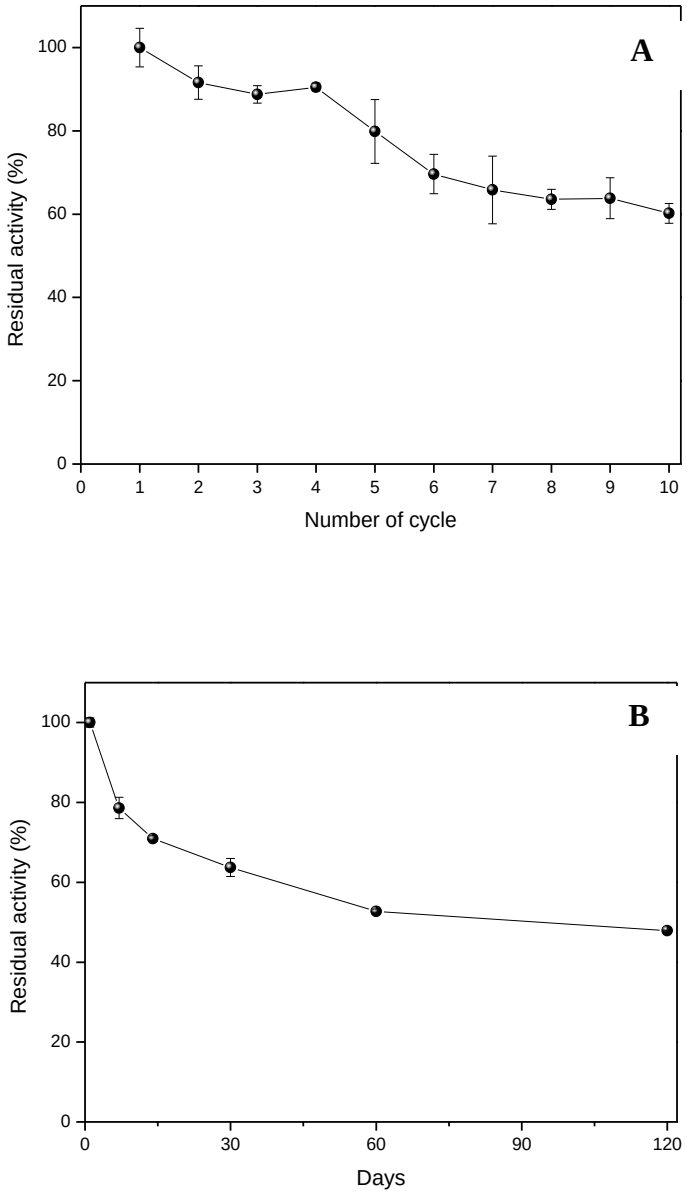

Figure 5. Reusability of the mDE-invertase in short (A) and long term (B).

4. Conclusion

Invertase was successfully immobilized on mDE and hydrolysis process of sucrose was optimized through 2^4 factorial experimental design. The experimental conditions that maximized the invertase catalytic activity in the sucrose hydrolysis were pH 4.5, temperature of 45°C, 0.25M sucrose concentration and 0.05 mg mL^{-1} invertase concentration. Thereafter, this immobilized derivative showed a better thermal stability when compared with the free enzyme. At 35°C and 45°C the free invertase was inactivated at a much faster rate than the mDE-invertase. In addition, immobilized

invertase on mDE showed excellent storability with a good retained activity (above 80%) after 120 days storage period. The activity of mDE-invertase was stable for 10 cycles retaining more than 60% of residual activity. And the mDE-invertase stored at 4°C had a long term reuse of 120 days with about 50% of residual activity. These results obtained allow concluding that mDE particles can also be easily applied for immobilization of other industrial enzymes as well as the mDE-invertase to be used in the production of inverted sugar.

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Capítulo 7

5.6. Artigo a ser submetido ao periódico *Journal of Colloid and Interface Science*

Título: Polyaniline coating on magnetic diatomite: Characterization and its use as matrix for protein immobilization

Autores: Mariana Cabrera, Sílvia Guedes Braga, Taciano França da Fonseca, Raquel Varela Barreto de Souza, Jackeline da Costa Maciel, Fernando Soria, David F.M. Neri, Luiz B. Carvalho Jr.[1,*]

Polyaniline coating on magnetic diatomite: Characterization and its use as matrix for protein immobilization

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Abstract

A magnetic diatomite covered with polyaniline (mDE-PANI) was prepared in two-steps. Firstly the magnetite was synthetized on diatomite surface and after the magnetic diatomite was treated with aniline to form a layer of polyaniline by oxidative polymerization. This hybrid material was characterized by particle size analysis, X-ray diffraction (XRD), scanning electron microscopy (SEM), surface area measurements, Fourier transform infrared (FTIR), Mössbauer spectroscopy (MS) and magnetization measurements. The mDE-PANI had a particle size of 2.43 μm and exhibited mesoporous and superparamagnetic behavior. The coating process no affected the magnetic property of the material. The optimum pH and temperature profile of the immobilized invertase were the same than that of free enzyme. The immobilized derivative showed a marked increase in K_m and V_{max} around of 3-fold and 6- fold, respectively, when compared with the free enzyme. However, the thermal stability of the immobilized invertase was much higher than that of free enzyme. In addition, the mDE-PANI-invertase was reused for 10 times and retained 55% of its initial activity. The hybrid material obtained has potential commercial applications, i.e. as a support for the covalent immobilization of invertase and this immobilized biocatalyst can be used successfully in the production of invert syrup.

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Keywords: Hybrid material; Diatomaceous earth; Magnetite; Polyaniline; Immobilization

1. Introduction

Enzymes are important biomolecules for industrial applications and its main advantage is a better thermal stability and reuse through the immobilization process. Today there are many studies of enzymes immobilized as well as matrix based different materials. Huang et al [1] reported that the support usually has significant effects on the performances of the catalysts due to the synergistic effect between the support and the catalytic species. Thus to work with hybrid materials, composed by two or more compounds of organic and inorganic origin, could be a good and interesting proposal as matrix for biomolecule immobilization. It is very important not only to combine the properties of the parent components but to develop specific new and useful properties for intended purpose. Of all the materials, clays have attracted much attention due to their high cation exchange capacity, large surface area, low cost and ready availability [2, 3]. Diatomite or diatomaceous earth (DE) is a lightweight sedimentary rock composed mainly of silica ($SiO_2.nH_2O$) microfossils of aquatic unicellular algae. This clay mineral readily available in nature, consists of a wide variety of shape and sized diatom units (DU), typically 10-200 μm, in a structure containing up to 80-90% porosity [4]. Due to a high surface area and porous structure, small particle size, chemical inertness, low thermal conductivity, low density as well as low cost the diatomite has a big number of industrial applications as filtration media for various beverages, inorganic and organic chemicals, adsorbent, catalytic support and biological support [5, 6] . The diatomite-supported catalysts showed promising applications in catalysis [7].

Magnetite (Fe_3O_4) particles of nanometer and micrometer dimensions and composites of this material have attracted increasing research interest in the fields of catalysis as a catalytic support of several biomolecules, environmental remediation, the biomedical field and sensing devices, cell labeling and immunomagnetic separations, magnetic resonance imaging, targeted drug delivery and bio-imaging in the last years [8-15]. The magnetite has the property of being easily separated and collected by an external magnetic field. The bare magnetic particles tend to easily aggregate and therefore their use for bioanalytical purpose can be difficult. The surface these particles can be functionalized with different organic or inorganic coatings in a core-shell format or be prepared in a composite from using various synthetic polymers, like polyaniline [8, 16]. Thereby, these magnetic particles can either be used for biomolecules immobilization, or functionalized or encapsulated in polymers or silica materials to fabricate hybrid composites with increased biocompatibility and added functionalities. To our knowledge, little attention has been paid to the biomolecule immobilization on modified diatomite as a matrix [17-21], especially when this hybrid material is covered with polyaniline (PANI). Besides, due to the unique combination of physical and chemical

properties the diatomite mineral together with the magnetite and polyaniline, should be a hybrid material promising as good matrix for the immobilization process, as required for industrial applications using enzymes immobilized.

The objective of this work was to prepare a hybrid support composed by magnetite, diatomite and aniline to be used as a matrix for biomolecule immobilization, using in this work the invertase as model enzyme. The magnetite was synthesized on diatomite surface and after this material was treated with aniline to form a layer of polyaniline over the magnetic diatomite particles. The magnetic diatomite covered with polyaniline (mDE-PANI) was characterized by particle size analysis, X-ray diffraction (XRD), scanning electron microscopy (SEM), surface area measurements, Fourier transform infrared (FTIR), Mössbauer spectroscopy (MS) and magnetization measurements and were also carried out assays for the characterization of immobilized derivative.

2. Materials and methods

2.1. Materials

Diatomaceous earth (DE) was kindly supplied by TAMER S.A. (Salta, Argentina). A process of water washing and repeated sedimentation was applied to purify the raw DE. Invertase from Baker's yeast, aniline, glutaraldehyde and bovine serum albumin were purchased from Sigma Aldrich Chemicals (St. Louis, USA). All other chemicals were of high purity available commercially.

2.2. Synthesis of the magnetite/diatomite composite

The synthesis of magnetic DE was performed according to Amaral et al [22] with the next modifications: (a) incubation temperature of DE with $FeCl_3.6H_2O/FeCl_2.4H_2O$ by 30 minutes was extended from 80ºC to 100ºC; (b) final pH magnetization was 11.0 adjusted with ammonium hydroxide (7.6 M). The magnetic diatomaceous earth (mDE) obtained was washed with distilled water until pH 7.0 and recovered by a magnetic field (Ciba Corning; 0.6 T). The mDE was dried at 50 ºC overnight and then sieved.

2.3. Magnetite/diatomite composite covered with polyaniline

The magnetic particles of diatomite were treated with aniline to form a layer of polyaniline by oxidative polymerization. This process was studied through a design of experiment (2^4 complete factorial design) where four variables (oxidative agent type, aniline concentration, time of polymerization and temperature of polymerization) were analyzed as function of enzymatic activity as dependent variable. In this work will be displayed the best conditions obtained in the DOE above

mentioned. Magnetite/diatomite particles were treated with 0.1 M $KMnO_4$ solution at 25 °C for 1 hour, washed with distilled water and immersed into 0.25 M aniline solution prepared in 2.0 M HCl. The polymerization was allowed to occur for 30 minutes at 4°C. The mDE-PANI was successively washed with distilled water, 2.0 M HCl and distilled water to remove residual aniline. Finally, it was dried at 50°C for 4 h.

2.4. Immobilization process of invertase

mDE-PANI particles (0.01 g) were treated with 2.5 % v/v (2 mL) prepared in 0.2 M sodium acetate buffer (pH 5.0) for 1 h under stirring at 25°C. Activated mDE-PANI was washed several times with distilled water and 0.2 M sodium acetate buffer, pH 5.0 until the washings became colorless. The treated particles were recovered using magnetic field (0.6 T). Invertase (0.1 mg protein/mL) was incubated with mDE-PANI particles (0.01 g) for 12 h at 4°C under mil stirring. Afterwards the mDE-PANI particles were washed five times with 0.2 M sodium acetate buffer, pH 5.0. The invertase immobilized on mDE-PANI (mDE-PANI-invertase) was collected by the magnetic field and the supernatants including the first two washings were used for protein determination. The amount of immobilized protein was calculated by the difference between the offered protein amount and that found in the supernatants and washings. The mDE-PANI-invertase was stored in sodium acetate buffer at 4 ºC for further use.

2.5. Enzyme assay

Invertase activity was determined by using 0.15 M sucrose (10 mL) prepared in sodium acetate buffer (0.2 M, pH 4.5). After exactly 15 min of incubation at 25ºC, 20 µl the sample was withdrawn and added to 2.0 mL of working solution in order to measure released glucose using a glucose oxidase-peroxidase (GOD/POD) enzymatic kit (Doles, Goiás, Brazil). The enzyme activity unit (U) was defined as the amount of enzyme releasing 1 µmol of glucose per minute under the assay conditions.

Protein determination was carried out according to Lowry et al[23] using bovine serum albumin as the standard protein.

2.6. Characterization

The mDE-PANI particles obtained were characterized by particle size analysis, X-ray diffraction (XRD), scanning electron microscopy (SEM), surface area measurements, Fourier transform infrared (FTIR), Mössbauer spectroscopy (MS) and magnetization measurements. The size and size distribution were determined with a Microtrac S3500 particle size analyzer. The X-ray

diffractograms were recorded on a Siemens D5000 X-ray diffractometer using CuKα radiation (λ= 1.5406 Å). SEM images were obtained with a scanning electron microscope (FEI Model QUANTA 200 FEG). Surface area and porosity were determined with a Micromeritics ASAP 2420 porosimeter. The isotherms were obtained at 77 K using N_2 as an adsorbate. The specific surface area was calculated using the Brunauer-Emmett-Teller (BET) model. Pore size distribution and pore volume were determined from the desorption branch of the isotherms using the Barrett-Joyner-Halenda (BJH)-plot method. FTIR spectra in the 4000-400 cm^{-1} range were recorded in a BRUKER instrument model IFS 66. Mössbauer spectra were recorded at 4.2 K in a transmission geometry using a conventional ^{57}Fe Mössbauer spectrometer employing a 50 mCi ^{57}Co/Rh source. The spectra were analyzed using least squares method assuming Lorenzian line shapes. The isomer shift (IS) values are relative to α-Fe at room temperature. Magnetization measurements were performed at 298 K in magnetic fields varied from 0 to 50 KOe (5.0 T) using a SQUID magnetometer (Quantum Design Model MPMS-5S).

2.7. pH and temperature profile

Invertase activity was assayed over the pH range of 3.0-7.0 (0.2 M sodium acetate buffer for pH 3.0-5.5 and 0.2 M sodium phosphate buffer for pH 5.5-7.0) at 25°C for 15 min. For temperature profile study, the activity was assayed at different temperatures (30, 40, 45, 50, 55, 60 and 70°C) for 15 min at pH 4.5.

2.8. Kinetic studies

The activity of the free and immobilized invertase was assayed at 25°C with different final concentrations of sucrose, ranging from 0.025 to 0.25 M. The determinations were repeated twice and the respective kinetic parameters, including K_m and V_{max}, were obtained by non-linear regressions, using the PRISM software of GraphPad, USA.

2.9. Thermal stability

For thermal stability, the free and immobilized invertase was incubated at different temperatures (45, 50 and 55°C) and times (30, 60, 90 and 120 min) in a temperature-controlled water bath. Thereafter, the treated samples were cooled at 25°C. Then the residual activity was determined using sucrose as above described.

3.0. Reuse of mDE-PANI-invertase

The mDE-PANI-invertase was stored in sodium acetate buffer (0.2 M, pH 5.0) at 4°C and was reused 10 times at 30 minutes interval. The residual activity (%) was measured with sucrose (0.25 M) as substrate. After assay, immobilized preparation was washed with sodium acetate buffer and magnetically collected for the next activity cycle.

3. Results and discussion

3.1. Characterization of mDE-PANI

Initially, the magnetic composite particles formed by magnetite and diatomite had a particle size of 1.49 ± 0.27 μm. And when the mDE was covered with PANI, the hybrid material had a particle size of 2.43 ± 0.07 μm. Can be observed that the formation of PANI layer caused an increase in the particle size as result of the aggregation of inorganic particles surrounded by a PANI, this result is according with other authors [24-26]. Jaramillo-Tabares et al [24] reported that the oxidative polymerization method has demonstrated to be one of the more efficient techniques to get good polymer-particle interaction. Moreover, major properties are improved using this technique mainly due to a higher contact area and lower interphase resistance between the components.

The XRD analyses were used to further probe the phase identification and crystalline structures of the samples. X-ray diffraction patterns of the magnetite, mDE and mDE-PANI are presented in Fig. 1. The main characteristic peaks of the magnetite particles (Fig. 1A) located at 2θ= 30.32°, 35.75°, 43.32°, 53.89°, 57.34° and 62.96° are attributed to the crystal planes of magnetite at 220, 311, 400, 422, 511 and 440, respectively [27]. The XRD patterns of mDE and mDE-PANI (Fig. 1B and 1C) are in good agreement with that of the referenced amorphous silica, characteristic of a broad peak centered at ca. 21.8° [28]. By analyzing the XRD patterns it is observed that the magnetite is the most predominant crystalline phase in all samples. Furthermore, is possible to observe that the intensity of the magnetite peaks was not changed after cover process with polyaniline over the magnetite/diatomite composite. Polyaniline has also some degree of cristallinity and its maximum peak at 25° can be assigned to the scattering from polyaniline chains at interplanar spacing [29]. The XRD patterns of mDE and mDE-PANI are similar indicating that polyaniline has no effect on crystallization performance of diatomite composite [30].

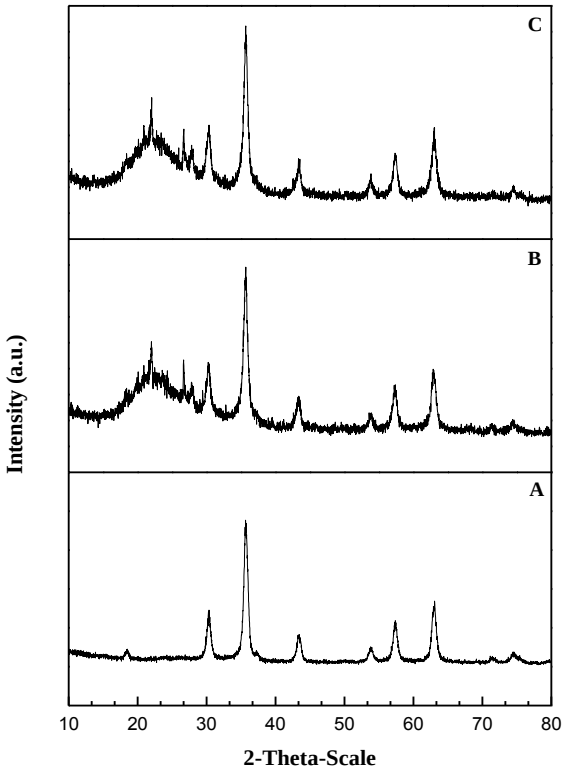

Figure 1. X-ray diffraction plots of magnetite (a), mDE (b) and mDE-PANI (c).

SEM images of DE, mDE and mDE-PANI are given in Fig. 2. From Fig. 2a, it is found that the structure of diatomite shell is destroyed and can be also observed irregular particles. The magnetization process of diatomite allowed changing the morphology of the material (Fig. 2b). However, the coating process of magnetic composite showed a similar texture than the mDE, but with layers more irregular and a high surface roughness (Fig. 2c). Probably, this surface alteration can be attributed at aggregates of magnetite on diatomite surface as well as the polymeric material of PANI covering the mDE particles.

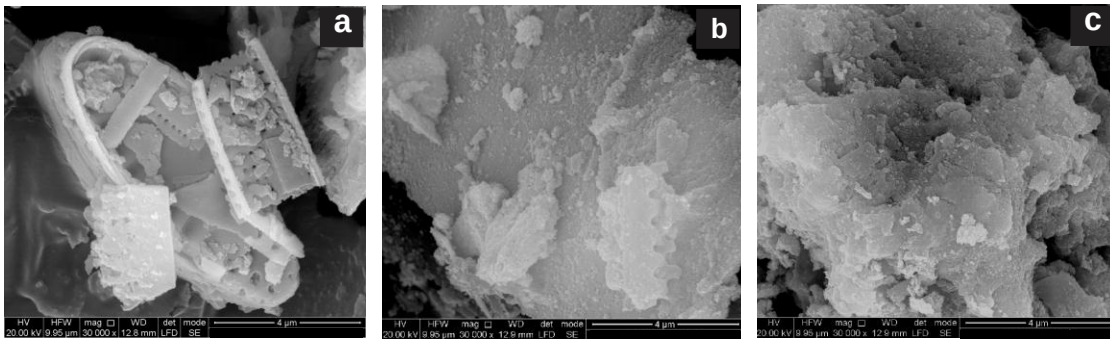

Figure 2. SEM images of DE (a), mDE (b) and mDE-PANI (c).

Surface area, pore volume and pore size of DE, mDE and mDE-PANI particles are described in Table 1. As a result of the magnetization process, the mDE particles presented a higher surface area and pore volume when compared to DE particles. This increase can be indicative of the creation of open pores on the diatomite backbone surface, as a consequence of magnetite deposits on surface of diatomite. Yuan et al [28] also reported this behavior with magnetic diatomite particles. However, after polymerization process, the mDE-PANI particles showed a decrease in the surface area and pore volume. It can be attributed to the PANI penetrates in porous regions of magnetite [31]. The values of pore size for DE, mDE and mDE-PANI are in the range of mesoporous solid (between 2-50 nm) according to IUPAC-classification.

Table 1. Surface area, pore volume and pore size of DE, mDE and mDE-PANI.

Sample	S_{BET} ($m^2\ g^{-1}$)	Pore Volume ($cm^3\ g^{-1}$)	Pore Size (nm)
Magnetite	121.8	0.35	9.3
DE	67.4	0.14	6.8
mDE	94.0	0.23	8.3
mDE-PANI	51.9	0.12	8.5

Fig. 3. shows FTIR spectra of magnetite, mDE and mDE-PANI. The magnetite spectrum exhibited absorption bands at around 631 and 566 cm^{-1} characteristic of the Fe-O bond [26, 32]. The band at 1638 cm^{-1} is ascribed to the hydroxyl characteristic peak of water adsorbed on the surface [33]. The spectral band intensities of mDE were around 1097, 797 and 470 cm^{-1}. The band at 1097 cm^{-1} (strong and broad) is mainly due to siloxane (Si-O-Si) stretching, while the bands at 797 and 470 (strong and narrow) cm^{-1} are due to (Si-O) stretching of silanol group and (Si-O-Si) bending vibration, respectively [34, 35]. After magnetization process, the mDE spectrum showed the same absorption bands of Fe-O bond at around 637 and 569 cm^{-1}, which supported the presence of magnetite particles. The coating with PANI on magnetite/diatomite surface was visible at 1482 cm^{-1}, the absorption band corresponding to C=C stretching mode for the benzenoid rings [30, 36]. These results revealed the presence of magnetite and PANI coating on the diatomite particles. Thereby, hybrid material (mDE-PANI) obtained with magnetic property and functional groups available can be used as a matrix in the biomolecules immobilization by covalent binding.

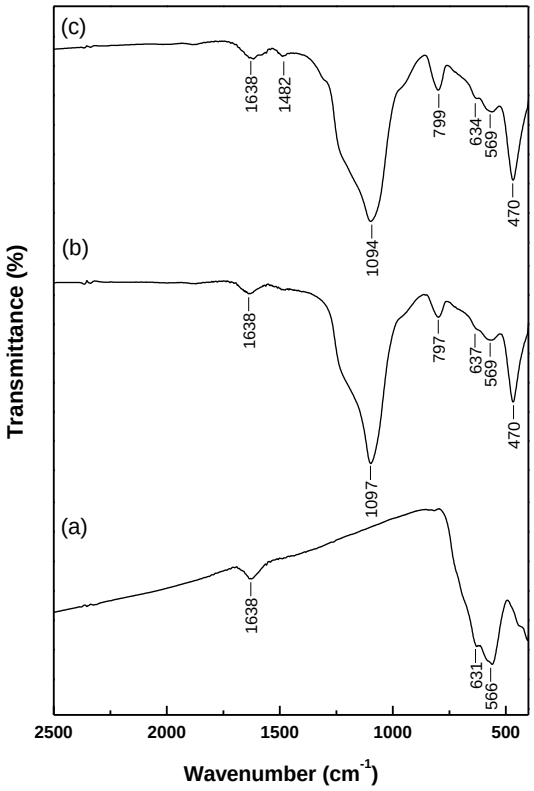

Figure 3. FTIR of magnetite (a), mDE (b) and mDE-PANI (c).

Mössbauer spectra of magnetite, mDE and mDE-PANI are shown in Fig. 4. The spectra were fitted using a two sextet model (sextet1 and 2) for all samples. For the magnetite, the first one (A sites) has a hyperfine magnetic field, δ_{hf} = 47.5 T, and an isomer shift, IS = 0.31 mm/s; assigned to Fe^{3+} ions; the second sextet (B sites) has a, δ_{hf} = 43.2 T, and IS = 0.33 mm/s; this sextet correspond to the mixed Fe^{2+} –Fe^{3+} ions [37]. These values are similar to the bulk material (sextet 1: δ_{hf} = 49.0 T and IS = 0.26 mm/s and sextet 2: δ_{hf} = 46.0 T and IS = 0.67 mm/s), but slightly lower [38]. The deviation in the ideal area ratio (1:2) of the iron in tetrahedral and octahedral position obtained from the subspectra area is due to the smaller particle size compared to their bulk counterpart [39]. The hyperfine magnetic fields for mDE (sextet 1 equal to 52.3 T and sextet 2 equal to 50.3 T) and mDE-PANI (sextet 1 equal to 52.8 T and sextet 2 equal to 50.4 T) showed slightly higher values than pure magnetite (Fig.4b-c and Table 2). The mDE spectrum show an increase in the A sites compared to the spectrum for pure magnetite, whereas the intensity of B sites (Fe^{3+}) decreases. For mDE-PANI spectrum was observed a decrease in the B sites compared to the spectrum for pure magnetite, whereas the intensity of A sites increases. In the latter case, this behavior represents a clear solid-state electronic interaction between magnetite and PANI and may be attributed to an electron

transfer process from the B sites (Fe^{2+} –Fe^{3+}) of magnetite to PANI as also observed by Jaramillo-Tabares et al [24].

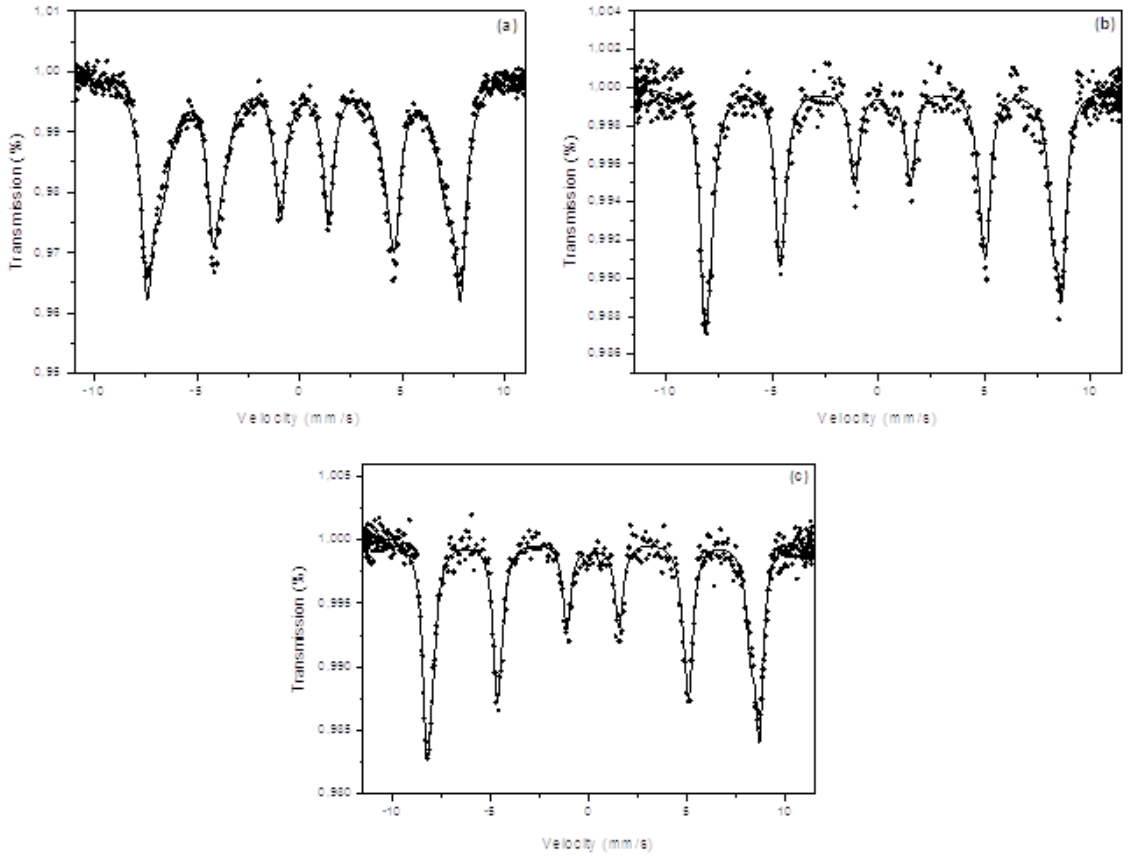

Figure 4. Mössbauer spectra of magnetite (a), mDE (b) and mDE-PANI (c).

Table 2. Hyperfine parameters. IS: isomer shift; δ_{hf}: hyperfine field; Area: relative area. Uncertainty in IS is 0.02 mm/s, while that is δ_{hf} is 0.5 T. Area is accurate within 2%.

Sample	Component	IS (mm s^{-1})	δ_{hf} (T)	Area (%)
Magnetite	Sextet 1	0.31	47.5	49.0
	Sextet 2	0.33	43.2	51.0
mDE	Sextet 1	0.33	52.3	49.5
	Sextet 2	0.28	50.3	50.5
mDE-PANI	Sextet 1	0.32	52.8	61.7
	Sextet 2	0.28	50.4	37.3

The magnetic properties of magnetite, mDE and mDE-PANI particles were measured by applying an external magnetic field at 298 k. Fig. 5. shows the magnetization curves for all samples indicating that there was neither remnant magnetization (Magnetization equal to zero for Magnetic field equal to zero) no coercivity. The saturation magnetization of the magnetite, mDE and mDE-PANI whose values were 60, 12 and 10 emu g^{-1}, respectively, are also observed in Fig. 5. The decreased saturation magnetization for the mDE and mDE-PANI particles can be attributed to surface effects, such as magnetically inactive layer producing disordered surface [40]. Therefore, we can conclude that the magnetization of magnetic composite is lower than pure magnetite due to difficult alignment of magnetic dominions in the material as also reported by Neri et al [41] and Maciel et al [26]. On the other hand, the coating with PANI does not affect the magnetization of mDE particles. In addition, the magnetite, mDE and mDE-PANI particles exhibited ferromagnetic and superparamagnetic behavior.

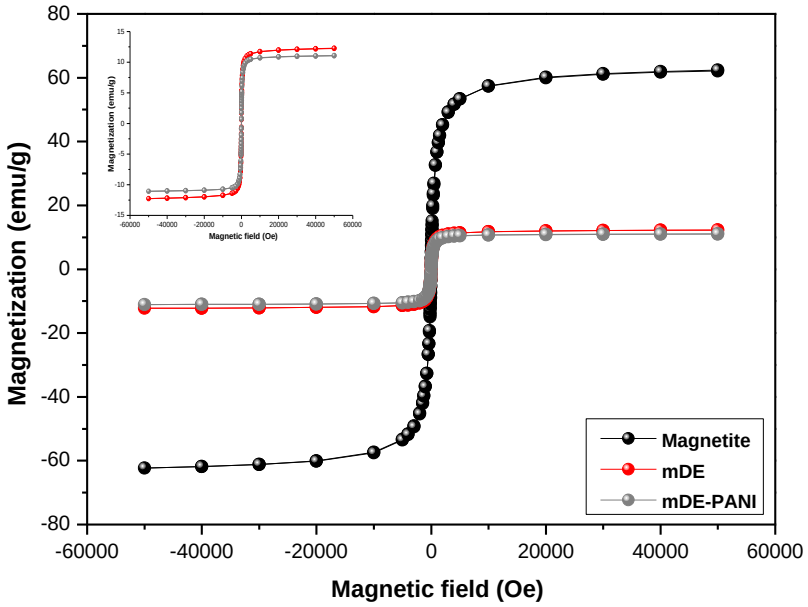

Figure 5. Magnetization curves of magnetite, mDE and mDE-PANI. The insets are the enlarged magnetization curves of the mDE and mDE-PANI.

3.2. Characterization of mDE-PANI-invertase

3.2.1. Effect of pH and temperature on invertase activity

The most studies about of characterization of invertase have reported an optimum pH in the range of 4–5.5 and an optimum temperature in the range of 45–65°C for the enzyme activity [42-49]. Our results are presented in the Table 3 and the properties of immobilized derivative are not different of

the free enzyme, indicating that the immobilization process not produced significant alterations of the physical and chemical properties of the enzyme. Furthermore, the no increase in optimum pH and temperature can be explained due to covalent bond formation might also reduce the conformational flexibility of the enzyme and make it more stable against pH and temperature change. These results are in agreement with the previous reports that invertase presents the same values for pH and temperature after immobilization process [18, 45, 49, 50].

3.2.2. Kinetic parameters

The effect of substrate concentration on the enzymatic activity of the free and immobilized invertase on mDE-PANI was analyzed for the same range of sucrose concentrations (0.025 to 0.25 M). The apparent kinetic parameters of the free and immobilized invertase are presented in Table 3. The invertase immobilization led to an increase in the K_m value around of three-fold, which indicated that the formation of the enzyme-substrate complex was more difficult for the immobilized enzyme. A decrease in the substrate affinity is generally observed after invertase immobilization on different supports [51-53]. The V_{max} value of mDE-PANI-invertase and the free form was 3828 and 630 U mg^{-1} enzyme, respectively, thus indicating the catalytic activity of the mDE-PANI-invertase increased six-fold compared to the free form of the enzyme. Thereby, we concluded that the K_m and V_{max} values were significantly affected after covalent immobilization of invertase on mDE-PANI particles. These changes in the affinity, is probably caused by the lower accessibility of the substrate to the active site of the immobilized invertase.

Table 3. Properties and kinetic parameters of free and immobilized enzyme on mDE-PANI.

	pH	T (ºC)	K_m (mM)	V_{max} (U mg^{-1} enzyme)
Free enzyme	4.5	50	73.5	630
Immobilized enzyme	4.5	50	198.8	3828

3.3. Thermal stability

Thermal stability studies of free and immobilized invertase were carried out by enzyme incubation in the absence of substrate at different temperatures (45, 50 and 55°C) and times (30, 60, 90 and 120 min). Fig. 6 shows the heat inactivation curves of free and immobilized invertase. At 45°C, the activity of the immobilized invertase and free enzyme retained their activities about 75 and 35%, respectively after 120 min for the same incubation time. At 50°C, the activities of the immobilized and free enzymes were retained at levels of 61 and 33%, respectively. The immobilized form (mDE-PANI-invertase) was inactivated at a much slower rate than the free form. At 55°C, the

profile for both preparations was similar. The immobilized and free enzymes retained its activity about 38 and 26% after 120 min. These results suggest that the thermostability of immobilized invertase increased as a result of covalent immobilization on mDE-PANI particles. Similar observations have been previously reported for various immobilized systems [46, 54, 55].

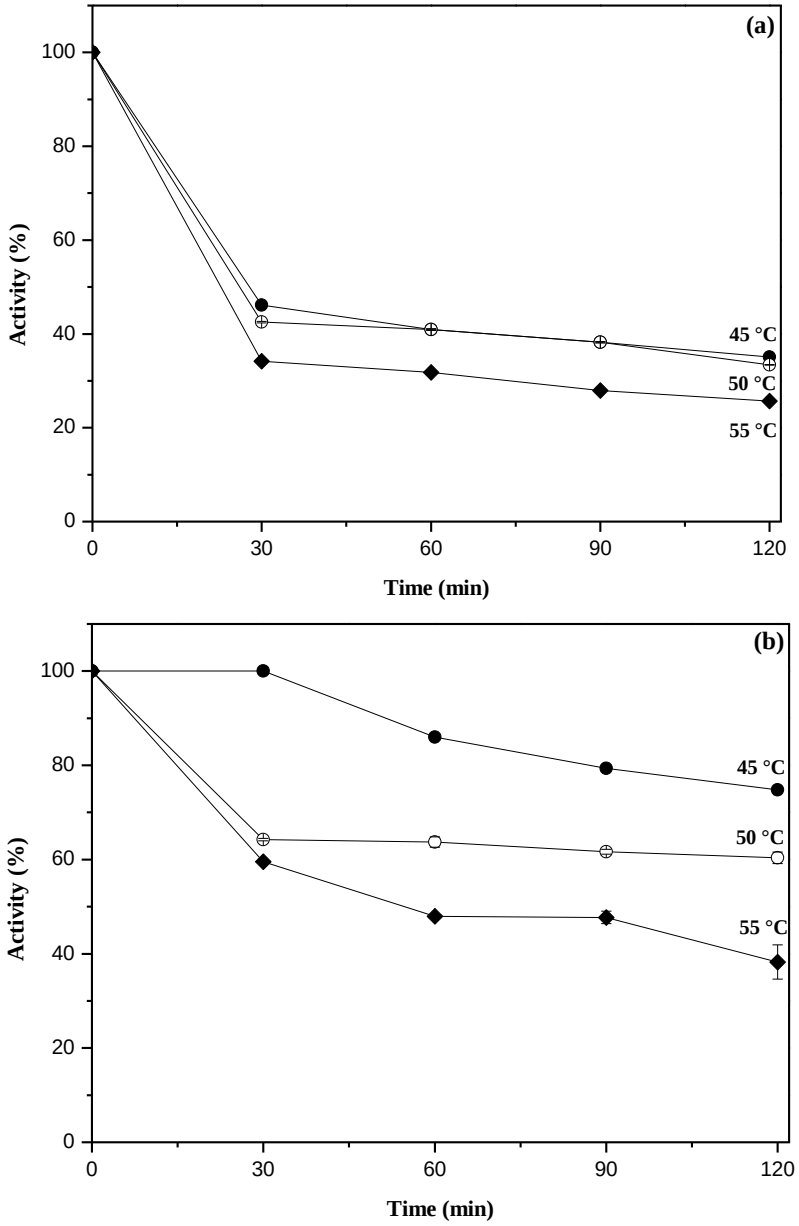

Figure 6. Influence of temperature on the stability of free invertase (a) and mDE-PANI-invertase (b).

3.4. Reuse

Immobilized invertase was used repeatedly 10 times and the residual activities (%) are presented in Fig. 7. After the 10^{th} use, immobilized invertase on mDE-PANI retained 55% of its initial activity. Raj et al [56] reported the appreciable reusability of the immobilized invertase on nanogel-matrix up to eight cycles, but the relative activity (%) decreased to 11.03% after the 9th cycle. Probably,

decrease of activity of mDE-PANI-invertase can be attributed to loss of magnetic composite particles between each cycle. Thereby, our results show that the hybrid material (mDE-PANI) and immobilization protocol are efficient for the reuse of the enzyme. And this could create a very important economic advantage in industrial applications such as an enzyme reactor for production of invert syrup.

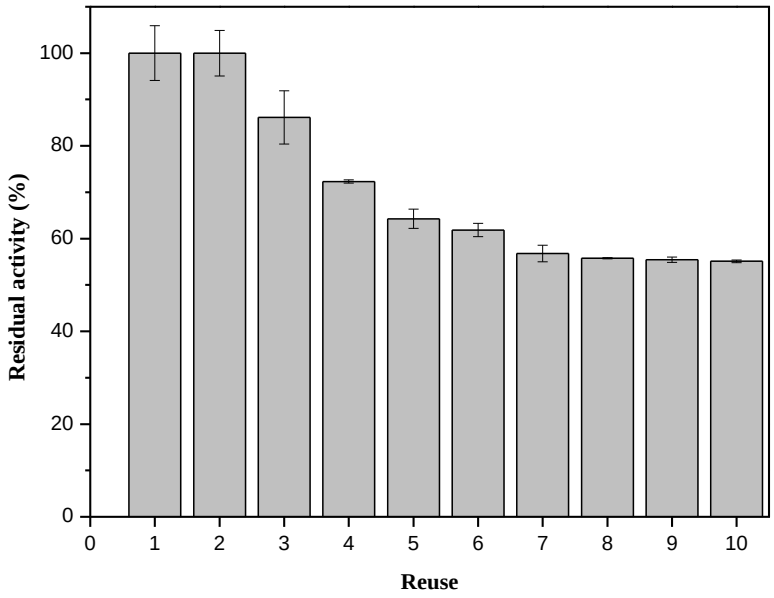

Figure 7. Effect of reuse on the activity of mDE-PANI-invertase.

4. Conclusion

The magnetic diatomite coated with polyaniline (mDE-PANI) was produced by magnetite co-precipitation on raw diatomite surface and after magnetization process was carried out the coating with PANI of mDE particles by oxidative polymerization of aniline. The hybrid material was characterized by several techniques and the mDE-PANI particles exhibited mesoporous and superparamagnetic behavior. The coating process did not affect the magnetic property of the material. In this study, mDE-PANI particles were used as a support for invertase immobilization. As previously mentioned, the optimum pH and temperature profile of the immobilized invertase were the same than that of free enzyme. The immobilized derivative showed a marked increase in K_m and V_{max} around of 3-fold and 6- fold, respectively, when compared with the free enzyme. However, the thermal stability of the immobilized invertase was much higher than that of free enzyme. In addition, the mDE-PANI-invertase was reused for 10 times and retained 55% of its initial activity. The immobilized invertase on mDE-PANI can be used successfully in the production of invert syrup.

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6 Conclusões

- A α-L-ramnosidase imobilizada covalentemente nos suportes ferromagnéticos: Dacron-hidrazida, POS/PVA e quitosana, reteve 36,3%; 40,4% e 4,1% de sua atividade inicial, respectivamente, quando comparada com a enzima livre. Os perfis de pH e temperatura para todas as enzimas imobilizadas não mostraram diferença em relação à enzima livre, exceto o derivado de quitosana que apresentou maior temperatura máxima. O derivado enzimático Dacron-hidrazida mostrou melhor desempenho que a enzima livre para hidrolisar a naringina 0,3% (91% e 73% após 1 h, respectivamente) e na síntese de ramnósidos (0,116 e 0,014 mg narirutina após 1 h, respectivamente);
- Os compósitos magnéticos a partir de minerais (mMMT, mTD e mTD-PANI) apresentaram tamanho de partícula inferior a 20 μm e comportamento de materiais mesopororos e superparamagnéticos. As medidas de magnetização revelaram uma redução de seis vezes nas medidas de saturação da magnetização, provavelmente devido aos efeitos de superfície, tal como uma camada magneticamente inativa produzindo uma superfície desordenada. O processo de revestimento com polianilina não afetou a propriedade magnética do suporte mTD. Os espectros Mössbauer dos compósitos magnéticos realizados a 4,2 K mostraram uma mistura de magnetita e maguemita em igual proporção na mMMT, e uma fase de magnetita pura nas amostras de mTD e mTD-PANI. Estes resultados foram confirmados por DRX;
- A mMMT-invertase apresentou igual pH ótimo, maior temperatura máxima e estabilidade térmica quando comparada com a enzima livre, e manteve 91% da sua atividade inicial após 7 ciclos consecutivos de reutilização;
- O processo de imobilização da invertase em mTD foi estudado através de um planejamento fatorial fracionário $2^{7-2}_{IV.}$ As condições operacionais escolhidas para o processo de imobilização foram: concentração de APTES 2,5%, tempo de contato com APTES 2 h, concentração de glutaraldeído 10%, tempo de contato com glutaraldeído 1 h, tempo de imobilização 12 h, pH de imobilização 5,5 e concentração de invertase 0,15 mg/mL. O processo de hidrólise da sacarose pela mTD-invertase foi estudado por um planejamento fatorial completo 2^4, foram encontradas as seguintes condições experimentais: pH 4,5; temperatura de 45°C; concentração de sacarose 0,25 M e concentração de invertase 0,05 mg mL^{-1}. A mTD-invertase mostrou bom desempenho quanto à estabilidade de armazenamento, tempo de prateleira e reuso com atividade retida de 88% (2 meses), 50% (4 meses) e 60% (10 reutilizações), respectivamente;

- A mTD-PANI-invertase apresentou igual pH ótimo e temperatura máxima, e maior termoestabilidade que a enzima livre, e manteve 55% da sua atividade inicial após 10 ciclos consecutivos de reutilização;
- Os derivados imobilizados mMMT-invertase, mTD-invertase e mTD-PANI-invertase mostraram 83,0%; 92,5% e 81,2% de atividade retida, respectivamente, quando comparada com a enzima livre;
- Portanto, os resultados obtidos demonstram que os compósitos magnéticos produzidos a partir de matérias orgânicos e inorgânicos (minerais de baixo custo e altamente disponíveis na natureza) são matrizes promissoras para a imobilização covalente de α-L-ramnosidase e invertase, bem como para a imobilização de outras biomoléculas.

7 Perspectivas

Os resultados obtidos foram de elevada importância e superaram as expectativas. Os diversos compósitos magnéticos formados a partir de Dacron-hidrazida, POS/PVA, quitosana, argila montmorilonita, terra de diatomáceas, magnetita e polianilina, poderão ser utilizados como matrizes para a imobilização covalente de biomoléculas. Pretende-se também iniciar a preparação de mais quatro (04) artigos científicos relacionados às enzimas tripsina e β-galactosidase imobilizadas em terra de diatomáceas magnética (mTD) e terra de diatomáceas magnética revestida com polianilina (mTD-PANI). Além disso, se dará inicio as atividades do Pós-Doutorado, projeto intitulado "SÍNTESE, CARACTERIZAÇÃO E APLICAÇÕES BIOMÉDICAS DE QUATUM DOTS (QDS) BIOMAGNÉTICOS", no Programa de Pós-Graduação em Biologia Aplicada à Saúde do Laboratório de Imunopatologia Keizo Asami – Universidade Federal de Pernambuco.

8 Anexos

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Abstract:	Magnetic particles as matrix for enzyme immobilization have been used due to the enzymatic derivative can be easily removed from the reaction mixture by a magnetic field. This work presents a study about the synthesis and characterization of iron phases into magnetic montmorillonite clay (mMMT) and magnetic diatomaceous earth (mDE) by 57Fe Mössbauer spectroscopy (MS), magnetic measurements and X-ray diffraction (XRD). Also these magnetic materials were assessed as matrices for the immobilization of invertase via covalent binding. Mössbauer spectra of the magnetic composites performed at 4.2 K showed a mixture of magnetite and maghemite about equal proportion in the mMMT, and a pure magnetite phase in the sample mDE. These results were verified using XRD. The residual specific activity of the immobilized invertase on mMMT and mDE were 83% and 92.5%, respectively. Thus, both magnetic composites showed to be a promising matrix for covalent immobilization of invertase.

8.3 Trabalho publicado em periódico

Soria, F., Ellenrieder, G., Oliveira, G.B., Cabrera, M., Carvalho Jr., L.B., 2012. α-L-Rhamnosidase of Aspergillus terreus immobilized on ferromagnetic supports. Applied Microbiology and Biotechnoloy. 93, 1127-1134.

8.4 Trabalhos apresentados em congressos

Cabrera, M., Soria, F., Carvalho Júnior, L.B. Invertasa inmovilizada en montmorillonita ferromagnética. **I Workshop Internacional em Biotecnologia, III Encontro ALFA-VALNATURA e III Jornada Científica do LIKA. 2008.** Recife, Brasil.

Cabrera, M., Soria, F., Carvalho Júnior, L.B. **Matrizes Naturais para a Imobilização de Invertase. II Simpósio de Inovação em Ciências Biológicas. 2009.** Recife, Brasil.

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Soria, F., Cabrera, M., Robin, J., Macoritto, A., Blanco, S., Geronazzo, H. Hidrólisis de naringina con α-L-ramnosidasa inmovilizada en arcillas regionales ferromagnéticas. **XVI Congreso Argentino de Fisicoquímica y Química Inorgánica. 2009.** Salta, Argentina.

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Zotelo, J.J.R., Soria, F., Cabrera, M.P., Destefanis, H. Inmovilización de α-amilasa *A. Niger* en silicoaluminatos naturales. **XXVIII Congreso Argentino de Química y 4to. Workshop de Química Medicinal. 2010.** Buenos Aires, Argentina.

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Souza, R.V.B., Fonseca, T. F., Cabrera M. P., Carvalho Jr, L. B. Optimization of invertase immobilization on diatomaceous earth magnetic particles. **III Simpósio Internacional em Diagnóstico e Terapêutica e VI Jornada Científica do LIKA. 2011.** Recife, Brasil.

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8.6 Orientações

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