UNIVERSIDADE TIRADENTES – UNIT

PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE PROCESSOS – PEP

ESTRATÉGIAS DE MODIFICAÇÃO DE SUPORTES MESO E NANOPOROSOS PARA IMOBILIZAÇÃO DE LIPASE DE *Burkholderia cepacia*

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ARACAJU, SE - BRASIL
FEVEREIRO DE 2017

Oliveira, Maria Vanessa Souza
O048e Estratégias de modificação de suportes meso e nanoporosos para imobilização de lipase de *Burkholderia cepacia* / Maria Vanessa Souza Oliveira ; orientação [de] Profº. Drº Álvaro Silva Lima; Profº. Drº. Álvaro Silva Lima; Profª. Drª. Cleide Mara Faria Soares – Aracaju: UNIT, 2017.

139 f. il ; 30 cm

Tese (Doutorado em Engenharia de Processos) - Universidade Tiradentes, 2017
Inclui bibliografia.

1. Lipase. 2. Líquidos iônicos próticos. 3. (eco)toxicidade. 4. Sílica modificada. 5. Nanotubos de carbono modificados I. Oliveira, Maria Vanessa Souza. II. Lima, Álvaro Silva. (orient.). III. Soares, Cleide Mara Farias. (orient.). IV. Universidade Tiradentes. V. Título.

CDU: 66.0973:577.15

SIB- Sistema Integrado de Bibliotecas

ESTRATÉGIAS DE MODIFICAÇÃO DE SUPORTES MESO E NANOPOROSOS PARA IMOBILIZAÇÃO DE LIPASE DE *Burkholderia cepacia*

Maria Vanessa Souza Oliveira

TESE SUBMETIDA AO PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE PROCESSOS DA UIVERSIDADE TIRADENTES COMO PARTE DOS REQUISITOS NECESSÁRIOS PARA A OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA DE PROCESSOS

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Aos meus amados pais, de sangue e de coração,
que sempre incentivam os meus passos
com amor, paciência e disciplina.

AGRADECIMENTOS

A Deus, por ter me concedido o dom da vida, saúde e perseverança para superar as dificuldades.

A meus familiares e ao meu namorado Denisson pelo carinho, amor, estímulo, paciência e apoio que foram essenciais para a minha vida pessoal e para a minha formação. Em especial ao meu pai Vicente (*in memoriam*) que agora é uma lembrança recheada de saudade nos nossos corações

Aos meus professores orientadores Dr. Álvaro Silva Lima e Dra. Cleide Mara Faria Soares pelos ensinamentos, confiança, amizade, oportunidades e idéias. Sem a ajuda de vocês este sonho não teria se tornado realidade. Vocês foram insuperáveis!

Aos professores Dr. Rui Silva, Dr. Filipe Oliveira, Dra. Silvana Mattedi e Dra. Rebeca Y. Cabrera-Padilla pelos valiosos conhecimentos transmitidos.

Aos colegas do laboratório, colegas do programa de pós-graduação e amigos adquiridos durante esta jornada, tanto em Aracaju como em Salvador e Portugal, pelo tempo de convívio harmonioso, ajuda, ouvido atencioso e momentos de descontração.

Ao Intituto de Tecnologia e Pesquisa e todos os seus funcionários

A Universidade Tiradentes, ao Programa de Pós Graduação em Engenharia de Processos e a sua coordenação.

Ao Cnpq, Capes e Fapitec.

A todos aqueles, sem exceção, que contribuíram de maneira direta ou indireta durante a minha caminhada e tornaram possível a realização deste trabalho de um modo ou de outro.

Resumo da Tese apresentada ao Programa de Pós-graduação em Engenharia de Processos da Universidade Tiradentes como parte dos requisitos necessários para a obtenção do grau de Doutor em Engenharia de Processos.

ESTRATÉGIAS DE MODIFICAÇÃO DE SUPORTES MESO E NANOPOROSOS PARA IMOBILIZAÇÃO DE LIPASE DE *Burkholderia cepacia*

A imobilização de enzimas em suportes sólidos está associada a vantagens tais como produção contínua, estabilidade e fácil remoçao do catalisador, as quais são atrativas em diversos processo. Desta forma, biocatalisadores foram preparados com a lipase de *Burkholderia cepacia* imobilizada em suportes mesoporosos (sílica modificada com líquido iônico prótico – LIPs) e nanoporosos (nanotubos de carbono – CNTs de diâmetros diferentes) e a (eco)toxicidade e biodegradabilidade de alguns LIPs foi estudada. Bactérias, leveduras, fungos filamentosos e semente de alface foram utilizados nos testes com os LIPs (m-2-HEAA, m-2-HEAPr, m-2-HEAB, m-2-HEAP) e os resultados mostraram que, em geral, o alongamento da cadeia alquílica dos LIPs aumenta o impacto negativo destes ompostos em vários micro-organismos testados. Os suportes de sílica foram sintetizados pela técnica sol-gel utilizando os seguintes LIPs como agentes de modificação: 2HEAP, m-2HEAP, BHEAP. Uma nova técnica de lavagem por soxleht seguida por secagem em estufa foi utilizada para retirar os agentes modificadores (LIPs). Foi realizada análise morfológica, física e química destes suportes e as propriedades enzimáticas da lipase livre e imobilizada foram determinadas. Os métodos de imobilização utilizados foram Adsorção (ADS) e ligação covalente utilizando glutaraldeído (LCG) ou epicloridrina (LCE). Os espectros de FTIR demonstraram que os LIPs foram removidos dos suportes modificados. A análise termogravimétrica mostrou que os suportes modificados apresentaram menos moléculas de água em comparação com o suporte puro. As análises morfológicas revelaram diferenças de porosidade destacando o suporte modificado com o LIP mais hidrofóbico (m-2HEAP) por apresentar maior volume e tamanho de poro em relação aos outros suportes de sílica modificados. Os biocatalisadores imobilizados por ADS apresentaram ótimos valores de estabilidade térmica ($t_{1/2}$ de 231 até 309 h), estabilidade operacional (alta atividade relativa após 40 reciclos) e baixos valores de K_m. Os maiores valores de rendimento de recuperação de atividade total foram obtidos para os biocatalisadores imobilizados no suporte modificado com m-2HEAP-S (ADS) e m-2HEAP-S (LCE). Os espectros de FTIR apresentaram a banda característica das enzimas (amida I) para todos os sistemas imobilizados. O máximo rendimento em ésteres etílicos foi obtido com o biocatalisador m-2HEAP-S (LCE) para a reação de transesterificação assistida por ultrassom. Adicionalmente o $t_{1/2}$ para este biocatalisador sob cavitação ultrassônica foi de 21,1 h. Os suportes nanoporosos (CNTs) obtiveram a superfície modificada por carboxilação seguida de aminação e posteriormente a lipase foi imobilizada por ADS. As imagens de SEM, análise termogravimétrica e análise elemental evidenciaram diferenças de pureza, área superficial e diâmetros entre os CNTs utilizados como suportes. A análise de FTIR demonstrou diferenças entre os CNTs modificados. As imagens de TEM e FTIR confirmaram a imobilização da lipase nos CNTs e o biocatalisador imobilizado com o menor diâmetro e de superfície modificada (CNT NC 7000 – NH_2) exibiu o maior rendimento de recuperação de atividade total 65,98 % e reuso de 7 ciclos.

Palavras chave: Lipase, líquidos iônicos próticos, (eco)toxicidade, sílica modificada, nanotubos de carbono modificados.

Abstract of the thesis presented to the Post-graduation Program in Process Engineering of Tiradentes University as part of the requirements for the Doctorate degree in Engineering Processes

STRATEGIES FOR THE MODIFICATION OF MESO AND NANOPOROUS SUPPORTS FOR IMMOBILIZATION OF Burkholderia cepacia LIPASE

Enzymes immobilization on solid support is associated with advantages such as continuous production, stability and easy removal of the catalyst, which are attractive in several processes. Thus, biocatalysts were prepared with *Burkholderia cepacia* lipase immobilized on mesoporous supports (modified silica with protic ionic liquid - LIPs) and nanoporous supports (carbon nanotubes - CNTs of different diameters) and the (eco) toxicity and biodegradability of some LIPs was studied. Bacteria, yeast, fungi and lettuce seeds were used in the tests with LIPs (m-2-HEAA, m-2-HEAPr, m-2-HEAB, m-2-HEAP) and the results showed that, in general, alkyl chain tends to increase the negative impact of the PILs towards all organisms and biological systems under study. Silica supports were synthesized by the sol-gel technique using the following LIPs as modifying agents: 2HEAP, m-2HEAP, BHEAP. A new soxleht wash technique followed by drying was used to remove the modified agents (LIPs). Morphological, physical and chemical analysis of these supports were performed and the enzymatic properties of free and immobilized lipase were determined. The immobilization methods used were Adsorption (ADS) and covalent binding using glutaraldehyde (CBG) or epichlorohydrin (CBE). FTIR spectra demonstrated that the LIPs were removed from the modified supports. Thermogravimetric analysis showed that the modified supports presented less water molecules compared to the pure support. Morphological analysis revealed differences in porosity highlighting the modified support with the most hydrophobic LIP (m-2HEAP) because it presents greater pore volume and pore size than other modified silica. Biocatalysts immobilized by ADS presented excellent thermal stability ($t_{1/2}$ from 231 to 309 h), operational stability (high relative activity after 40 cycles) and low K_m values. The highest total activity recovery yield were obtained for the biocatalysts immobilized on modified support with m-2HEAP-S (ADS) and m-2HEAP-S (CBE). FTIR spectra presented the characteristic band of the enzymes (amide I) for all immobilized systems. The maximum ethyl esters yield was obtained with the biocatalyst m-2HEAP-S (CBE) for transesterification reaction assisted by ultrasound. In addition the $t_{1/2}$ for this biocatalyst under ultrasonic cavitation was 21.1 h. Nanoporous supports (CNTs) obtained the surface modified by carboxylation followed by amination and subsequently lipase was immobilized by ADS. SEM images, thermogravimetric analysis and elemental analysis showed differences of purity, surface area and diameters between the CNTs used as supports. The FTIR analysis showed differences between the modified CNTs. TEM images and FTIR confirmed lipase immobilization on CNTs and the immobilized biocatalyst with the smallest diameter and surface-modified (CNT NC 7000 - NH_2) exhibited the highest 65.98% total activity recovery yield and reuse of 7 cycles.

Palavras chave: Lipase, protic ionic liquids, **(**eco)toxicity, modifed silica, modified carbon nanotubes.

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LIs - Líquidos Iônicos

LIPs - Líquidos Iônicos Próticos

CNTs – Nanotubos de carbono

ADS – Adsorção Física

LCG - Ligação Covalente utilizando Glutaraldeído como agente de ativação

LCE – Ligação Covalente utilizando Epicloridrina como agente de ativação

TG – Análise Termogravimétrica

SEM – Microscopia Eletrônica de Varredura

TEM – Microscopia Eletrônica de Transmissão

FTIR – Espectroscopia de Infravermelho com Transformada de Fourier

XRD - Difração de Raios-X

BET – Método de Brunauer–Emmett–Teller

m-2-HEAA – Acetato de N-metil-2-hidroxietilamônio

m-2-HEAPr – Propionato de N-metil-2-hidroxietilamônio

m-2-HEAB – Butirato de N-metil-2-hidroxietilamônio

m-2HEAP - Pentanoato de N-metil-2-hidroxietilamônio

2HEAP - Pentanoato de 2-hidroxietilamônio

BHEAP - Pentanoato de bis(2-hidroxietil)amônio

2HEAP-S – Suporte de sílica modificado com Pentanoato de 2-hidroxietilamônio

m-2HEAP-S – Suporte de sílica modificado com Pentanoato de N-metil-2-hidroxietilamônio

BHEAP-S – Suporte de silica modificado com Pentanoato de bis(2-hidroxietil)amônio

CBG – Ligação Covalente utilizando Glutaraldeído como agente de ativação

CBE – Ligação Covalente utilizando Epicloridrina como agente de ativação

APTS - 3-aminopropiltrietoxisilano

CAPÍTULO I

1. INTRODUÇÃO

O desenvolvimento de novos materiais com propriedades adequadas para a imobilização de enzimas é uma área emergente de pesquisa e de fundamental importância para a área de bioprocessos, pois a aplicação de biocatalisadores utilizando enzimas imobilizadas atendem à demanda por processos menos poluentes e mais seletivos.

Vários artigos indicam o progresso no uso de materiais mesoporosos, como a sílica, e suas contribuições correspondentes ao processo de imobilização de enzima (SOARES et al., 2006; CARVALHO et al., 2015; BARBOSA et al., 2016). Os materiais de sílica mesoporosa apresentam como vantagens como elevada área superficial, estabilidade química, térmica e mecânica, distribuição de poros altamente uniformes, capacidade de adsorção elevada e tamanho de poro sintonizável. Não obstante, o recente interesse em nanotecnologia tem proporcionado uma diversidade de materiais que podem ser utilizados para imobilizar enzimas devido as suas potenciais aplicações. Dentre eles os nanotubos de carbono (CNTs) se destacam por fornecer os limites máximos determinantes da eficiência enzimática, como a relação superfície/volume, a capacidade de carregamento da enzima e a resistência à transferência de massa.

Contudo, melhorias nas propriedades finais de biocatalisadores imobilizados, como, por exemplo, maior estabilidade e eficiência catalítica, são relatadas a partir da modificação superficial de suportes antes do procedimento de imobilização, por meio da utilização de agentes modificadores. Na literatura há alguns exemplos de suportes nano e mesoporosos modificados superficialmente por meio da inserção de grupos funcionais e mudanças nas propriedades morfologicas, tais como: CNTs modificados por tratamentos oxidativos (TAVARES *et al*., 2015; PRLAINOVIC *et al*., 2016) e sílica com superfície modificada com líquidos iônicos – LIs (HU *et al*., 2012; ZOU *et al*., 2014). Assim, estudos de modificação de suportes nano e mesoporosos é fundamental para preparação de biocatalisadores imobilizados eficientes com futura utilidade para os pesquisadores em varias aplicações biocatalíticas.

Os líquidos iônicos possuem várias propriedades características, o que os torna uma classe única de produtos químicos. Uma das propriedades mais importantes é sua

não-volatilidade, tornando-os alternativas "verdes" aos compostos orgânicos voláteis. No entanto, os LIs podem contribuir para a poluição das águas pois são amplamente solúveis em água significando que podem ser liberados em ecossistemas aquáticos. Desta forma, informações acerca da toxicidade dos LIs e conhecimento dos seus efeitos sobre a estrutura, estabilidade e atividade de biocatalisadores imobilizados, ainda são escassas e necessárias devido ao grande número de líquidos iônicos existentes.

LIs tem sido introduzidos em materiais de sílica com o objetivo de melhorar a formação dos mesoporos durante a obtenção do suporte e alguns estudos do nosso grupo de pesquisa apontam tendencias em potencial para o desenvolvimento deste campo (BARBOSA *et al*., 2014; MARTINS *et al*., 2016). Líquidos iônicos próticos (LIPs) foram utilizados por SOUZA *et al*. (2013) durante o processo de imobilização por encapsulamento e os autores obtiveram rendimentos de imobilização superiores a 1000%.

OLIVEIRA *et al*. (2014) aplicaram o sistema imobilizado com adição de LIPs em reação de transesterificação, o qual demonstrou que o biocatalisador apresenta eficiência catalítica para produzir ésteres etílicos a partir do óleo de babaçu refinado e etanol. A partir destas constatações, surgiu o interesse do grupo em estudar os possíveis efeitos dos LIPs aplicados como agentes modificadores de sílica com o intuito de produzir biocatalisadores adequados para posterior aplicação em reações biocatalíticas de grande interesse.

Embora haja informações sobre a utilização de líquidos iônicos próticos como aditivos no processo de imobilização, estudos empregando LIPs como agentes modificadores de suportes de sílica e posterior remoção destes agentes para aplicação da sílica modificada em imobilização de enzimas não foram encontrados na literatura.

O desenvolvimento de métodos mais eficientes, com o intuito de eliminar desvantagens como baixas taxas de conversão e longos tempos de reação, tem sido muito pesquisado. A literatura indica que reações químicas podem ser aceleradas por meio do uso de aquecimento não convencional (micro-ondas e ultrassom). As micro-ondas representam uma radiação não ionizante que influenciam os movimentos moleculares tais como migração de íons ou rotações de dipolos sem alterar a estrutura molecular. O ultrassom consiste na cavitação, fenômeno de colapso de bolhas em tempos muito curtos resultando em alta turbulência e dispersão do material contido no sistema reacional.

Desta forma este trabalho visa contribuir para a ampliação dos conhecimentos referentes a bioprocessos propondo estratégias de modificação de materiais nano e mesoporosos que fornecem características fundamentais para serem empregados como suportes na imobilização de lipase, bem como a caracterização e obtenção de parâmetros relacionados a aplicabilidade dos sistemas imobilizados em processos biocatalíticos assistidos por aquecimento não convencional (micro-ondas e ultrassom) para obtenção de produtos de valor agregado.

CAPÍTULO II

2. OBJETIVOS

2.1.OBJETIVO GERAL

Aplicar estratégias de modificação de suportes mesoporosos (sílica obtida por meio da técnica sol-gel) e nanoporosos (nanotubos de carbono) para utilização na imobilização da lipase de *Burkolderia cepacia*, assim como estudar parâmetros relacionados aos biocatalisadores e empregar em reações de transesterificação assistida por aquecimento convencional e não convencional (micro-ondas e ultrassom).

2.2.OBJETIVOS ESPECÍFICOS

- Avaliar a toxicidade e biodegradabilidade de LIs: acetato de N-metil-2-hidroxietilamônio (m-2-HEAA), propionato de N-metil-2-hidroxietilamônio (m-2-HEAPr), butirato de N-metil-2-hidroxietilamônio (m-2-HEAB), e pentanoato de N-metil-2-hidroxietilamônio (m-2-HEAP);

- Sintetizar os suportes de sílica por meio da técnica sol-gel utilizando os agentes modificadores: 2HEAPS (pentanoato de 2-hidroxietilamônio), m-2HEAPS (pentanoato de N-metil-2-hidroxietilamônio) e BHEAPS (pentanoato de bis(2-hidroxietil)amônio);

- Avaliar a remoção dos líquidos iônicos próticos nos suportes de sílica obtidos pela técnica sol-gel por meio de um novo método de lavagem utilizando o soxleht;

- Imobilizar a lipase de *Burkholderia cepacia* nos suportes de sílica por meio de dois métodos de imobilização, adsorção física e ligação covalente utilizando glutaraldeído ou epicloridrina como agentes de ativação;

- Avaliar o efeito do cátion (amina) dos líquidos iônicos próticos na modificação do suporte por meio de análises de caracterização físico-química e morfológica;

- Avaliar parâmetros relacionados a imobilização por meio da obtenção do rendimento de imobilização e estabilidade operacional;

- Aplicar os biocatalisadores imobilizados mais eficientes em reações de transesterificação utilizando óleo de coco residual e etanol como fonte de matérias primas sob aquecimento convencional e não convencional (micro-ondas e ultrassom);

- Funcionalizar a superfície dos suportes nanoporosos (nanotubos de carbono – CNTs) por carboxilação seguida de aminação;

- Imobilizar a lipase de *Burkholderia cepacia* nos CNTs por meio de método de adsorção física;

- Avaliar os efeitos da funcionalização e da imobilização nos CNTs por meio de análises de FTIR, TEM e obtenção de rendimento de imobilização;

CAPÍTULO III

3. REVISÃO BIBLIOGRÁFICA

Neste tópico será apresentado um levantamento das informações relevantes para o desenvolvimento deste trabalho, com enfoque na imobilização de enzimas, suportes adequados para a imobilização de enzimas, com destaque para sílica mesoporosa obtida pela técnica sol-gel, líquidos iônicos, nanotubos de carbono, reação de transesterificação e aquecimento não convencional com ênfase em micro-ondas e ultrassom.

3.1.IMOBILIZAÇÃO DE ENZIMAS

O termo "enzimas imobilizadas" refere-se a "enzimas fisicamente confinadas ou localizadas em uma determinada região do espaço definido, com retenção da sua atividade catalítica, e que pode ser usada repetidamente e de forma contínua" (GUISAN, 2006). A imobilização confere uma estabilidade adicional para uma variedade de enzimas contra a desnaturação (BAYRAMOGLU *et al*., 2002), assim como uma fácil separação entre o biocatalisador e o meio reacional, permitindo sua reutilização, o que representa uma excelente abordagem de química verde, reduzindo o custo e a quantidade de biocatalisadores (MINOVSKA *et al*., 2005; ANDRADE *et al*., 2012).

A imobilização é, em muitos casos, associada a uma diminuição da atividade da enzima ou um agravamento de outras características catalíticas. No entanto, a imobilização de uma enzima pode também melhorar a sua atividade, a especificidade e seletividade. Assim, a construção de uma grande biblioteca de biocatalisadores preparados por diferentes estratégias de imobilização pode permitir encontrar soluções para uma diversidade de situações visando a melhoria das propriedades da enzima (RODRIGUES *et al*., 2013).

Para uma imobilização ótima é necessário um estudo cuidadoso das condições de imobilização e do suporte, e ainda assim o sucesso da imobilização pode depender de diversos fatores e de requisitos específicos de um processo particular e industrial. Uma

condição importante para a imobilização de enzimas é que o suporte proporcione um ambiente inerte e biocompatível, isto é, que não interfira com a estrutura nativa da proteína (ANSARI e HUSAIN, 2012). As propriedades das enzimas imobilizadas são governadas pelas propriedades tanto da enzima como do material do suporte (Figura 3.1). A interação entre os dois confere uma enzima imobilizada com propriedades físico-químicas e cinéticas específicas que podem ser decisivas para a sua aplicação prática (KRAJEWSKA, 2004).

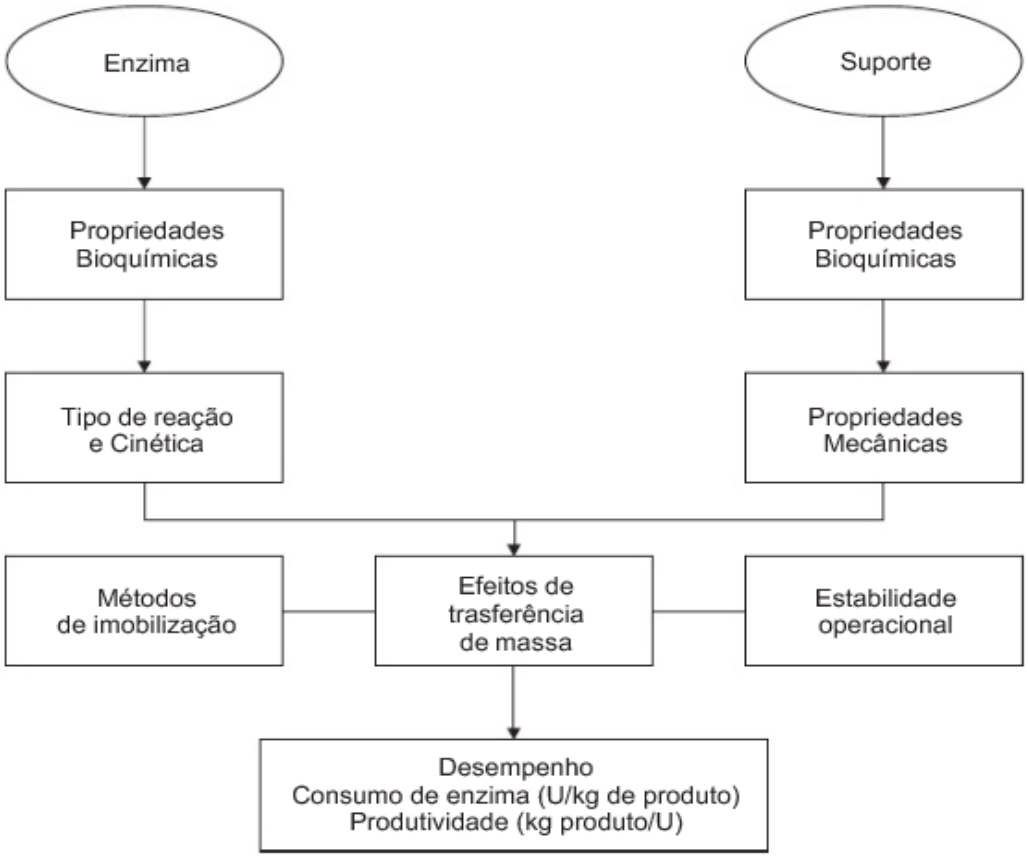


Figura 3.1: Fluxograma da interação entre suporte e enzima.

Fonte: BON *et al*. (2008).

Dentre as enzimas utilizadas para imobilização, as lipases (triacilglicerol hidrolases de ésteres, EC 3.1.1.3) se sobressaem por sua capacidade de catalisar a quebra de gorduras e óleos com subsequente liberação de ácidos graxos livres, diacilgliceróis, monogliceróis e glicerol. Estas enzimas são estudadas tanto pelo seu potencial de aplicações industriais como pelo seu grande interesse no desenvolvimento de métodos recorrentes à melhoria de suas propriedades catalíticas. Além disso, possuem

disponibilidade de grande número de preparações comerciais, ampla especificidade e estabilidade relativamente melhor em meios contendo solventes orgânicos (em comparação com outras enzimas) e são eficientes em reações diversas, como esterificação, transesterificação e aminólise em solventes orgânicos. A Figura 3.2 apresenta um esquema das reações catalisadas por lipases dependentes dos reagentes de partida empregados (VILLENEUVE *et al*., 2000; CASTRO *et al*., 2004; KAPOOR e GUPTA, 2012).

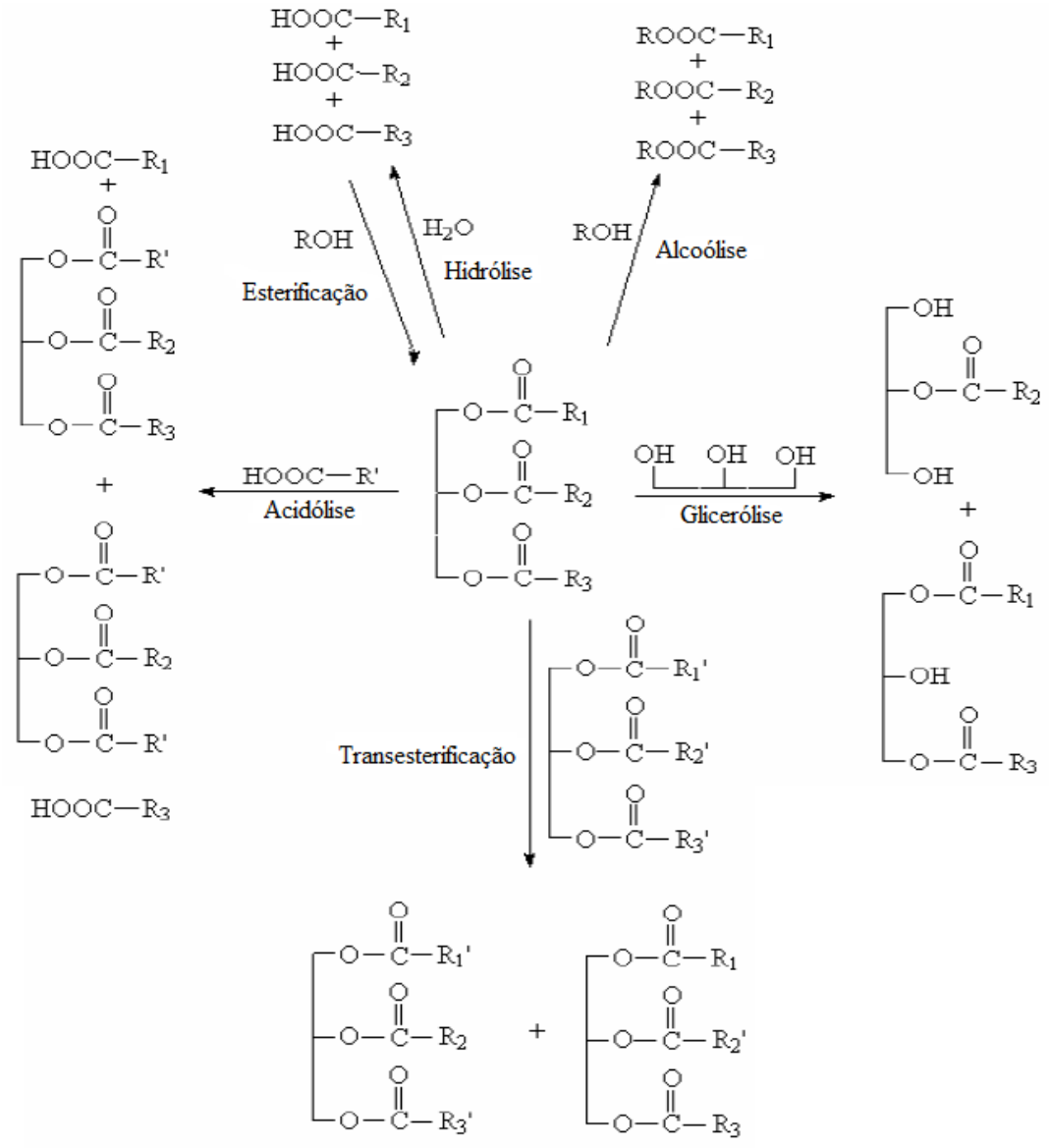


Figura 3.2: Reações catalisadas por lipases.

Fonte: CASTRO *et al*., 2004.

Alguns estudos relatam que a lipase de *Burkholderia cepacia* (LBC - anteriormente denominada *Pseudomonas cepacia*) é uma das lipases mais úteis por ser versátil e especialmente adequada para reações em solventes orgânicos (HARA *et al*., 2008; ABDULLA e RAVINDRA, 2013; CARVALHO *et al*. 2014; OLIVEIRA *et al*., 2014). A estrutura cristalina desta lipase (Figura 3.3) se destaca por apresentar algumas características incomuns a outras lipases como um sítio de cálcio que, supostamente, estabiliza a tríade catalítica e a presença de um ácido carboxílico adicional que pode servir como uma alternativa para aceitação de prótons (KIM *et al*., 1997; SCHRAG *et al*., 1997).

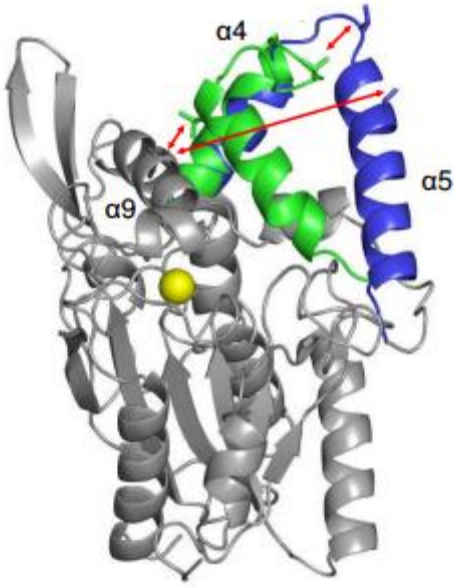


Figura 3.3: Representação da estrutura da lipase *Burkholderia cepacia*. A tampa da estrutura cristalina aberta da LBC está representada de cor azul e modelo fechado de cor verde e a esfera do íon Ca^{2+} de cor amarela. A tríade catalítica que consiste em Ser, His, Asp está na cor vermelha.

Fonte: TRODLER *et al*. (2009).

A lipase de *Burkholderia cepacia* é amplamente utilizada na biorrefinaria, para a síntese de biocombustíveis, e em uma grande variedade de reações em fases aquosas e não aquosas tornando-a alvo para processos de imobilização (KE *et al.* 2014). Segundo Da Rós *et al*. (2010), as condições estabelecidas para a maximização da atividade hidrolítica da lipase de *Burkholderia cepacia* livre foram pH 7,5 e 50 °C. No entanto, estas condições podem variar para sistemas imobilizados em virtude do suporte e do método de imobilização. Neste contexto, o material do suporte e a estratégia de

imobilização são dois importantes fatores para se atingir em sistemas imobilizados ativos e estáveis.

Em relação à imobilização de enzimas, desde 1960 uma extensa variedade de técnicas tem sido desenvolvida para imobilizar biomoléculas em virtude das vantagens provenientes da imobilização. Apesar dos inúmeros protocolos, os vários métodos de imobilização são principalmente classificados em quatro categorias: adsorção, ligação covalente, ligação cruzada e encapsulamento (BIRGIT *et al*., 1999; KANDIMALLA *et al*., 2006; ABDULLA e RAVINDRA, 2013).

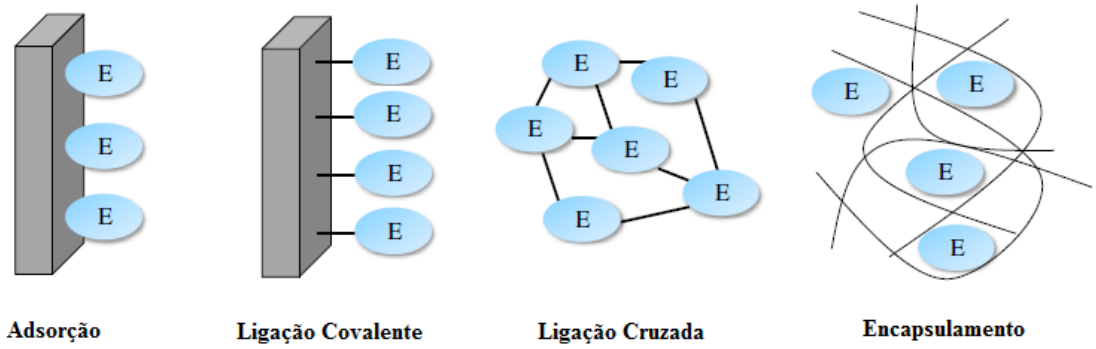


Figura 3.4: Representação esquemática dos métodos de imobilização: adsorção, ligação covalente, ligação cruzada e encapsulamento. **E**: enzima.

Fonte: Adaptado de SASSOLAS *et al*. (2012).

A adsorção consiste na fixação da lipase na superfície do suporte por forças fracas, tais como de van der Walls, ligação de hidrogênio, interações hidrófobas ou forças de dispersão. Este método permite preparações sob condições suaves, sem grande perda de atividade e o processo associado é relativamente simples e de baixo custo. Além do mais, o suporte pode ser facilmente recuperado para repetição da reação enzimática. Em virtude destas vantagens, a adsorção é ainda o método mais utilizado para a imobilização de lipases (TAN *et al*., 2010; JESIONOWSKI *et al*., 2014). Embora este método de imobilização não envolva qualquer funcionalização do suporte, o que gera pouca ou nenhuma inativação da enzima, esta técnica apresenta alguns inconvenientes: as enzimas são fracamente ligadas ao suporte e pode ocorrer a dessorção da enzima resultante de variações na temperatura, pH e força iônica (SASSOLAS *et al*., 2012).

Para que a adsorção da enzima ocorra de forma bem sucedida, algumas condições que promovam uma afinidade enzima-suporte devem ser atendidas. A

imobilização por adsorção física em suportes hidrofóbicos é explicada por meio de uma propriedade importante das lipases: a ativação interfacial na presença da interface hidrofóbica. A lipase tem uma cadeia de oligopéptideo (domínio tampa) que cobre os seus sítios ativos, tornando-os inacessíveis aos substratos. Na ausência da interface, o sítio ativo é isolado do meio de reação, mostrando uma "conformação fechada". No entanto, na presença da interface hidrofóbica, importantes rearranjos conformacionais ocorrem, produzindo a "conformação aberta". Neste caso, as lipases são fortemente adsorvidas para as interfaces hidrofóbicas por meio da "tampa" que cobre o seu sítio ativo. Assim, as lipases reconhecem estas superfícies de modo semelhante aos dos seus substratos naturais (gotas de óleo), obtendo-se estruturas abertas "imobilizadas" (MENDES *et al*., 2012).

Na imobilização por ligação covalente a enzima é ligada covalentemente a um material insolúvel em água por meio da reação entre os grupos funcionais da proteína e grupos reativos do material ativado. Este método se baseia na formação de uma ligação forte entre a enzima e o suporte (SANTOS *et al*., 2008). Portanto enzimas imobilizadas desta forma são geralmente estáveis e não se dissociam do suporte na presença do substrato ou de soluções de alta concentração iônica (BON *et al*., 2008).

Este método de ligação entre a enzima e o suporte sólido é geralmente realizado por ativação inicial da superfície do suporte utilizando reagentes multifuncionais (a exemplo do glutaraldeído e da epicloridrina), seguido pela ligação da enzima ao suporte ativado, e posteriormente, a etapa de remoção do excesso de biomoléculas não ligadas é realizada (SASSOLAS *et al*., 2012). A ativação do grupo ligante é frequentemente realizada no suporte com o propósito de reduzir o risco da diminuição da atividade catalítica da enzima (BON *et al*., 2008).

Na literatura há muitos relatos da aplicação desses dois métodos de imobilização utilizando enzimas e suportes diversos: híbrido SiO_2-PVA (SANTOS *et al.;* 2008; FREITAS *et al*., 2009), PHBV (CABRERA-PADILLA *et al*., 2014), sílica aerogel (BARBOSA *et al*., 2014), sílica meso/macroporosa e zircônia (ZIVKOVIC *et al.*, 2015) e matriz de sílica porosa (CARVALHO *et al*., 2013).

3.2. SUPORTES

O desenvolvimento e a descoberta de novos materiais com propriedades adequadas para a imobilização de enzimas é um campo emergente de pesquisa e de crucial importância em muitas áreas, como por exemplo a biocatálise, por oferecer vantagens em vários processos, tais como produção contínua e estabilidade (ISPAS *et al*., 2009 e DICOSIMO *et al*., 2013; POURZOLFAGHAR *et al.*, 2016).

Visto que a seleção do suporte é um dos fatores fundamentais a serem considerados no desenvolvimento de biocatalizadores imobilizados, a busca por materiais apropriados ainda continua sendo amplamente estudada (MOHAMAD *et al*., 2015). O suporte é considerado ideal quando fixa a enzima de maneira irreversível, sem afetar sua atividade e sem interferir na reação em que será aplicado (MALCATA *et al*., 1990).

Embora seja reconhecido que não há nenhum suporte universal para todas as enzimas e suas aplicações, certo número de características desejáveis devem ser comuns a qualquer material considerado para a imobilização de enzimas. Para a escolha do suporte, que pode ser de origem orgânica (naturais e sintéticos) ou inorgânica (minerais e fabricados), alguns exemplos são apresentados na Tabela 3.1, dentre os parâmetros que devem ser considerados destacam-se: elevada afinidade para proteínas, disponibilidade de grupos funcionais reativos para reações diretas com enzimas e para modificações químicas, resistência mecânica, estabilidade química e física, balanço hidrofóbico/hidrofílico, capacidade de carregamento da enzima, custo, dentre outros (KRAJEWSKA, 2004; CARVALHO *et al.*, 2006). De acordo com Canilha *et al*. (2006) os suportes inorgânicos são mais vantajosos que os orgânicos pela durabilidade, densidade, estabilidade e controle de porosidade.

A morfologia, tamanho e distribuicão dos poros são propriedades do suporte que podem influenciar diretamente os efeitos difusionais causados pela transferência de massa entre os substratos e os biocatalisadores imobilizados. Desta forma, a morfologia e porosidade são fatores importantes que devem ser levados em consideração para escolha do suporte adequado, principalmente devido à possibilidade de imobilização da enzima na sua superfície e/ou no seu interior (CARVALHO *et al*., 2015).

Inúmeros suportes são relatados na literatura e muitos estão em fase de desenvolvimento, mas o interesse em sílica mesoporosa e em nanomateriais (nanotubos de carbono) são o foco desta pesquisa, a qual pretendeu estudar o potencial destes materiais como suporte para imobilização de lipase.

Tabela 3.1: Exemplos de suportes aplicados no processo de imobilização de enzimas.

Suporte	Exemplos
Inorgânico	Sílica e derivados, celite, suportes com base em alumínio, diatomácea, vidro pororso
Orgânico	Polissacarídeos, celulose, quitosana, amido, polímeros sintéticos, silicones, resinas e nanotubos de carbono

Fonte: Adaptado de MILETIC *et al*. (2012) e JESIONOWSKI *et al*. (2014).

3.2.1. Sílica: Técnica Sol-gel

As matrizes porosas de sílica podem ser adquiridas comercialmente ou sintetizadas por diferentes técnicas. Dentre elas, a mais utilizada para imobilização de enzimas é a sílica sintetizada por meio da técnica sol-gel (CARVALHO *et al*., 2015).

O método sol-gel se baseia na formação de uma suspensão coloidal (sol), a qual, por meio do processo de policondensação forma uma matriz sólida (gel) (REETZ *et al*., 1996; FRANKEN *et al*., 2002). A técnica sol-gel envolve um ácido ou uma base na catálise da hidrólise de tetra-alcóxisilanos [$Si(OR)_4$]. O precursor-silano e os demais reagetes iniciam a hidrólise e, posteriormente, uma solucão básica é adicionada para policondensação com formação de uma matriz de SiO_2 (GUISAN *et al.*, 2006).

A reação de polimerização do processo sol-gel pode ser dividida em etapas descritas pelas reações 1-3: (1) hidrólise do grupo alcóxido com a formação de grupos reativos do tipo silanol, (2-3) reações de condensação do grupo silanol a qual leva inicialmente à formação sol e, eventualmente, ao gel e por fim a policondensação completa dos grupos silanois (ALFAYA e KUBOTA, 2002).

$$Si(OR)_4 + nH_2O \rightarrow Si(OR)_{4-n}(OH)_n + nROH \quad (1)$$

$$\equiv Si—OH + HO—Si\equiv \rightarrow \equiv Si—O—Si\equiv + H_2O \quad (2)$$

$$\equiv Si—OR + HO—Si\equiv \rightarrow \equiv Si—O—Si\equiv + ROH \quad (3)$$

Benvenutti *et al*. (2009) descreveram características interessantes do método sol-gel, relacionando como vantagens a possibilidade de obtenção de componentes orgânicos e inorgânicos altamente dispersos; reações de gelificação processadas a baixas temperaturas permitindo inserir matriz sólida de biomoléculas como enzimas, proteínas e anticorpos e a possibilidade de obter materiais sob diferentes configurações. Como desvantagens foram destacadas o alto custo de alguns precursores; o número limitado de precursores disponíveis comercialmente e longos tempos de processamento, onde a reprodutibilidade nas propriedades finais dos materiais somente é alcançada com um controle minucioso das condições experimentais de síntese.

As propriedades únicas de materiais de sílica mesoporosa têm atraído interesse substancial para o uso como suportes de imobilização de enzimas. Estas características incluem elevada área de superfície, estabilidade química, térmica e mecânica, distribuição de poros altamente uniformes, tamanho de poro ajustável, capacidade de adsorção elevada e uma rede porosa ordenada que facilita a difusão livre de substratos e produtos da reação (ALOTHMAN, 2012; MA *et al*., 2016).

Em função de suas vantagens, esses materiais foram identificados como suportes promissores para a imobilização de enzimas, com aplicação em reações de biocatálise a exemplo de: hidrólise (SOARES *et al*., 2006; CARVALHO *et al*., 2013; BARBOSA *et al*., 2014), polimerização (ZHANG *et al*., 2013), reações de transesterificação com acetato de vinila (URSOIU *et al*., 2012) e reações de transesterificação para produção de biodiesel (OLIVEIRA *et al*., 2014).

No entanto, as enzimas imobilizadas em materiais mesoporosos normalmente apresentam uma atividade mais baixa do que as das enzimas livres. Em virtude desta circusntância, a modificação superficial de suportes mesoporosos tem recebido muita atenção recentemente. Além disso, a modificação da superfície também reduz parcialmente as aberturas dos poros e a superfície externa alivia os problemas de lixiviação (ZOU *et al*., 2010). Assim, a estrutura, composição e tamanho dos poros desses

materiais pode ser adaptada durante a síntese, pela adição de agentes modificadores, tais como os líquidos iônicos.

3.2.1.1. Líquidos iônicos (LIs)

Os líquidos iônicos (LIs) são líquidos não compostos de moléculas, mas de íons, um cátion orgânico e um ânion que pode ser orgânico ou inorgânico. Uma vez que são compostos de unidades carregadas, possuem baixa pressão de vapor e são considerados não-voláteis. Os LIs geralmente são líquidos viscosos com uma grande capacidade de dissolver substâncias inorgânicas e orgânicas e podem ser ajustados de acordo com as exigências de sua aplicação. Desta forma os LIs podem ser referidos como "designers solvents " (MEDEIROS *et al*., 2013; SANTOS *et al*., 2014; GOUVEIA *et al*., 2014).

Estes compostos são utilizados como solventes no campo da biocatálise em função das suas propriedades, como viscosidade, hidrofobicidade, polaridade, miscibilidade, entre outras. Por meio da alteração do cátion, ânion e substituintes ligados, os LIs podem ser otimizados e, com isso podem assegurar melhor seletividade e estabilidade operacional quando comparados a métodos tradicionais (YANG e PAN, 2005; HARTMANN e PEREIRA, 2016).

Os LIs podem ser classificados em Líquidos Iônicos Apróticos (LIAs) e Líquidos Iônicos Próticos (LIPs). Os LIs constituídos principalmente dos cátions orgânicos a base de imidazólio, piridino, tetraalquilamônio e tetraalquilfosfónio, são os chamados líquidos iônicos apróticos, os quais possuem sua aplicação industrial dificultada por apresentarem elevados custos de síntese (GREAVES e DRUMMOND, 2008; ÁLVAREZ *et al.*, 2010; ZARCULA *et al.*, 2010).

Os LIs sintetizados por transferência de prótons a partir de um ácido de Bronsted para uma base de Bronsted são chamados líquidos iônicos próticos (LIPs), os quais apresentam baixo custo, simplicidade de síntese e diferentes aplicações, o que favorece o interesse industrial (ÁLVAREZ *et al.*, 2010; SANTOS *et al*., 2014).

Algumas enzimas possuem a capacidade de realizar atividades catalíticas iguais ou até mesmo superiores em meios contendo LIs em comparação aos meios contendo solvetes convencionais (PETERS *et al*., 2007). No entanto, o uso de LIs como meio de reação dificultou o seu potencial de aplicação devido à grande quantidade requerida e alto custo (RIISAGER *et al*., 2006). Tais problemas podem ser contornados mediante adição dos LIs ao suporte (ZOU *et al*., 2010). Segundo Mehnert *et al*. (2002) o uso de líquido iônico em suporte combina as vantagens deste material com o material do suporte sólido, permitindo o uso de quantidades significantemente reduzidas de LIs.

Estudos sobre o confinamento de líquidos iônicos foram inicialmente relatados por Dai *et al*. (2000), que introduziram os líquidos iônicos em gel de sílica porosa. Os autores relataram que o líquido iônico ficou aprisionado na rede covalente de sílica, uma vez que foi ligado quimicamente à matriz inorgânica. Trabalhos com foco na utilização de líquidos iônicos como aditivos durante a preparação de biocatalisadores imobilizados por meio da técnica sol-gel têm sido relatados por Lee *et al*. (2007), Zarcula *et al*. (2010), Kato *et al*. (2011), Souza *et al*. (2013), Barbosa *et al*. (2014).

Contudo, um dos maiores problemas associado ao uso de líquidos iônicos corresponde a capacidade destes compostos de diminuir ou destruir a camada de água em torno da superfície das enzimas, ou impedir o acesso ao sítio ativo diminuindo a sua atividade e/ou estabilidade (VENTURA *et al*., 2012). Neste contexto, considerando as propriedades dos líquidos iônicos e o propósito de melhorar a performance do biocatalisador imobilizado em sílica, surgiram estudos promissores em relação à utilização de líquidos iônicos como modificadores de suporte de sílica, relatando melhorias nas propriedades das enzimas imobilizadas.

A literatura descreve que sistemas imobilizados com sílica mesoporosa SBA-15 com superfície modificada com líquidos iônicos demonstraram aumento na atividade enzimática, na capacidade de reutilização e na estabilidade térmica (ZOU *et al*. 2010; HU *et al*. 2012; YANG *et al*. 2013). Martins *et al*. (2016) avaliaram a utilização de líquidos iônicos próticos na preparação de híbridos de sílica e PHBV para imobilização de lipase de *Burkholderia cepacia* por ligação covalente e obtiveram como resultado melhorias das características morfológicas e físico-químicas, maior estabilidade térmica e operacional.

Apesar destes relatos e considerando que os LIs podem influenciar a capacidade das enzimas, vale ressaltar que a literatura não retrata a modificação de suporte de sílica por meio do emprego de líquidos iônicos próticos com remoção completa dos agentes modificadores (LIPs) antes da aplicação em imobilização de enzimas.

Com base neste contexto, é notório que, nos últimos anos, o interesse nos LIs tem aumentado de forma considerável e, consequetemente, a sua aplicação. Portanto, a investigação da sua toxicidade é necessária para prever seu impacto no meio ambiente e na saúde do ser humano (CUNHA *et al*., 2015; ANVARI *et al*., 2016).

3.2.1.2. Toxicidade dos LIs

A utilização dos líquidos iônicos em grande escala, inevitavelmente, provoca a liberação destes produtos químicos no meio ambiente e a sua excelente estabilidade química e térmica pode levar à sua acumulação, causando danos ao ecossistema e afetando os organismos vivos. Os LIs não representam ameaça para o ar, em função do seu valor baixo de pressão de vapor. Porém ecossistemas aquáticos e solo são destinos potenciais dessas substâncias, que podem ser liberadas na forma de água pós-industrial (esgoto). Portanto, é necessário investigar a toxicidade e a ecotoxicidade dos LIs para compreender seu impacto no meio ambiente e na saúde humana (KUDŁAK *et al*., 2015; CUNHA *et al*., 2015; FAN *et al*., 2016).

Em geral, existem várias maneiras de estabelecer as toxicidades de LIs por meio de testes em algas, bactérias e peixes de água doce (FOULET *et al*., 2016). De fato, já foi demonstrado que alguns LIs, tais como aqueles baseados em imidazólio, são mais tóxicos que alguns compostos orgânicos voláteis já utilizados na indústria química, tais como metanol e diclorometano (GARCIA *et al*., 2005; GOUVEIA *et al*., 2014). No entanto, em função de sua estrutura mais simples, o potencial de impacto ambiental dos líquidos iônicos próticos é esperado para ser menor do que o impacto proveniente dos líquidos iônicos apróticos (PERIC *et al*., 2013)

A toxicidade dos LIs depende, na maioria dos casos, da sua lipofilicidade, do comprimento da cadeia alquílica, e da presença de grupos polares no cátion (principalmente oxigênio ou um átomo de nitrogênio). Alguns estudos indicam que os primeiros fatores estão associados com o aumento da toxicidade, enquanto o último resulta na diminuição das propriedades tóxicas (PHAM *et al*. 2010; GRZONKOWSKA *et al*. 2016).

Yu e Nie *et al*. (2011) investigaram a ecotoxicidade de líquidos iônicos (LIs) com três espécies de bactérias (*Escherichia coli*, *Staphylococcus aureus* e *Bacillus subtilis*) e seis LIs. Todas as concentrações dos LIs demonstraram alguma toxicidade para os três micro-organismos e o aumento no comprimento da cadeia alquílica correspondeu ao aumento na toxicidade dos LIs.

Fan *et al*. (2016) estudaram os efeitos de doze líquidos iônicos na atividade da tripsina. Os resultados indicaram que a presença dos LIs inibiu a atividade da tripsina e o grau de inibição dependeu da estrutura química dos LIs (ambos ânion e cátion).

Entretanto, a toxicidade dos líquidos iônicos pode ser reduzida por meio da manipulação adequada das estruturas químicas dos LIs durante sua síntese. Ventura *et al*. (2012) estudaram a toxidade de líquidos iônicos aromáticos e não aromáticos e descreveram que o uso de cadeia alquílica do cátion com dois átomos de nitrogênio não parece ter um impacto sobre a toxicidade enquanto que a utilização de cadeia ramificada pode ser relevante. Além do mais, cátions não aromáticos demonstraram uma capacidade significativa para aumentar a hidrofobicidade enquanto reduz a toxicidade.

Zhao *et al.* (2014) fizeram uma relação entre extrutura e toxicidade de vários LIs e evidenciaram que a toxicidade nem sempre aumenta com o aumento da cadeia alquílica destes compostos, reforçando a possibilidade de sintetizar LIs com o intuito de alcançar melhores propriedades físico-químicas e LIs ambientalmente seguros.

3.2.2. Nanotubos de carbono (CNTs)

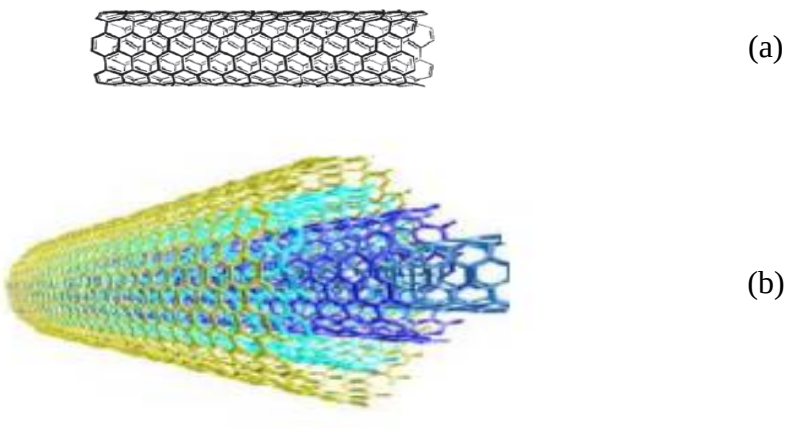
Nanotubos de carbono (CNTs) são alótropos do carbono que possuem a forma tubular de folhas de grafeno e comprimentos na faixa de algumas centenas de nanômetros a vários microns. Vários são os procedimentos disponíveis para a síntese dos nanotubos de carbono, no entanto, deposição química de vapor (do inglês chemical vapour deposition - CVD) é a técnica mais empregada. Geralmente os CNTs são impuros devido à presença de diferentes sub-produtos indesejáveis durante sua síntese (como por exemplo carbono amorfo e metal catalisador), o que torna um desafio sua produção com elevada pureza e tamanho controlado (em comprimento e diâmetro) de uma forma reprodutível e econômica (SAIFUDDIN *et al*., 2013; HERRERO-LATORRE *et al*., 2015).

Contudo, os nanotubos de carbono oferecem características atrativas que determinam a eficiência de biocatalisadores, incluindo alta área de superfície, resistência à transferência de massa, elevada capacidade de adsorção e carregamento enzimático eficaz. Desta forma, esses nanomateriais (CNTs) podem servir como excelentes materiais de suporte para imobilização de enzimas (FENG e JI, 2011; SILVA *et al*., 2014a).

Atualmente são relatados dois tipos de CNTs (Figura 3.5): os de parede simples (SWCNT, do inglês Single Wall Carbon Nanotubes), e os de paredes múltiplas (MWCNT, do inglês Multiwall Carbon Nanotubes), que consistem de folhas SWCNT coaxiais. O diâmetro dos CNTs depende da formação dos nanotubo em camadas simples ou múltiplas (SWCNT: 1-3 nm, MWCNT: 5 a 100-200 nm). Esses dois tipos de CNTs têm sido utilizados para imobilizar enzimas, no qual MWCNT são mais desejáveis em virtude do seu baixo custo (FENG e JI, 2011; MAJJED *et al*., 2013; HERRERO-LATORRE *et al*., 2015).

É importante ressaltar que os nanotubos de carbono oferecem a possibilidade de serem funcionalizados, modificando, assim, as suas propriedades e melhorando a sua eficiência como suportes (SILVA *et al*., 2014a). Segundo Yu *et al*. (2015) pesquisas demonstraram que o design específico de nanomateriais por funcionalização da superfície adaptados com grupos de carga (como por exemplo -COOH, $-NH_2$, $-SO_3H$, entre outros) podem ser uma ferramenta para controlar forças motrizes de adsorção de proteínas

(Figura 3.6). No entanto, métodos de funcionalização da superfície dos CNTs têm suas próprias vantagens e limitações, e a escolha destes métodos é ditada pelas aplicações necessárias a partir dos materiais nanocompósitos gerados (MITTAL, 2011).



(a)

(b)

Figura 3.5: Esquema representativo dos tipos de CNTs. (a) SWCNT e (b) MWCNT.
Fonte: Adaptado de MAJEED *et al*. (2013) e MEHRA e PALAKURTHI (2015).

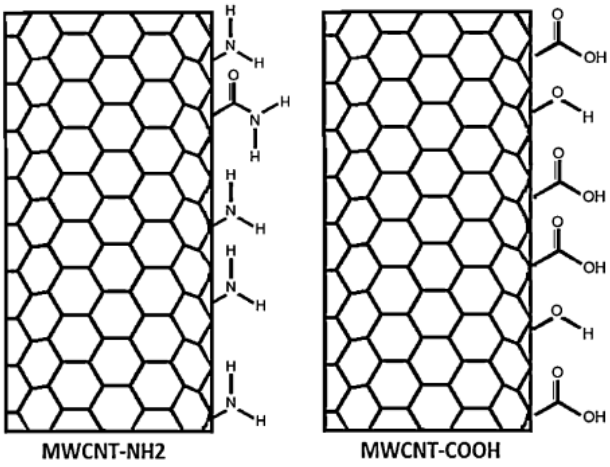


Figura 3.6: Tipos de nanotubos de carbono com superfície funcionalizada.
Fonte: RASTIAN *et al*. (2014).

Enzimas podem ser imobilizadas nos nanotubos de carbono por meio de abordagens covalente e não-covalente. Comparando os dois métodos, o método não covalente é geralmente preferido, pois é realizado sem aditivos químicos e preserva a conformação

nativa da enzima (TAVARES *et al*., 2015). Cang-Rong e Pastorin (2009) relataram que sistemas imobilizados com amiloglucosidase (AMG) em nanotubos de carbono exibiram melhor eficiência catalítica quando adsorvido fisicamente em comparação a amostras imobilizadas covalentemente.

Conforme Feng e Ji (2011), no método de adsorção física direta, a força da interação entre a enzima e os CNTs é predominantemente hidrofóbica. As regiões hidrofóbicas na parte externa da enzima podem interagir com a parede dos CNTs por meio das interações hidrofóbicas. Outros fatores como interação de empilhamento π-π entre as paredes laterais dos nanotubos de carbono, presença de anéis aromáticos, interações eletrostáticas e pH desempenham papel importante na adsorção (NEPAL e GECKELER, 2006).

Como exemplo de aplicação biocatalisdores preparados com enzima imobilizada em nanotubos de carbono, citam-se capacidade de catalisar reações de hidrólise (PRLAINOVIC *et al*., 2016) e reações de transesterificação com N-butirato de vinila (ZNISZCZOŁ *et al*., 2016) e óleo de *Jatropha curcas* (DEEP *et al*., 2015).

3.4. REAÇÃO DE TRANSESTERIFICAÇÃO

Biocatalisadores enzimáticos são amplamente empregados em reação de transesterificação com o intuito de produzir produtos de interesse industrial como ésteres flavorizantes (SUN *et al*., 2012) resveratrol (KUO *et al.,* 2013) e biodiesel (FREITAS *et al*., 2009; TONGBORIBOON *et al*., 2010; AZÓCAR *et al*., 2010; RODRIGUES e AYUB, 2011; DA RÓS *et al*., 2014).

Para a produção de biodiesel a reação de transesterificação resume-se em uma reação química envolvendo matéria-prima renovável como óleos vegetais e gorduras animais (fonte de triglicerídeos) e um álcool de cadeia curta como metanol ou etanol utilizando substâncias homogêneas ou heterogêneas como catalisador para produzir biodiesel e glicerol (MEHER *et al*., 2006; ATADASHI *et al*., 2012). O processo geral de transesterificação envolve uma sequência de três reações consecutivas e reversíveis, na qual primeiramente os triglicerídeos são convertidos em diglicerídeos, posteriormente os

diglicerídeos são convertidos em monoglicerídeos e no terceiro passo os monoglicerídeos formam as moléculas de glicerina (Figura 3.6) (CHRISTOPHER *et al*, 2014).

Estequiometricamente, são necessários três moles de álcool para a conversão de uma mol de triglicerídeo em ésteres, mas geralmente altas quantidades de álcool são adicionadas para conduzir a reação na direção de formação dos produtos (GULDHE *et al*., 2015). Contudo, é importante evidenciar que alguns fatores como a quantidade de álcool, a concentração do catalisador, o tempo e a temperatura da reação podem afetar a produção de biodiesel (LEUNG *et al*., 2010).

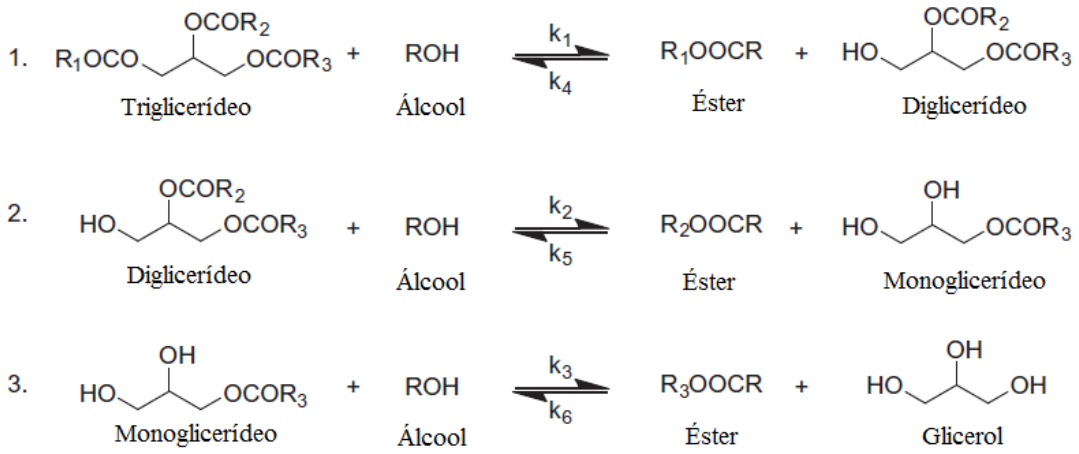


Figura 3.7: Esquema geral para reação de transesterificação (R refere-se a um grupo alquil pequeno, R_1, R_2 e R_3 referem-se a cadeias de ácido graxo e k_1, k_2, k_3, k_4, k_5, k_6 são catalisadores enzimáticos ou químicos).

Fonte: Adaptado de CHRISTOPHER *et al*, 2014.

A transesterificação pode ser catalizada por ácido/base ou por via enzimática (ANITESCU e BRUNO, 2012; DA ROS *et al*., 2012). Embora a catalise enzimática apresente alguns inconvenientes, associados com menores taxas de reação, custos mais elevados e perda de atividade ou inibição enzimática, a rota enzimática para a produção de biodiesel é considerada como uma alternativa ambientalmente amigável, tornando o processo mais limpo, uma vez que permite uma fácil recuperação do glicerol, reuso do biocatalisador, não exige etapa de neutralização, consome menos energia e reage em temperaturas baixas (ANTCZAK *et al*., 2009; CHRISTOPHER *et al*., 2014).

Entretanto, o principal obstáculo para a comercialização do biodiesel é o seu elevado custo, visto que a disponibilidade e os tipos de matéria-prima são contribuintes

para o custo global de produção de biodiesel (CHEN *et al*., 2012; RAMACHANDRAN *et. al*., 2013). Portanto, o uso de óleos ou gorduras não comestíveis ou residuais contribuem para tornar a geração deste biocombustível economicamente viável (ANTCZAK *et al*., 2009; QIUL *et al*. 2011).

A utilização de óleo de coco residual como matéria-prima para a reação de transesterificação pode permitir a transformação de resíduos produzidos a partir da indústria do óleo do coco em produtos valiosos, que são essenciais para reduzir o custo da produção de biodiesel associada com o processamento de matéria prima e técnicas de conversão através de abordagens sustentáveis (OLIVEIRA *et al.*, 2010;. TUPUFIA *et al.*, 2013, WANG *et al.*, 2014).

O coqueiro (*Cocos nucifera L*.) encontra-se naturalizado em longas áreas da costa nordestina brasileira, cujo os principais produtores são a Bahia, Sergipe e o Rio Grande do Norte. As variedades mais comuns no Brasil são o coqueiro gigante, o qual é predominante no país, o anão e o híbrido, o qual é proveniente do cruzamento natural ou artificial entre as variedades gigante e anão. O coco é uma fonte oleaginosa perene de grande potencial, uma vez que o conteúdo de óleo na polpa é superior a 60 %, o que equivale a uma produção de 500 a 3000 kg de óleo/ha. O óleo de coco apresenta como componente majoritário o ácido láurico (em torno de 40 %), os quais possuem cadeias curtas que promovem uma interação mais efetiva com os agentes transesterificantes, permitindo a obtenção do produto (biodiesel) com excelentes características físico-químicas (LIMA *et al.* 2007; SILVA *et al.* 2014b).

No entanto, os triglicerideos e álcoois são imiscíveis, gerando baixas taxas de reação provenientes dos efeitos difusionais relacionados à transferência de massa entre os reagentes. Além do mais, o efeito de aquecimento por meio do método convencional depende da condutividade térmica, do calor específico e da densidade dos materiais o que resulta em temperaturas de superfície elevadas, não uniformes e, consequentemente, em transferência de calor a partir da superfície exterior para o volume interno da amostra (SALAMATINIA *et al*., 2012; RAMACHANDRAN *et al*. 2013; GUDE *et al*., 2013).

Considerando este princípio, pesquisadores têm investigado técnicas distintas para intensificação da interface óleo e álcool resultando em altas conversões de biodiesel, conhecidas por técnicas não convencionais, como micro-ondas e ultrassom, as quais

também apresentam como vantagens: rápido aquecimento e arrefecimento; redução de custos devido à energia, tempo e economia de espaço de trabalho; processamento preciso e controlado; redução no tempo de processamento; entre outros (GUDE *et al.* 2013; ADEWALE *et al*., 2015; MICHELIN *et al*., 2015).

3.5. MICRO-ONDAS

Micro-ondas é uma forma de energia eletromagnética com a faixa de frequência de 300 MHz a 300 GHz e comprimento de onda correspondente entre 1 mm e 1 m. As micro-ondas possuem os comprimentos de onda mais longos e menor energia quântica disponível do que as outras formas de energia eletromagnética como luz visível, raios ultravioleta ou infravermelhos (OGHBAEI e MIRZAEE, 2010). A região de micro-ondas situa-se entre a região do infravermelho e as ondas de rádio no espectro eletromagnético, como é mostrado na Figura 3.8 (SANSEVERINO, 2002; SOUZA e MIRANDA, 2011).

Micro-ondas é considerada uma tecnologia sustentável, devido à sua característica amigável ao meio ambiente e ao seu consumo de energia reduzido, requerendo menos energia em comparação com métodos convencionais de aquecimento e efetuando as reações de forma mais rápida, eficiente e segura (TEIXEIRA *et al.*, 2014)

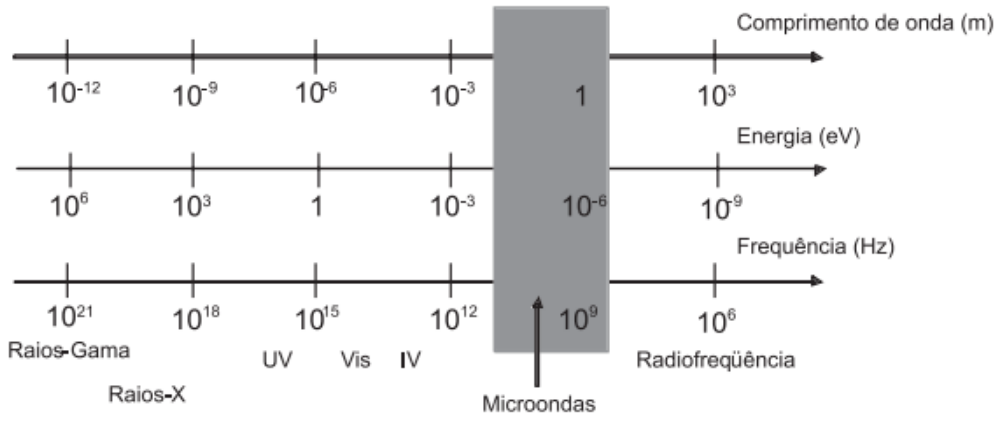


Figura 3.8: Faixa de frequência e comprimento de onda de micro-ondas.

Fonte: SOUZA e MIRANDA (2011).

O aquecimento de líquidos usando micro-ondas pode ser explicado pela interação da matéria com o campo elétrico da radiação incidente, promovendo o movimento de íons, assim como o alinhamento das moléculas (que tem dipolos permanentes ou induzidos) com o campo elétrico aplicado (chamado aquecimento dielétrico). Se uma molécula possui um momento dipolar, quando ela é exposta a irradiação de micro-ondas o dipolo tenta alinhar com o campo elétrico aplicado (Figura 3.9). Como o campo elétrico é oscilante, os dipolos constantemente tentam realinhar e continuam a seguir este movimento. Esta reorientação contínua das moléculas resulta em atrito e consequentemente em calor. Na condução iônica, os íons se movem e ao mesmo tempo colidem uns nos outros gerando calor. Desta forma, a energia interage com as moléculas a uma taxa muito rápida e como as moléculas não possuem tempo para relaxar, haverá aquecimento localizado. Com base neste contexto, conclui-se que o reator micro-ondas fornece energia diretamente para os reagentes (SANSEVERINO 2002; ENCINAR *et al.*; 2012; GUDE *et al*., 2013).

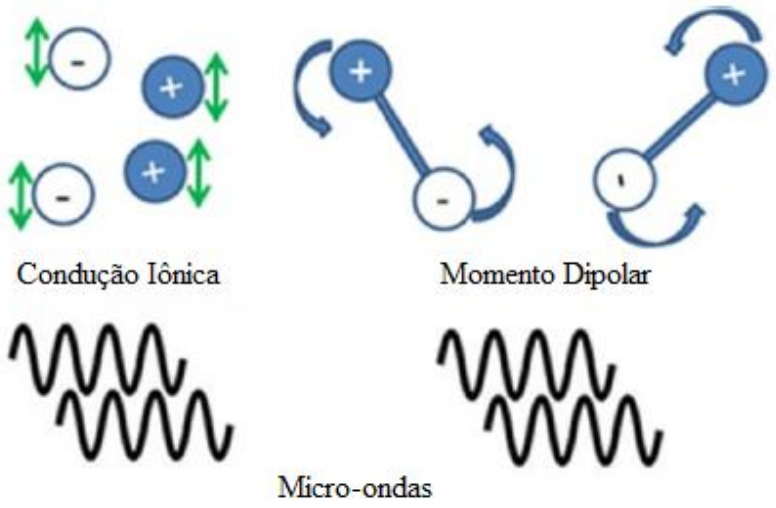


Figura 3.9: Condução iônica e momento dipolar sob micro-ondas.

Fonte: Adaptado de GUDE *et al*., 2013.

A energia de micro-ondas baseada na síntese química tem vários méritos e é importante do ponto de vista tanto científico como da engenharia. Sua aplicação em escala laboratorial para a produção de biodiesel tem mostrado o potencial desta tecnologia para obter resultados superiores em relação às técnicas convencionais, como curto tempo de reacção, produtos de reação mais limpa e redução dos tempos de separação e purificação (GUDE *et al*., 2013).

O mecanismo de interações das micro-ondas com os sistemas enzimáticos ainda não está claramente definido. No entanto, estudos de Réjasse *et al*. (2004) mostraram que a estabilidade enzimática da lipase *Candida antarctica* imobilizada foi maior sob aquecimento proveniente das micro-ondas do que sob aquecimento térmico convencional. Além disso, o ganho da estabilidade enzimática sob aquecimento por micro-ondas parece ser mais elevado em um solvente mais polar, que interage fortemente com o campo de micro-ondas.

Em virtude de seu potencial, a literatura tem relatado a eficiência do aquecimento por micro-ondas em reações de transesterificação enzimática. Nogueira *et al*. (2010) investigaram o efeito da irradiação sobre a taxa de transesterificação enzimática usando óleo de macaúba e etanol como matérias-primas. Apesar de conseguirem rendimentos abaixo de 50% para *Novozyme* 435 e *Lipozyme* IM, os autores fizeram uma comparação da atividade catalítica das enzimas sob aquecimento convencional e irradiação de micro-ondas notando um aumento significativo na atividade de ambas as enzimas na presença desta forma de aquecimento não convencional.

Da Rós *et al*. (2012), em seu trabalho de transesterificação enzimática de biodiesel a partir de sebo bovino, demonstraram por meio de seus dados que o meio reacional, quando submetido à irradiação por micro-ondas, promoveu um aumento significativo na velocidade da reação, em comparação com o sistema de aquecimento por meio convencional.

Queiroz *et al*. (2015) investigaram o efeito da energia de micro-ondas na metanólise de óleos de soja e girassol catalisadas por Novozyme 435. Os autores indicaram que o uso das micro-ondas melhorou a velocidade de reação na ordem de aproximadamente 1,5 vezes quando comparado ao aquecimento convencional.

3.6. ULTRASSOM

Ultrassom é uma frequência superior a da audição humana, que é usada para uma variedade de fins em diversas áreas (RAMACHANDRAN *et. al*., 2013). A Figura 3.10

mostra uma ideia da frequência do ultrassom em relação à sensibilidade do ouvido humano (16 Hz a 16 KHz). A frequência característica do ultrassom, em geral, está no intervalo de 16 KHz a 1 MHz, aquelas com valores entre 1 a 10 MHz correspondem à região de alta frequência (MARTINES *et al*., 2000).

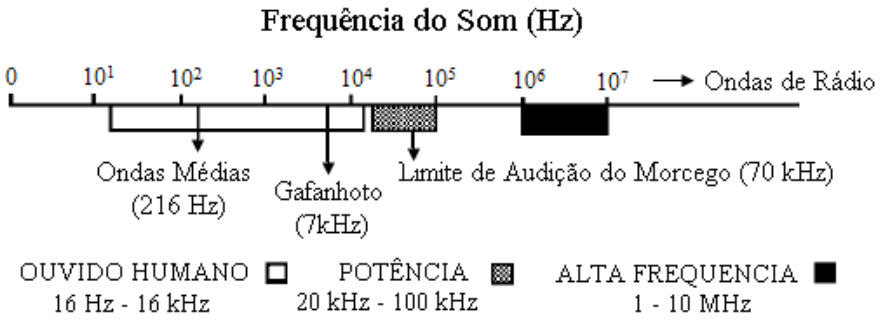


Figura 3.10: Faixa de frequência de ondas sonoras.
Fonte: Adaptado de MARTINES *et al*., 2000.

É considerada uma tecnologia 'verde' em virtude de sua alta eficiência, baixos requisitos instrumentais, tempo de processo reduzido significativamente e desempenho economicamente viável. Os efeitos do ultrassom ocorrem a partir do fenômeno de cavitação: formação, crescimento e colapso implosivo de cavidades em líquidos que liberam grandes quantidades de energia altamente localizada (ROKHINA *et al*., 2009).

A irradiação de ultrassom provoca bolhas de cavitação próximo ao limite entre a as fases do sistema, e como resultado, micro bolhas são formadas. O colapso assimétrico das bolhas de cavitação perturba o limite das fases causando emulsificação por jatos de ultrassons que incidem de um líquido para o outro. Uma vez que tal emulsão é formada, a área da superfície disponível para a reação entre as duas fases aumenta significativamente. A cavitação pode também levar a um aumento localizado da temperatura no limite da fase enriquecendo a reação de transesterificação (GOLE E GOGATE *et al*. 2012; VELJKOVIC *et al*. 2012; JIN *et al*., 2015).

Muitos são os benefícios oriundos da aplicação da cavitação em processos químicos, tais como: redução do tempo de reação; aumento do rendimento da reação; uso de menos condições energéticas (temperatura e pressão) em comparação com as rotas convencionais; redução do período de indução da reação desejada; possíveis mudanças nas vias reacionais, resultando no aumento da seletividade; aumento da eficácia do

catalisador utilizado na reação (GOGATE *et al*., 2006; RAMACHANDRAN *et al*., 2013). Em relação à produção de biodiesel, o aumento na taxa de formação de éster sob condições de mistura ultrassônica é resultante de fatores como emulsificação e transferência de massa eficiente induzidos pelas condições de turbulência e pela circulação do líquido no reator gerados pelo emprego da cavitação (KELKAR *et al*., 2008; HSIAO *et al*., 2010).

Contudo, pesquisadores relatam que a atividade das enzimas pode ser melhorada por meio da aplicação da cavitação, mas a influência da radiação sônica sobre a atividade e a estabilidade das enzimas depende dos parâmetros de sonicação e da preparação enzimática específica, ressaltando que sistemas compostos por ultrassom de alta potência podem provocar a interrupção da função da enzima em estudo e ruptura das células ao contrário do emprego das baixas potências (KWIATKOWSKA *et al*. 2011; DELGADO-POVEDANO e CASTRO, 2015). Desta forma, o ultrassom tem sido usado em processos biológicos, a Tabela 3.2 apresenta de forma resumida alguns estudos empregando do ultrassom como forma de aquecimento não convencional em reações de transesterificação enzimática.

Tabela 3.2: Reações enzimáticas realizadas por meio do emprego do ultrassom.

Biocatalisador	Reação	Considerações	Referências
Lipozyme	Transesterificação de óleo de soja com etanol	O ultrassom é uma alternativa potencial para elevação do rendimento da reação de transesterificação	Batistela *et al*. (2012)
Novozym 435	Transesterificação de óleo residual e 1-propanol em oleato de propila	O uso do ultrassom melhorou a atividade enzimática e acelerou a velocidade de difusão, obtendo 95% de rendimento em 50 minutos de reação a 40°C e potência de 100 W.	Wang *et al*. (2007)
Novozym 435	Transesterificação enzimática de 1-gliceril benzoato	O ultrassom é uma perspectiva promissora para ultrapassar as limitações de transferência de massa resultantes da utilização de glicerol como substrato.	Ceni *et al*. (2011)
Novozym 435	Produção de biodiesel com óleo de cozinha usado e carbonato de dimetil	O uso do ultrassom aumentou a conversão da transesterificação enzimática para o mesmo tempo de reação com o método de agitação convencional	Gharat e Rathod (2013)
Novozym 435	Transesterificação enzimática para síntese de acetato de cinamilo	A aplicação do ultrassom reduziu o tempo obtendo conversão máxima em 20 min em comparação ao aquecimento convencional	Tomke e Rathod (2015)

CAPÍTULO IV

4. ARTIGOS

Este capítulo está apresentado na forma de artigos científicos (Artigo I, II e III) e está constituído por introdução, materiais e métodos utilizados no desenvolvimento dos artigos, resultados obtidos e sua discussão, e, por fim, a conclusão e as referências.

Artigo I - **(Eco)toxicity and biodegradability of protic ionic liquids** estudou a toxicidade contra vários micro-organismos e a biodegradabilidade de quatro PILs, nomeadamente, acetato de N-metil-2-hidroxietilamônio (m-2-HEAA), propionato de N-metil-2-hidroxietilamônio (m-2-HEAPr) butirato de N-metil-2-hidroxietilamônio (m-2-HEAB), e pentanoato de N-metil-2-hidroxietilamônio (m-2-HEAP). O artigo foi publicado no periódico Chemosphere.

Artigo II - **Effect of hydrophobicity of the cationic portion of protic ionic liquids in silica modification for lipase immobilization** estudou o efeito dos líquidos iônicos próticos com diferentes porcões catiônicas (amina) na modificação de sílica para serem empregadas como suporte na imobilização de lipase de *Burkholderia cepacia* por adsorção física e ligação covalente e posteriormente aplicadas em síntese de ésteres etílicos. O artigo será possivelmente submetido ao periódico Microporous and Mesoporous Materials.

Artigo III - **Surface modification of two differents multi-wall carbon nanotubes for generation of efficient immobilized system with *Burkholderia cepacia* lipase** estudou o efeito de biocatalisadores imobilizados por adsorção física em diferentes CNTs com a superfície modificada no rendimento de imobilização. Para fins de comparação as amotras foram caracterizadas antes e após a imobilização da lipase de *Burkholderia cepacia*. O artigo será possivelmente submetido ao periódico Journal of Nanotechnology.

4.1. ARTIGO I

(ECO)TOXICITY AND BIODEGRADABILITY OF PROTIC IONIC LIQUIDS

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Abstract

Ionic liquid (ILs) are considered as "environmentally friendly" compounds. However, their potential toxicity to organisms of different trophic levels has still been investigated. This study aims to evaluate the toxicity against various microorganisms and the biodegradability of four protic ILs (PILs) namely, N-methyl-2-hydroxyethylammonium acetate, m-2-HEAA; N-methyl-2-hydroxyethylammonium propionate, m-2-HEAPr; N-methyl-2-hydroxyethylammonium butyrate, m-2-HEAB; and N-methyl-2-hydroxyethylammonium pentanoate, m-2-HEAP, according to standard protocols. The antimicrobial activity was determined against: two bacteria, *Sthaplylococcus aureus* ATCC-6533 and *Escherichia coli* CCT-0355; the yeast *Candida albicans* ATCC-76645; and the fungi *Fusarium* sp. LM03. The toxicity of all PILs was tested against the aquatic luminescent marine bacterium *Vibrio fischeri* using the Microtox$^{@}$ test. The impact of the PILs was also studied regarding their effect on lettuce seeds (*Lactuta sativa*). The biodegradability of these PILs was evaluated using the ratio between biochemical oxygen demand (BOD) and chemical oxygen demand (COD). The results show that the elongation of the alkyl chain tends to increase the negative impact of the PILs towards all organisms and biological systems under study (bacteria, yeast, fungi and lettuce seeds). According to these results, m-2-HEAA and m-2-HEAP are the less and most toxic PILs studied in this work, respectively. Additionally, all the PILs have demonstrated low biodegradability.

Keywords: Protic ionic liquids, toxicity evaluation, antimicrobial activity, Microtox®, phytotoxicity, biodegradability.

1. INTRODUCTION

Ionic liquids (ILs) are molten salts at low temperature which properties can be tuned for a specific application, by an adequate combination of the cation/anion/alkyl chain length and functionalization (Hussey, 1988). Because of their unique properties, including non-volatility and non-flammability, variable solubility, high chemical and thermal stability (Domanska, 2006) , high ionic conductivity and wide electrochemical potential window, ILs have been widely studied, used and recognized as promising alternatives for various applications some of them with high industrial potential (Wasserscheid and Keim, 2000). In this scenario, ILs have been applied as promising alternatives in organic synthesis (Wang et al. 2003), catalysis (Souza et al. 2003), electrochemical (Jiang et al. 2004) and chemical separation (Ishikawa et al. 2006), biocatalysis (Ventura et al. 2012b, Sintra et al. 2014), enzyme immobilization (Souza et al. 2013, Oliveira et al. 2014) and in various extraction processes (Freire et al. 2012, Ventura et al. 2012c, Ventura et al. 2012a).

Recently protic ILs (PILs) have been the principal focus of several studies (Chen et al. 2014, Huang et al. 2014, Kusano et al. 2014, Peric et al. 2014, Santos et al. 2014, Thawarkar et al. 2014). They are synthesized by proton transfer from a Brønsted acid to a Brønsted base (Anouti et al. 2008, Mirjafari et al. 2013). A key property of PILs is their capacity to promote hydrogen bonds, in which proton acceptance and proton donation are included (Austen et al. 2012). The interest in this class of ILs stems from their simple synthesis, low cost of preparation and purification, and also their claimed biodegradable and low toxic nature (Hussey 1988). They have been applied in organic synthesis (Hangarge et al. 2002), chromatography techniques (Poole 2004), as proton conducting electrolytes (Menne et al. 2013), self-assembly media (Atkin 2005), catalysts (Jiang et al. 2004), and solvents (Achinivu et al. 2014). PILs are normally considered as of good technical performance (Greaves et al. 2006), however the current European Union environmental legislation concerning the registration, evaluation, authorization and restriction of chemicals (REACH, 2006) imposes the the safety assessment of new chemicals in which (eco)toxicological and biodegradation demands are included. Despite the widespread idea that PILs should be less toxic than the aprotic ILs (Peric et al. 2014), they are poorly studied and new structures require new studies. Many authors have reported the importance of investigating the toxicity of protic ionic liquids, according to Egorova & Ananikov, (2014), it's very important to consider the toxicity of the

compounds before applying them in any process. The toxicity of the newly investigated ILs is not yet known. George et al. (2015) report that the protic ionic liquid to have a construction based on a reaction acid / base, its toxicity is similar to a parent amine or an acid. The main objective of this work is the toxicity and biodegradability evaluation of four PILs, in which we have applied experimental tests to determine *i)* their antimicrobial activity, towards a Gram-negative and a Gram-positive bacteria, one fungus and one yeast, *ii)* their toxicity *via* Microtox®, *iii)* their phytotoxicity tested in lettuce seeds and *iv)* their biodegradability in water.

2. EXPERIMENTAL SECTION

2.1. Materials

In this study were used two antibiotics as positive control: Tetracycline (purity of 95-100%) and miconazole (purity of 99.77%) for bacteria and fungus, respectively, and NaCl (purity of 99%) as a negative control solutions. Tetracycline, miconazole, NaCl and the lettuce seeds were purchased at DEG Farmacêutica, Genix Farmacêutica, Quimex and in the Central Market from Aracaju, Sergipe, Brazil, respectively.

In this work, four PILs were used, namely the N-methyl-2-hydroxyethylammonium acetate (m-2-HEAA), the N-methyl-2-hydroxyethylammonium propionate (m-2-HEAPr), the N-methyl-2-hydroxyethylammonium butyrate (m-2-HEAB) and the N-methyl-2-hydroxyethylammonium pentanoate (m-2-HEAP), whose chemical structures are depicted in Figure 4.1. Those were synthesized at our laboratory, by reacting equimolar amounts of the amine and the respective organic acids, as detailed elsewhere (Matzke et al. 2010). All IL were used in this study in pure form (99%).

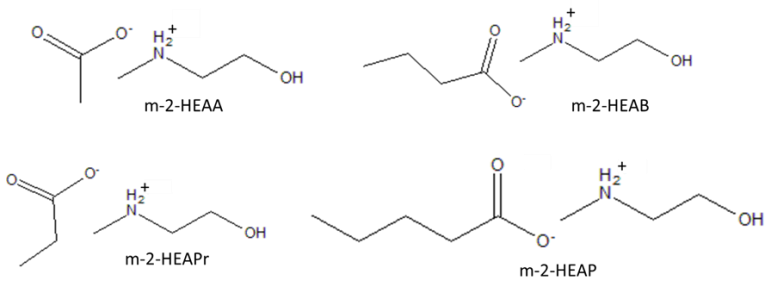

Figure 4.1. Chemical structure and respective abbreviation names for the PILs studied

2.2. (Eco)toxicity evaluation

2.2.1. Antimicrobial activity tests

To test the antimicrobial activity of the PILs (Ventura et al., 2012d), miconazole (50 µg.L^{-1}) and tetracycline (50 µg.L^{-1}), aqueous solution, were used as reference compounds and positive control for fungi and bacteria, respectively. The negative control used in this work was an aqueous solution of NaCl at 0.9% (w/v) . The microorganisms, *Escherichia coli* CCT-0355 (Gram-negative bacteria), *Staphylococcus aureus* ATCC-6533 (Gram-positive bacteria), *Fusarium* sp. LM03 (mold) and *Candida albicans* ATCC-76645 (yeast). These microorganisms were grown in Bushell-Hass medium (total composition, g.L^{-1}: $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; $(NH_4)_2HPO_4$, 1.0; KNO_3, 1.0; and $FeCl_3$, 0.05) until an optical density of 1.0, taking into account the MacFallen scale. Suspensions of 1 mL of these microorganisms were uniformly spread on the plates [with Nutrient Agar (Total composition g.L^{-1} Meat extract 3.0; Peptone 5.0; Agar 15; pH: 6.8 ±0.2) and Sabouraud (Total composition g.L^{-1} Peptone 10; Dextrose 40; Ágar 15; pH: 5.6±0.2) medium for bacteria and fungi, respectively], and wells of 6 mm of diameter were punched under a sterilized ambient, with a sterile glass tube. Samples of each one of the PILs (50 µL) were then placed into the wells, previously inoculated by the target microorganisms. The plates were then incubated at 37 ^{o}C. The growth inhibition halo was measured using a caliper after 24 or 48 h, depending of the microorganism. Each PIL was tested in triplicate, being the halo of inhibition presented as the average of the replicates.

2.2.2. Microtox® test

Standard Microtox® liquid-phase assays (Ventura et al. 2010) were used to evaluate the luminescence inhibition of the bacteria *Vibrio fischeri* (strain NRRL B-11177) following exposure to each compound at 15ºC. The bacteria was exposed to a range of geometrically diluted aqueous solutions (normally from 0 to 81.9%, geometric factor = 2) of each PIL, where 100% of IL corresponds to a known concentration of a stock solution previously prepared (Azur 1998, Azur 1999). After 5 and 15 minutes of exposure time to each PIL, the light output of the luminescent bacteria is measured and compared with the light

output of a blank control sample for the estimation of the corresponding 5 min- and 15 min-EC_{50} values (Ventura et al. 2012b) (EC_{50} being the estimated concentration yielding 50 percent effect), and the corresponding 95 percent confidence intervals were estimated for each IL through Microtox® Omni™ Software version 4.1.25 (Azur, 1999).

The toxic units (TU) were also determined at 15 minutes of exposure time by the calculation of the inverse of each EC_{50} value (Eq. 1) (Chang e al. 2013).

$$TU = \frac{1}{EC_{50}} \times 100 \qquad \text{Eq. 1}$$

2.2.3. Phytotoxic tests

The phytotoxicity study aimed at assessing the toxicity level of the PILs on lettuce seeds (*Lactuta sativa*). The seeds were pre-treated by being washed with tap water for 4 hours, and then placed on Petri dishes (12 x 12 cm). Each dish contained three paper filters saturated with 9 mL of different aqueous solutions of each PILs studied. Each PIL was tested at six concentrations, 1.0×10^{-7}, 1.0×10^{-6}, 1.0×10^{-5}, 5.0×10^{-3}, 1.0×10^{-3} and 1.0×10^{-2} ($g.L^{-1}$). For the treatments and the control (water), 30 undamaged seeds of identical size were placed evenly on the filter papers placed in the Petri dishes and then incubated in the dark at 28 °C. The control test was used to investigate the stability and reproducibility of the germination capacity of the lettuce seeds in absence of xenobiotics. Three repetitions for each experiment (meaning each Lethal Dose - LD concentration selected) were done. After three days of incubation, the final germination percentages (FG - %) were obtained according to standard protocols described in literature (Biczak et al. 2014). The cells were also analyzed with light optic microscope. Cells in interphase and undergoing division were examined to assess the induction of chromosome and nuclear aberrations, such as abnormal anaphases (multipolar, with bridges, delayed, etc.), fragments and loss of chromosomes, C-metaphases, micronuclei and multinucleated cells (Souza et al. 2013).

2.3. Biodegradability

The chemical and biochemical oxygen demands (abbreviated in this work as COD and BOD) were determined using the experimental methodology described in the Standard Methods Analysis of Water and Wastewater. The biodegradability of the PILs tested at different concentrations were determined after 48 h incubation (APHA 2012).

Chemical Oxygen Demand (COD) is a parameter that measures the amount of organic matter capable of being oxidized by chemical means exist in a liquid sample. It is expressed in mg $O_2.L^{-1}$. The method measures the concentration of oxidized organic material. However, there may be interference due to which there are inorganic substances that can be oxidized (sulfides, sulfites, halide, etc.). Its measurement allows evaluating quantization parameters organic matter in aqueous compounds; may be waste, rivers and groundwater, it is also used in the solid waste sector, in particular in sludge. For potable water it is not easy its direct measurement due to low concentration of organic matter, which is why it is usually used oxidisability method with potassium biphthalate.

Since the Biochemical Oxygen Demand and Biological Oxygen Demand (BOD) is the amount of oxygen consumed in the degradation of organic matter in the aquatic environment by biological processes and is expressed in milligrams per liter ($mg.L^{-1}$). It is the most widely used yardstick to measure pollution. The method based on the inoculation of the sample in a sealed bottle and these are incubated at 20 °C for 05 days. The amount of oxygen is measured before and after incubation.

3. RESULTS AND DISCUSSION

3.1. (Eco)toxicity evaluation

3.1.1. Antimicrobial activity

The antimicrobial activity test was based on diffusion test agar recommended by Bauer et al (1966) where the paper disks are soaked in the compounds of interest and placed on Petri plates previously seeded with the microorganisms of interest.

Figure 4.2 shows the results of the antimicrobial activity of all PILs tested against different microorganisms. The results suggest that all PILs have activity against the microorganisms under investigation, namely for the yeast and mold. The criteria to analyze these results are: *i)* how the results obtained for the different PILs compare and depend on the increase in the alkyl chain length and *ii)* how the data obtained for the PILs compares with the growth inhibition halo obtained for the positive controls, tetracycline for the bacteria species and miconazole for the remaining two microorganisms. Both criteria can be analyzed in Figure 4.2, which show the behavior obtained for the PILs against all the microorganisms under study and allows the comparison between the results obtained for the PILs and controls.

The *E. coli* CCT-0355 results show a common behavior of an increase in toxicity when the alkyl chain length is elongated until the chain of four carbons (Figure 4.2), followed by the "*cut-off*" effect at the butyl chain. The data of *S. aureus* ATCC-6533 shows the lower toxicity of the PILs when compared with the control tetracycline used for the bacteria species, behavior also observed for *E. coli* CCT-0355. However, the common effects of "*alkyl side chain*" and "*cut-off*" effect are not as clear as for *E. coli* CCT-0355. The same tendency is not observed when the results of both yeast and fungi are analyzed against the data of miconazole. It can observe the same profile as already seen earlier, low toxicity of protic ionic liquids occurred when the alkyl chain contains two carbon atoms and increased toxicity with increasing alkyl chain. This behavior can be observed in previous studies (Pernak et al. 2001a, Pernak et al. 2001b, Pernak et al. 2003a, Pernak et al. 2003b, Pernak et al. 2004, Ventura et al. 2012a). However, it was noticed a strange behavior of the PIL with three carbons (m-2-HEAPr) for all microorganisms, except *E. coli* CCT-0355. Despite the morphologic differences between the microorganisms under study (Salyers, Whitt. 2001 ,Ventura et al. 2012a), the peculiar difference in behavior between ILs with three and four carbons in the alkyl chain is not new. Cho et al. (2007) studied the toxicity of four ionic liquids based on Br, as anion, and imidazolium as cation, against green alga *Selenastrum capricornutum* ATCC-22662; and they observed similar behavior to that seen in this work. A similar change in the alkyl side chain is observed between the ILs 1-propyl-3-methylimidazolium bromide [C_3mim]Br and 1-butyl-3-methylimidazolium bromide [C_4mim]Br, which means that for some organisms, these

two alkyl chains may promote some changes to the commonly observed "alkyl side chain" effect, which was not until now explained.

3.1.2. Microtox® tests

The bioluminescent bacterium *Vibrio fischeri* and the Microtox® technique have been used as part of a model standard methodology to test the toxicity of different chemicals (Ranke et al. 2007, Matze et al. 2010, Ventura et al. 2012a, Ventura et al. 2014). The four PILs were tested in terms of their effect against this marine luminescent bacterium for 5 and 15 minutes of exposure/toxic action. Table 4.1 shows the toxicity results for the luminescent bacteria and for two exposure times in the form of EC_{50} ($mg.L^{-1}$) values. From a brief analysis of the results, the toxicity of the PIL to the bacteria is ordered as m-2-HEAA < m-2-HEAPr < m-2-HEAB < m-2-HEAP, verified for both 5 and 15 minutes of exposure. This tendency is clearly explained by the increase in the alkyl chain length (Matze et al. 2010, Ventura et al. 2012, Ventura et al. 2012a). In the same table, the toxic units (TU) are also described. When Microtox® is applied, the TU data can be used as a measure of the toxic action of the chemicals (Chang et al. 2013). According to the results of TU and supported by the classification normally applied to identify the most and less toxic chemicals, it is safe to describe the PILs tested here as *non-toxic*. The same harmless nature of PILs was also reported by Peric et al. (2013). In this work, there was a minimal effect anion propionate front of the luminescent bacterium *Vibrio fischeri*. Opposite effect (maximum) has been reported for fungus *C. albicans* ATCC 76645 and *Fusarium* sp. LM03 and *S. aureus* ATCC 6533 and E. coli CCT 0355. This maximum effect was reported when the organism used was the microalgae *Pseudokirchenriella subcapitata* by Peric et al 2013. Moreover, while these PILs are considered as non-toxic, based on Microtox® tests, actually the ionic liquids m-2-HEAPr and m-2-HEAP are more toxic than the miconazol for the *Fusarium* sp. LM03.

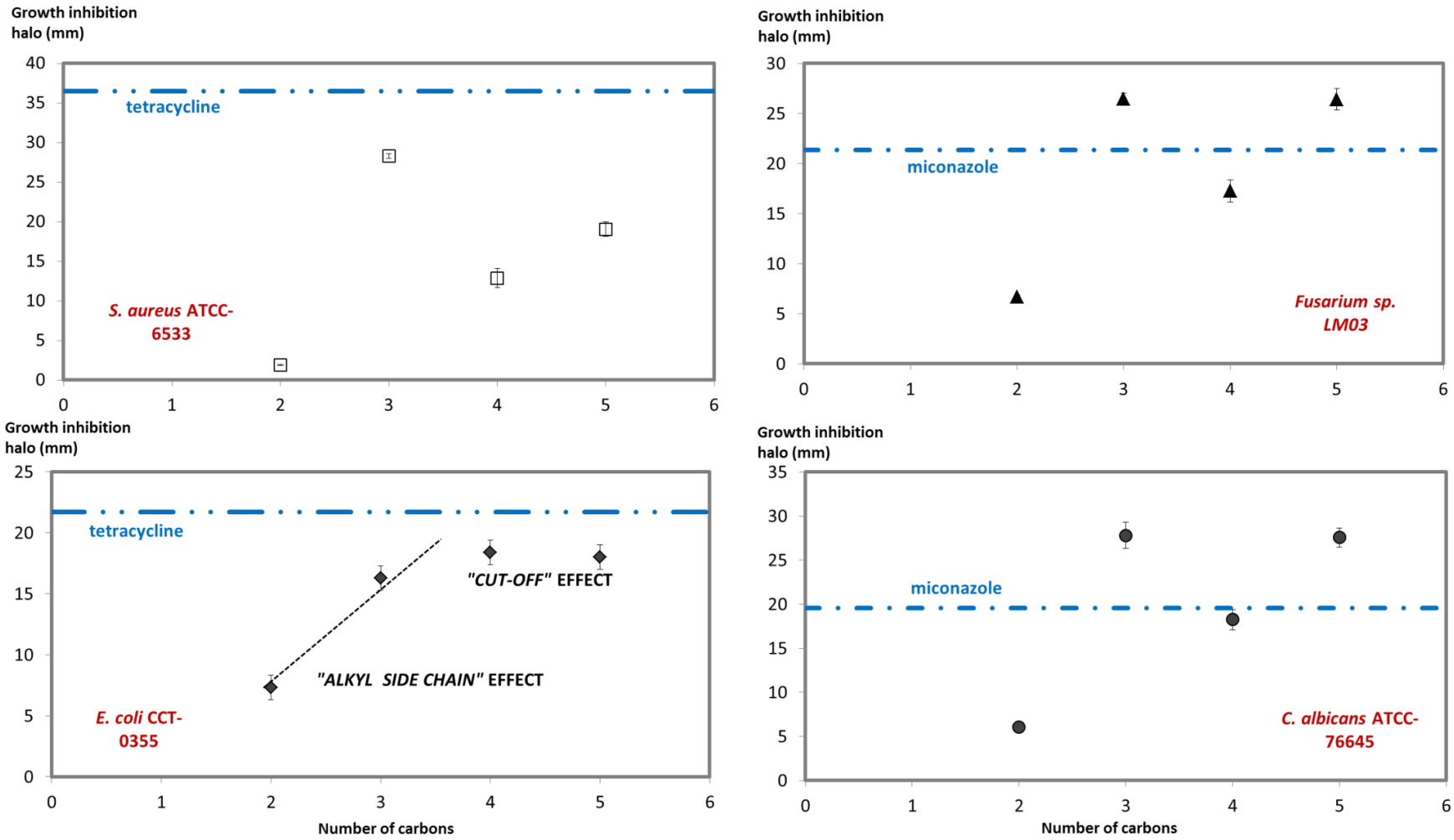

Figure 4.2. Growth inhibition halo (in mm) *versus* number of carbons in the alkyl chain; results of the positive controls (tetracycline and miconazole – blue lines): (□) *S. aureus* ATCC-6533, (♦) *E. coli* CCT-0355, (▲) *Fusarium* sp. LM03, (●) *C. albicans* ATCC-76645.

Table 4.1. Microtox® EC_{50} values ($mg.L^{-1}$) for the *Vibrio fischeri* after 5 and 15 min of exposure to the PILs, with the respective 95% confidence limits (in brackets).

ILs	EC_{50} ($mg.L^{-1}$) 5min (lower limit; upper limit)	EC_{50} ($mg.L^{-1}$) 15min (lower limit; upper limit)	TU*[(1)]
m-2-HEAA	900.63 (427.14; 1898.29)	962.54 (220.11; 4209.61)	0. 10
m-2-HEAPr	620.78 (304.29; 1266.62)	887.81 (104.83; 7504.40)	0.11
m-2-HEAB	591.33 (436.91; 799.76)	717.00 (30.33; 780.05)	0.14
m-2-HEAP	456.62 (162.59; 1282.46)	551.61 (142.02; 2142.46)	0.18

*****the TU data were calculated with the EC_{50} data at 15 minutes of exposure time.

[(1)] **Note:** the classification referred in literature describes the following: TU < 1 non-toxic; TU = 1-10 toxic; TU = 10-100 very toxic; TU > 100 – extremely toxic (Chang, Wang et al. 2013)

3.1.3. Phytotoxicity

The sensitivity of *Lactuta sativa* germination to the four PILs here investigated was also checked by determining the LD_{50} values**,** being the IL-m-2-HEAA more toxic to higher LD_{50} value (1.85 ± 0.01). The PIL m-2-HEAPr (1.18 ± 0.03) and m-2-HEAB (1.16 ± 0.01) had similar toxicity. Finally PIL m-2-HEAP proved to be the least toxic to LD50 = 0.45 ± 0.04. It is clearly seen the decrease of LD_{50} with the increase in the alkyl chain of the PIL and, consequently of the hydrophobic nature of the PIL tested, which is in accordance with recent literature (Studzinska, Buszewski, 2009, Cybulski et al. 2011, Biczak et al. 2014, Bubalo et al. 2014). Actually, the same tendencies are described for the marine luminescent bacteria (see results from Section 3.1.2 of this work).

To further support the phytotoxicity results found for the PILs, images of the mitotic cell division of *Lactuta sativa* seeds, when exposed to different PILs concentrations are presented in Figure 4.3. Different conditions were considered, namely the absence of PILs

(Figure 4.3A) and the presence of each PIL under analysis, from the shorter until the long alkyl chains, i.e. from m-2-HEAA (Figure 4.3B) to m-2-HEAP (Figure 4.3E). By comparison with Figure 4.3A, it is possible to observe that all PILs have some impact in the structure of the cell, although to a less extent for the m-2-HEAA (Figure 4.3B) and m-2-HEAPr (Figure 4.3C). However, the same behavior is not observed in Figures 4.3D and 4.3E, in which the seeds were placed in contact with the more hydrophobic PILs, namely m-2-HEAB (four carbons in the alkyl chain) and m-2-HEAP (five carbon atoms in the alkyl chain). Figure 4.3D shows a cell having micronuclei in prophase while in Figure 4.3E the cell was at interphase with chromosomal loss and presents micronuclei in prophase.

The data suggests that the toxicity or the negative impact of the presence of the PILs increases with the elongation of the alkyl chain. These changes in the aspect of the cells are attributed to the cell mutagenicity, as discussed elsewhere (Caritá, Marin-Morales, 2008).

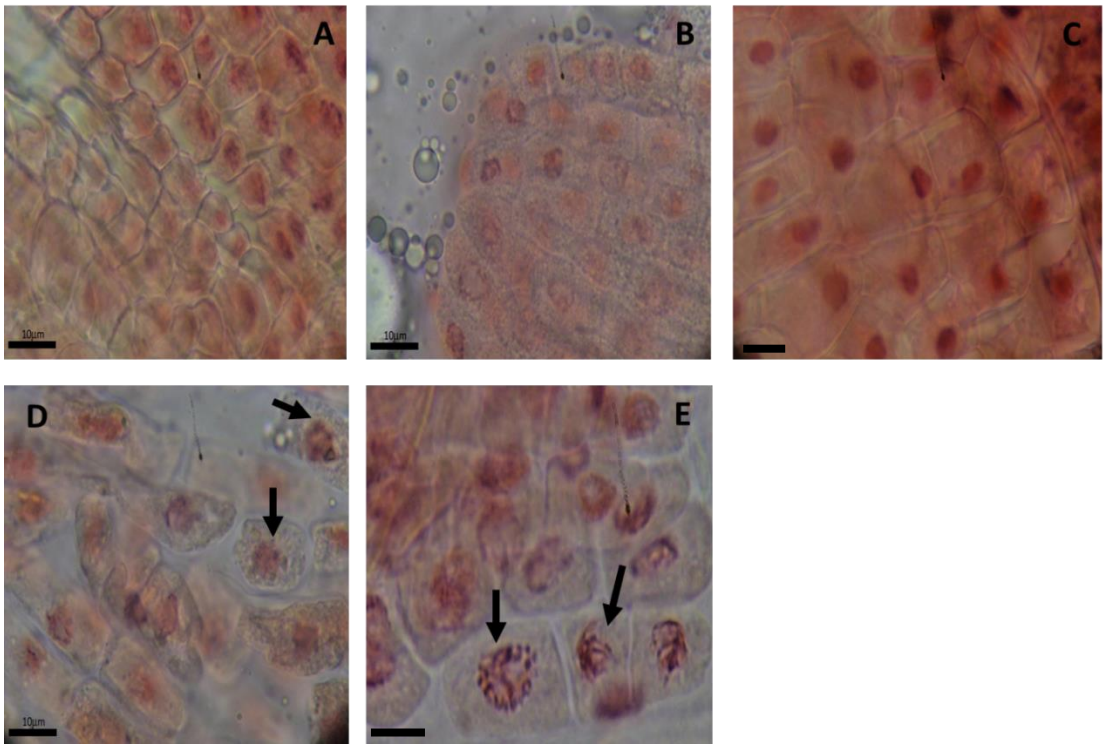

Figure 4.3. Mitosis phases of *Lactuta sativa* cells with and without the presence of PILs: (**A**)- control; (**B**) – seeds exposed to m-2-HEAA (1 x 10^{-6}) (**C**)- seeds exposed to m-2-HEAPr (1 x 10^{-6}); (**D**) – seeds exposed to m-2-HEAB (1 x 10^{-5}) and (**E**) – seeds exposed to m-2-HEAP (1 x 10^{-5})

3.2. Biodegradability evaluation

In contrast to chemical degradation, which requires the assistance of an oxidant for catalysis, biodegradation is the microbial breakdown of chemical compounds (Coleman, Gathergood 2010). Table 4.2 shows that all compounds showed values of BOD/COD lower than 0.5, indicating the possibility of anaerobic degradation. Another factor to be noted is the lowest biodegradable nature of m-2-HEAA. Some authors relate the radical size with the toxicity and biodegradability of the compound. Bubalo et al. (2014) reported that the smaller the radical chain added, the lower the toxicity of the tested imidazolium ILs. The biodegradation potential of ILs in general (protic and aprotic) in aqueous media has been addressed in some works (Wells, Coombe, 2006, Docherty et al. 2007, Romero et al. 2008, Coleman, Gathergood 2010, Peric et al. 2011, Stolte, Steudte 2012, Peric et al. 2013). Wells, Coombe (2006) investigated the biodegradability of quaternary ammonium, imidazolium, phosphonium and pyridinium compounds by measuring the BOD. They observed that the cations with short side chains (C4) were not biodegradable, which is in agreement with the results here reported, and that this main conclusion seems to be independent of the protic or aprotic nature of ILs. Moreover, the generality of the works reporting biodegradability studies describe the low biodegradable nature of ILs.

Table 4.2. Results for the chemical oxygen demand (COD – $mg.L^{-1}$), biochemical oxygen demand (BOD – $mg.L^{-1}$) and percentage of oxygen consumed (O_2 - %).

ILs	COD ($mg.L^{-1}$)	BOD ($mg.L^{-1}$)	COD/BOD	BOD/COD (% oxygen uptake or biodegradation)	%O_2 (%)
m-2-HEAA	28760.00	271.10	106.10	0.01	30.50
m-2-HEAPr	23280.00	333.00	69.90	0.01	36.70
m-2-HEAB	20540.00	322.20	63.70	0.02	35.30
m-2-HEAP	15060.00	255.00	59.00	0.02	28.00

4. CONCLUSIONS

In this study, the toxicity and biodegradability of four distinct PILs were tested and analyzed. From the main results of this study, it was found that the elongation of the alkyl chain of the information leaflets tested is increasing the negative impact of these chemicals for the various microorganisms tested, as expected. This trend was found for the different microorganisms studied. When bacteria were studied it was observed that with increasing chain alkyl toxicity PILs increased. When these tests were performed for the fungi was observed that PILs containing three and five carbon atoms in its alkyl chain were more toxic than the others. When the test organism was the luminescent marine bacterium *Vibrio fischeri* was observed that toxicity increased with increasing alkyl chain. When the phytotoxicity was conducted test was observed indicating formation of cytotoxicity structures in all samples *Lactuta sativa* exposed to protic ionic liquids. It was then concluded that m-2-HEAPr and m-2-HEAP represent the less and more toxic PILs studied in this work. Regarding the biodegradability study, the results suggest that these four chemicals showed low biodegradability.

ACKNOWLEDGMENTS

This work was developed in the scope of the project CICECO-Aveiro Institute of Materials (Ref. FCT UID /CTM /50011/2013), financed by national funds through the FCT/MEC and when applicable co-financed by FEDER under the PT2020 Partnership Agreement. The authors are grateful for the financial support from PIBIC-FAPITEC for the grant of Rafael L. F. de Barros. The authors also thank the financial support from Fundação para a Ciência e a Tecnologia under the project PTDC/AAC-AMB/119172/2010.

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4.2. ARTIGO II

EFFECT OF CATIONIC HYDROPHOBICITY OF PROTIC IONIC LIQUIDS BASED ON 2-HYDROXYETHYLAMMONIUM IN SILICA MODIFICATION FOR LIPASE IMMOBILIZATION

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Abstract

Protic ionic liquids (PILs) with different cations (pentanoic acid – anion) were used as modifier agents of silica and these materials were used as support for immobilization Burkholderia cepacia lipase by physical adsorption and covalent binding - CB (treating with γ-aminopropyltriethoxysilane followed of activating with glutaraldehyde or using epichlorohydrin as activating agent). The silica supports were characterized by nitrogen adsorption–desorption (BET), thermal analysis (DTA-TG), small-angle X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and immobilized biocatalysts were characterized by Fourier transform infrared spectroscopy (FTIR), total activity recovery yield, pH and optimal temperature, thermal and operational stability and kinetic parameters were obtained. The FTIR showed that the PILs excess were efficiently removed by soxhlet extraction from the modified silicas. The morphological characterization revealed changes in the structural properties of the modified silicas. The modified silica with a PIL based on the most hydrophobical cationic (m-2HEAP) showed the highest pore diameter, pore volume and total activity recovery yield of the 64.78 % when immobilized by physical adsorption. This bcatalysts presented good thermal stability ($t_{1/2}$ from 231 to 309 h) and reusability (the highest relative activity after 40 recycles). The immobilized biocatalysts by covalent binding using epichlorohydrin (CBE) presented excellent total activity recovery yield (from 182 to 235 %). FTIR analysis confirmed the presence of the enzyme for all immobilized systems by the presence of a band of amide I and 92 % of ethyl esters yield was obtained from transesterification reaction assisted by ultrasound. In addition, the ultrasound intensified the half-life of the immobilized biocatalysts by CBE on modified support with m-2HEAP. The results revealed that modification of silica with PILs was efficient method to improve the properties of immobilized lipase and the more hydrophobic PIL was beneficial for immobilization, since the industrial applications are favorable due to the easier synthesis, lower ecotoxicity and low cost.

Keywords: Protic ionic liquid; modified silicas; immobilization, transesterification.

1. **INTRODUCTION**

The field of mesoporous materials has been progressively growing in the last two decades, rapidly acquiring significant attention in many areas: materials science, chemistry and biotechnology (Gascón et al., 2014). Mesoporous silica offers many advantages due to properties such as large surface area, controlled pore size, and sufficient silanol groups for surface modification (Alothman et al., 2012). According to Dash et al. (2008) modified silica gel can host a wide variety of materials, can be applied in modern areas, are environmentally friendly, provide a long term service and can efficiently substitute traditional catalysts, particularly in liquid phase reactions. These characteristics make these materials one of the most promising supports for enzyme immobilization (Zou et al., 2010; Hu et al., 2012).

Physico-chemical and morphological modifications of the supports can produce immobilized biocatalysts with higher catalytic efficiency due to minimization of diffusional effects of substrates and products during the reaction, improved operating stability in continuous and discontinuous processes (Carvalho et al., 2015).

Some studies on surface modification of mesoporous materials have been carried out to improve the properties of the immobilized enzyme, describing the use of ionic liquid as an effective modified agent (Zou et al., 2010; Hu et al., 2012). However, among the ionic liquids, those synthesized by proton transfer from a Brønsted acid to a Brønsted base (so-called Protic Ionic Liquids - PILs) feature simplicity of synthesis, low cost of preparation and purification, favouring different applications and demonstrated to be biocompatible with lipases (Souza et al., 2013; Oliveira et al., 2016).

The use of PILs has been reported in the biocatalists preparation as additives during the immobilization process and hybrid support synthesis, however, the literature does not report the removal of the PIL from the supports before the immobilization process. Souza et al. (2013) used different protic ionic liquid (varied by the length of the alkyl chain) added in the immobilization process of the *Burkholderia capecia* lipase on silica sol-gel by encapsulaption. Immobilized biocatalyst in the presence of protic ionic liquids showed high activity yields. Oliveira et al., (2014) demonstrated that immobilized system by encapsulation with the addition of PIL has catalytic efficiency for producing esters from

refined babassu oil and ethanol. Martins et al. (2016) used PIL to hybrid support synthesis (PHBV and silica) and immobilized lipase on hybrid supports founded greater total activity recovery yield and good thermal and operational stability.

In this context, aroused interest in studying the effect of PILs (with different cations) in the silica modification to be used as support for lipase immobilization and apply in ethyl esters syntesis using coventional and non-conventional heating, highlighting the removal of PIL before immobilization process. Non-conventional heating (microwave and ultrasound) might be attractive to be considered sustainable technologies, improving the performance enzyme-catalyzed reactions, reducing reaction time and increasing the reaction yield (Da Rós et al. 2014; Adewale et al., 2015; Subhedar and Gogate, 2016).

The current research aims to analyze the effects of PILs with different cations (pentanoic acid - anion) in the modification of the silica by the achievement of the morphological and physico-chemical properties, to immobilize the *Burkholderia cepacia* lipase in the pure and modified silicas by physical adsorption and covalent binding and to characterize the biocatalysts. The application in ethyl esters synthesis using conventional and non-conventional heating was also investigated.

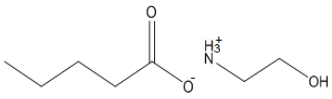
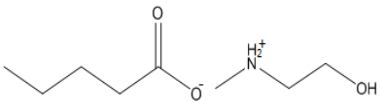
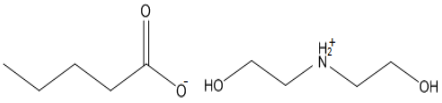
2. **MATERIAL AND METHODS**

2.1. Materials and reagents

Lipase from *Burkholderia cepacia* (Amano Lipase) with enzymatic activity of the 2521.15 $U.mL^{-1}$ was purchased from SIGMA – ALDRICH (Japan). The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (NJ, USA) and used without further purification. Ethanol (purity of 99%), ammonia (purity of 28%), hydrochloric acid (purity of 36%) and gum Arabic (purity of 85%) were obtained from Synth (São Paulo, Brazil). The protic ionic liquids - PILs were synthesized for this work, from acid-basic neutralization reaction according to Alvarez et al. (2010) and purified in order to achieve humidity values below 0.1%. After purification, the salt formation was confirmed by FTIR analysis. The PILs are show in Table 4.3 together with the log of P scale, which is

used to quantify the hydrobhobicity of Ionic Liquids (Naushad et al., 2012). Other chemicals were of analytical grade and used as received.

Table 4.3: Structure of protic ionic liquid (pentanoic acid - anionic portion) used in this work and log P of the cationic portion.

Nomenclature	PIL Structure	Log P (cationic portion)
2-hydroxyethylammonium pentanoate (2HEAP)		-1.32
N-methyl-2-hydroxyethylammonium pentanoate (m-2HEAP)		-0.88
bis(2-hydroxyethyl)ammonium pentanoate (BHEAP)		-1.57

2.2. Silica Syntesis by sol-gel technique

Silica was synthesized by sol-gel technique using the methodology applied by Oliveira et al. (2014) with some modifications. Briefly: 30 mL of TEOS was dissolved in 36 mL of absolute ethanol under an inert nitrogen atmosphere. Further, 0.22 mL of hydrochloric acid was dissolved in 5 mL of slowly added ultra-pure water. The mixture was stirred (200 rpm) for 90 min at 35 °C, and 1 % (w/v) of protic ionic liquids (2HEAP, m-2HEAP, and BHEAP) was added individually. Further, 1.0 mL of ammonium hydroxide dissolved in 6.0 mL of ethanol (hydrolysis solution) was added to the sol-gel reaction and the mixture was kept under static conditions for 24 h until complete polycondensation. The bulk gel was washed with hexane in soxhlet for 12 h and dried at 60 °C for 2 h. For comparison purposes, pure silica (PS) was prepared in the absence of the PIL. The modified silica materials were named: 2HEAP-S, m-2HEAP-S, BHEAP-S.

2.3. Immobilization of the lipase from *Burkholderia cepacia*

The lipase from *Burkholderia cepacia* was immobilized by physical adsorption and covalent binding (activating support with glutaraldehyde or epichlorohydrin) by three different methodologies as described below.

The lipase was immobilized by physical adsorption on silica support using the procedure from Cabrera-Padilla et al. (2013). Briefly, 10 mL of hexane was added to 1 g of the support with vigorous agitation at room temperature for 2 h, then 20 mL of the enzymatic solution (0.3 g of the enzyme solubilised in 20 mL of 0.1 M sodium phosphate buffer, pH 7.0) was added to the hexane and support suspension and agitated for another 2 h. The enzyme-support system was then incubated for 24 h at 4 °C. The immobilized lipase was recovered by vacum filtration coupled with repeated hexane wash and dried under vacuum at room temperature for 72 h. Filtrates and washes were collected and used for activity determination. The immobilized lipase was then stored at 4 °C.

The lipase was immobilized by covalent binding on silica support using glutaraldehyde according to Soares et al. (1999). The support was previously treated with γ-aminopropyltriethoxysilane (γ-APTS) and activated with glutaraldehyde. Buffer (100 mM sodium phosphate buffer, pH 7.0) or hexane were used as a dispersion medium. For each gram of dry support, 0.3 g of lipase was used. The enzyme as dissolved in 10 mL of 100 mM of sodium phosphate buffer, pH 7.0, and mixed with the support under low stirring during 2 h at room temperature. After this, 10 mL of 100 mM sodium phosphate buffer, pH 7.0, or 10 mL hexane was added to the mixture enzyme-support and coupling took place overnight at 4 °C. The derivative was filtered and thoroughly rinsed with 100 mM of sodium phosphate buffer or hexane and dried under vacuum at room temperature for 72 h.

The lipase was immobilized by covalent binding on silica support using epichlorohydrin according to methodology from Da Rós et al. (2014). Activation of silica supports particles was carried out with epichlorohydrin at 2.5 % (w/v) pH 7.0 for 1 h at room temperature, followed by exhaustive washings with distilled water. Silica particles were soaked into hexane under stirring (100 rpm) for 1 h at 25 °C. Then, the excess hexane was removed and lipase from *Burkholderia cepacia* was added at a ratio of 1 gram of enzyme to 4 gram of support. PEG-1500 was added together with the enzyme solution at

a fixed amount (5 mg/g of support). Lipase and support were maintained in contact for 16 h at 4 °C under static conditions. The immobilized lipase was filtered and thoroughly rinsed with hexane and dried under vacuum at room temperature for 72 h.

2.4. Effect of Triton X-100

In order to investigate the nature of interaction between lipase and silica support, immobilized preparations were incubated with 0.5 % (v/v) Triton X-100 at a ratio of 1 g:0.1 mL of triton solution per gram of biocatalyst immobilized. After 3 h, the material was centrifuged at 4000 rpm for 10 min and thoroughly rinsed with buffer (pH 7.0). The biocatalysts immobilized were assayed for lipolytic activity.

2.5. Lipase activity

Enzymatic activities of free and immobilized lipase were assayed by olive oil emulsion method according to modifications by Soares et al. (1999). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of free fatty acid per min under assay conditions (37 °C; pH 7.0; 10 min incubation). Analyses of enzymatic activities performed on the free and immobilized lipases were used to determine the total activity recovery yield (Ya) according to Eq. (1).

$$Ya\ (\%) = \frac{Us}{Uo} \times 100 \qquad (1)$$

in which U_S corresponds to the total enzyme activity recovered on the support and U_o represents the enzyme units offered for immobilization.

2.6. Biochemical Characterization

The effect of pH on the hidrolitic activity of the free and immobilized lipase was determined by incubating the biocatalyst between pH 2.0-10. The buffers used were 0.1 M citric acid-sodium citrate (pH 2.0-5.0), 0.1 M potassium phosphate (pH 6.0-8.0), and 0.1 M bicarbonate-carbonate (pH 9.0-10.5). The optimal temperature for activity of the immobilized lipase was determined in the 30-80 °C range in the same 0.1 M potassium phosphate buffer (pH 7.0).

2.7. Thermal stability

The thermostability of the free lipase and immobilized biocatalysts were investigated at 60 °C by pre-incubating the sample at optimal temperature and pH value (7.0) obtained by biochemical characterization or in organic medium (heptane) when submitted to microwave irradiation (about 100 W) and ultrasound (about 220 W). Samples were periodically withdrawn for activity assays up to get 50% of original activity. Residual activities were calculated as the ratio of the activity of enzyme measured after incubation for the maximal activity of the enzyme. The thermal inactivation constant (Kd) and half-life (t1/2) were calculated using Eqs. 2 and 3, respectively (Cabrera-Padilla et al., 2012).

$$A_{in} = A_{in0} \exp(-k_d \, . t) \quad (2)$$

$$t_{1/2} = \ln(0.5) / -k_d \quad (3)$$

where A_{in} is the residual activity after heat treatment for a period of incubation (U), A_{in0} is the initial enzyme activity (U), k_d is the inactivation constant (h^{-1}), and $t_{1/2}$ is the half-life (h).

2.8. Operational stability

The operational stability of the immobilized biocatalysts were determined by conducting hydrolysis reactions in consecutive batches (10 min, optimum temperature, and pH) using the same biocatalyst. After each batch, the immobilized enzyme was

washed with hexane once and reused for the next cycle of hydrolysis (Carvalho et al., 2013).

2.9. Kinetic parameters

The Michaelis–Menten (K_m) constant and maximum reaction velocity (V_{max}) hydrolysis, catalysed by the free and immobilized lipase, were determined. Systems were prepared containing fatty acids at concentrations ranging from 37 to 3348 mM, obtained from emulsions containing different proportions of olive oil (5–90 %) and an aqueous solution of gum arabic (7 % w/v). The apparent values of K_m and V_{max} were calculated by non-linear fitting using the programme Origin 8.0.

2.10. Morphological and physical-chemical properties

The samples of silicas supports synthesized were characterized by:

- X-ray diffraction (XRD) patterns (Philips X'Pert X-Ray Diffractometer).

- The surface areas of the pure silica and modified silicas were calculated using the Brunauer–Emmett–Teller (BET) method. Pore volume and average pore diameter estimation was based on the model developed by Barret, Joyner and Halenda (BJH) for mesoporous samples. Surface areas were evaluated according to the N_2 adsorption at 77 K using Micromeritics (Gemini V 2380) apparatus. Prior to analysis, samples were submitted to a thermal treatment at 100 °C for 48 h, to eliminate any water existing within the pores of the solids.

- Thermogravimetric and differential thermal analysis (Shimadzu DTG-60H/DTA-TG simultaneous apparatus), under a nitrogen atmosphere that started from room temperature and went up to 1000 °C, increasing at a heating rate of 20 °C min^{-1}.

- Scanning electron microscopy (SEM). The images were performed in a Hitachi SU-70 microscope operated in secondary electron mode at 15 kV.

- Transmission electron microscopy (TEM). Measurements were realized on a JEOL JEM-2200FS microscope operated at 200 kV. Samples for TEM measurements were prepared by dry adhesion of the samples to a holey carbon grid.

- FTIR analysis (spectrophotometer FTIR BOMEMMB-100). Spectra were obtained in the wavelength range from 400 to 4000 cm^{-1}. The samples of immobilized biocatalysts also were submitted to FTIR analysis.

2.11. Transesterification reactions

All transesterification reactions were performed with waste coconut oil and anhydrous ethanol at molar ratio of oil-to-ethanol of 1:12 and 40 °C without the addition of solvents. The biocatalysts were used at a fixed proportion (20 %) in relation to the total weight of reactants involved in the reaction (Da Rós et al., 2010). The transesterification reactions were assisted by Dubnoff Shaking Baths (conventional heating), microwave (Model Discover/University-Wave, Cem Corporation, and operation power 100 W) and ultrasound (Ultronique, Q 5.9 / 40A, operating frequency of 25 kHz and operation power of 220 W). Samples were quantified by gas chromatography (Agilent Technologies 7820-A GC coupled to the mass detector MSD 5975 and Supelcowax10 column).

3. **RESULTS AND DISCUSSION**

3.1. Removal of ionic liquid of silicas for immobilization

The use of ionic liquids in biocatalytic processes is known to influence the properties of lipases (Kazlauskas and Park, 2003; Souza et al., 2013). Ventura et al. (2012) reported that different ionic liquids and their concentration could increase or decrease the activity of the lipase B from *C. antarctica*. Thus, in order to avoid the influence of PILs on the enzymatic activity, modified silicas with PILs were treated by a new method using soxhlet extraction, consisting of washes with hexane to remove the ionic liquid excess present in the modified silicas. In adition, the removal of PIL can allowy the recycling these chemical compounds, making this important step in the in the support preparation process.

The FTIR spectra of all silica samples (pure - PS and modified – 2HEAP-S, m-2HEAP-S, BHEAP-S) show a broad band from 3100 to 3600 cm^{-1} attributed to O–H groups that indicates the presence of water incorporated in the silica (Figure S4.4). Characteristic bands at around 550, 800 and 1000-1250 cm^{-1} can be assigned to Si-O-Si vibration, confirming that a condensed SiOx network has formed. The band at 960 cm^{-1} is due to a stretching of the Si-OH vibration (Gao et al., 2010; Drozdz et al., 2013; Matteis et al., 2013).

FTIR spectra of hexane used in the soxhlet method (Figure S4.5) show characteristic bands of pure hexane and of the PILs: at 1380 cm^{-1} (CH_3), 1460 cm^{-1} (CH_2) and 2900 cm^{-1} (CH), which are common to the pure hexane; and bands between 3500-3300 cm^{-1} (OH and amine) and 1100 cm^{-1} (which can be combined CO and NH), common to the PILs, confirming that washing with hexane by soxhlet was capable of removing the excess of PILs of the modified silicas.

3.2. Thermal analysis – TG/DTA

The total mass loss observed in the TG plots (Figure S4.6) for pure silica (PS) was 25 % and for the modified silicas (2HEAP-S, m-2HEAP-S, and BHEAP-S) it shows a smaller value (20 %). Two obvious stages were observed in the DTA/TG curves. In the first stage, from room temperature up to about 200°C, a strong mass loss was observed, due to the evaporation of solvent and water molecules trapped in the materials. This is supported by the presence of an endothermic peak in the DTA curve around 100°C. Silica obtained by sol-gel technique contains many Si–OH groups and could easily absorb water (Yu et al., 2004; Wu et al., 2012).

The mass loss corresponding to the first stage was lower for modified silicas (12.5 %) in comparison with the PS (20 %), indicating less residual water and lower ethanol adsorption in the formation. This environment, of the modified silicas, can minimize the inactivation of lipase, once the lipase catalysis is characterized by interfacial activation and when lipases are in contact with an interface between water and an apolar phase, the lid opens allowing access to the active site. Thus, the increased hydrophobicity near the active site in the open conformation is the basis of preferential adsorption and

interfacial activation of lipases during adsorption on hydrophobic surfaces (Abahazi et al. 2014).

The second stage starts at about 250°C for pure silica and around 350°C for the modified silicas. This indicates that the modified silicas have higher thermal stability compared with the pure silica. The mass loss in this stage is due to the decomposition of the hybrid network and condensation of silanols (Velikova et al., 2013). There is a corresponding exothermic peak at about 350 °C much more pronounced for the modified silicas. Above 750 °C no further mass loss was noticed suggesting that at this temperature samples are already fully decomposed and thus the silica is free of any organic material (Tsiourvas et al., 2013; Souza et al., 2014).

3.3. XRD - Powder X-ray diffraction

Powder X-ray diffraction (XRD) pattern of the silica obtained by sol–gel technique (Figure S4.7) showed a broad peak at 22° (2θ) for all samples (pure silica – PS and modified silicas – 2HEAP-S, m-2HEAP-S, BHEAP-S) which is typical for the amorphous nature of silica and confirms the absence of an ordered crystalline structure (Tsiouvas et al., 2013; Zulkifli et al., 2013; Kiasat et al., 2014).

3.4. Specific surface area and porosity properties

The pore size of the silica and the structure of the ordered mesoporous silicas play an important role with respect to the activity of the immobilized enzymes (Zhou and Hartmann, 2013). All the samples presented relatively large surface area (Table 4.4), and all the pores in the pure and modified silicas are in the size range of mesopore. According to IUPAC classification, the pores with a diameter less than 2 nm are known as micropores, those having between 2 and 50 nm are known as mesopores, and those with greater than 50 nm are known as macropores (Gupta et al., 2013).

Table 4.4: Surface area and porous properties of the pure and modified silicas (2HEAP-S, m-2HEAP-S, BHEAP-S) obtained by sol-gel technique.

	PS	2HEAP-S	m-2HEAP-S	BHEAP-S
Surface area ($m^2.g^{-1}$)	917.15 (±43.13)	735.24 (±21.37)	780.58 (±28.36)	765.25 (±25.94)
Pore volume ($cm^3.g^{-1}$)	0.61 (±0.017)	0.65 (±0.007)	0.81 (±0.004)	0.59 (±0.010)
Pore size (nm)	3.37 (±0.170)	3.50 (±0.173)	3.90 (±0.205)	3.63 (±0.177)

Standard Deviation - SD

The use of PILs as modifier agents changed the morphological structure of the silica obtained by sol-gel technique, by decreasing the surface area and increasing the pore size relatively to the pure silica. In the literature, it is possible to observe a similar tendency of the surface area to decrease with the ionic liquid funcionalized mesoporous silica SBA-15 material (Zou et al., 2010; Hu et al., 2012). According to Souza et al. (2013) nitrogen adsorption‑desorption measurements clearly show that protic ionic liquids acted as agents of pore formation.

Carvalho et al (2015) reported that modifying agents can be used for porosity control and to adjust the hydrophilic/hydrophobic balance. The modified silica with the more hydrophobic PIL, the m-2HEAP (methyl group and cation with higher log P), displayed higher average pore size, pore volume and surface area, in comparison with the other modified silicas (Table 4.4). This suggests that the hydrophobicity of the PIL had a significant effect on the pore structure of the silica. Sievers et al. (2006) reported the influence of the nitrogen bound methyl group in the cation mobility. In this study, the cation mobility of the PIL with methyl group possibly favored the best porosity adjustment. Hu et al. (2012) also found that methyl-functionalized IL is a better modifier agent than other ILs used for SBA-15 silica, indicating that the decrease of textural parameters was connected with the kind of functional group of ILs.

Gupta et al. (2012) observed that, apart from pores accessible to BET analysis, there are also blind pores (or voids) filled with air or reactant vapor which are ‘inaccessible’ to BET. Because of the fast gelification, air or vaporizable solvent/reactants can remain

entrapped in the matrix. Such air or vapor filled regions deep inside the matrix give rise to such blind pores. Further, for long time storage, the entrapped vaporizable reactants will have a tendency to escape out of the voids creating cracks or a flaky structure matrix.

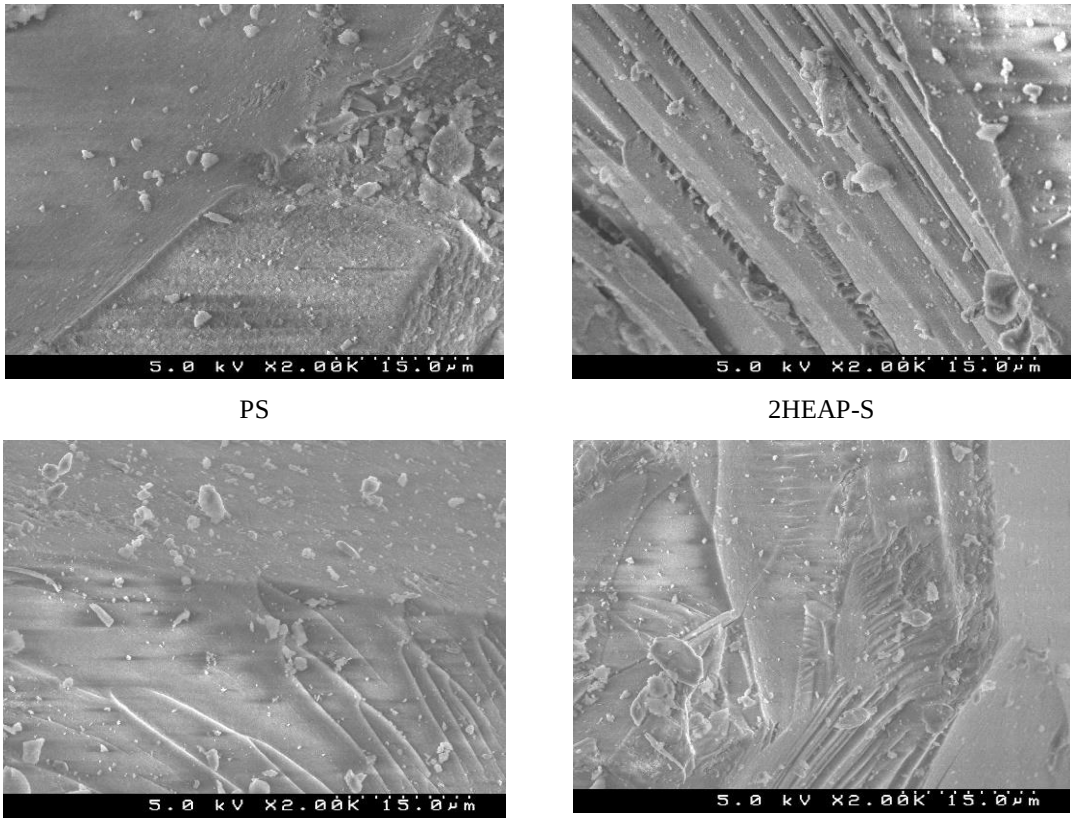
The pure and modified silicas show similar adsorption patterns (Figure S4.8). All samples display a isotherm type IV with a hysteresis loop, which is usually exhibited by mesoporous solids (Tajer-Kajinebaf et al., 2014; Nuntang et al., 2014). The results observed in the N_2 adsorption–desorption tests agree with those of the isotherms with H_2 hysteresis loops, which is a characteristic of mesoporous materials and generally associated with pores with narrow necks and wide bodies (Souza et al., 2013).

The hysteresis loops area of the samples and information of the table indicated that ionic liquid modification changed the surface pore of silicas (Zou et al., 2014). It is well known that the behavior of the sorption isotherms of porous materials is dependent on not only the pore size but also pore morphology (Li et al., 2008).

3.5. SEM - Scanning electron microscopy

SEM micrographs in Figure 4.9 show the surface appearance of irregularly shaped blocks of pure (PS) and modified silicas (2HEAP-S, m-2HEAP-S and BHEAP-S). Cleavage steps typical of the brittle fracture of ceramics and glasses are seen. This fracture pattern is consistent with the existence of small, strongly bound particles forming the agglomerates (Aramendía et al., 2001).

SEM analysis did not allow an obvious identification of the pore size and its distribution. These results are in agreement with those obtained by Souza et al. (2012) and Ursoiu et al. (2012). SEM studies only deliver information regarding the general morphology of the particles, and not on the actual conformation of the internal porous structure, but that was confirmed by analysis of nitrogen adsorption (Souza et al., 2013).



PS 2HEAP-S

m-2HEAP-S BHEAP-S

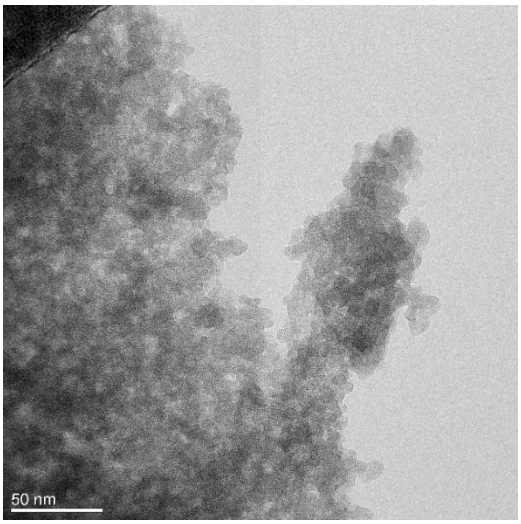
Figure 4.9: Scanning electron micrographs of pure (PS) and modified silicas (2HEAP-S, m-2HEAP-S, and BHEAP-S) obtained by sol-gel technique.

3.6. TEM – Transmission electron microscopy

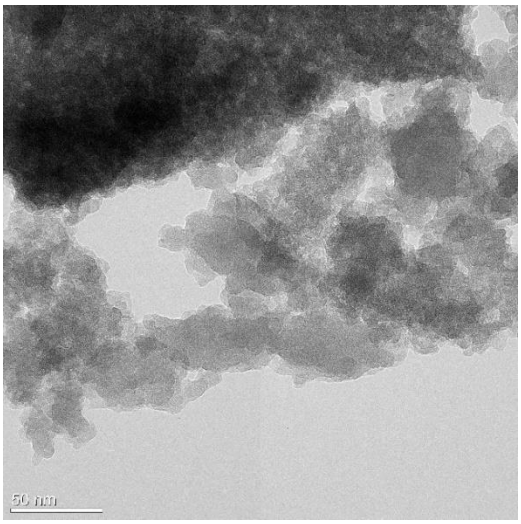
TEM was used to probe the real structure of the prepared silicas, namely the formed sol–gel network structure. The images obtained by TEM (Figure 4.10) for pure (PS) and modified silicas (2HEAP-S, m-2HEAP-S, and BHEAP-S) show dark regions corresponding to the silica while the brighter ones are pores.

The TEM images suggested that silica framework has a branched and uniform appearance being formed by agglomerates of the aggregative clusters, which were constituted by individual silica particles. That is, such primary particles were firstly aggregated into clusters, and these further agglomerated to yield the whole silica framework (Wu et al., 2012). In this work, it was observed that modified silicas (2HEAP-S, m-2HEAP-S, and BHEAP-S) had larger primary particles in comparison with pure

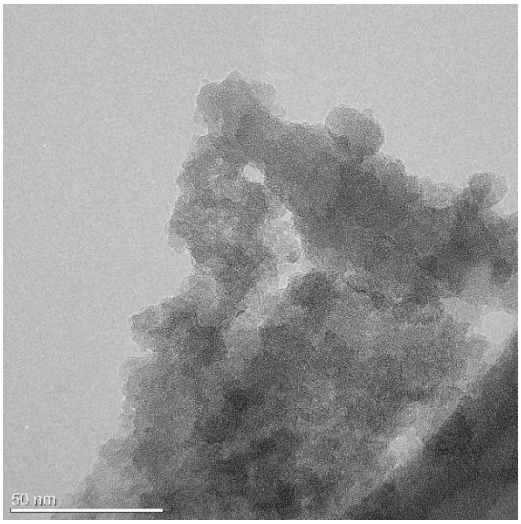
silica, which probably reduced the surface area of the modified silicas. This result is in agreement with the data obtained by BET analysis (section 3.4).



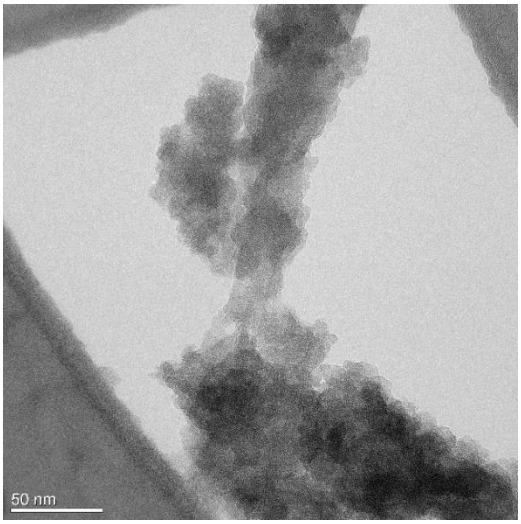
PS



2HEAP-S



m-2HEAP-S



BHEAP-S

Figure 4.10: TEM micrograph of pure (PS) and modified silicas (2HEAP-S, m-2HEAP-S, BHEAP-S) obtained by sol-gel technique.

3.7. Enzymatic activity

The synthesized silicas were used as support to immobilize lipase from *Burkholderia cepacia* by physical adsorption (ADS) and covalent binding using

glutaraldehyde (CBG) or epichlorohydrin (CBE) as activating agents. The use of PILs as modifier agent of the silica was founded to have different effects in the total activity recovery yield depending on the immobilization process, confirming that different immobilization protocols produced biocatalysts with different features.

Lipase from *Burkholderia cepacia* immobilized by physical adsorption on silica modified with more hydrophobic PIL (surface area, pore volume and pore size equal to 780.58 $m^2.g^{-1}$, 0.81 $cm^3.g^{-1}$ and 3.90 nm) presented the highest total activity recovery yield (64.78 %) in comparison with immobilized systems with other modified silicas with 2HEAP and BHEAP and pure silica, as showed in Figure 4.11. However, despite the pure silica has a higher surface area, the literature reports that enzyme activity can not be directly related to this property (Chen and Lin, 2003). The improvements of the total activity recovery yield may be related to the pore volume and size of the support, once that the modified silica with more hydrophobic LIP (m-2HEAP) shows greatest pore volume and pore size (Table 4.4). According to Jaladi et al. (2009) large pores facilitate substrate access to immobilized system and thus result in increased enzyme activity.

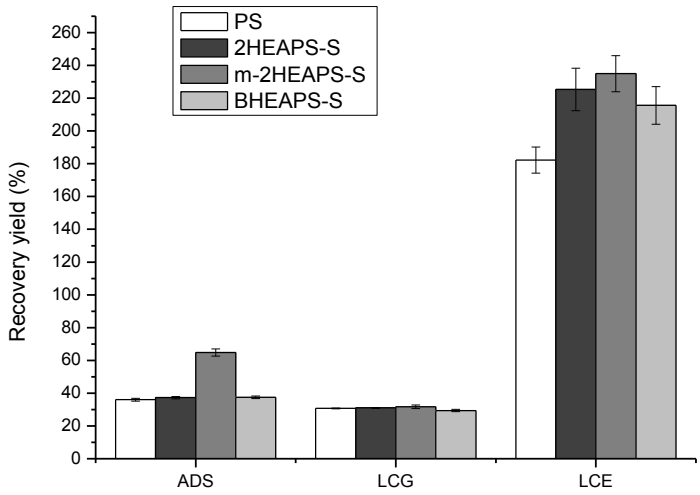


Figure 4.11: Total activity recovery yield for lipase from *Burkholderia cepacia* immobilized on pure (PS) and modified silicas (2HEAP-S, m-2HEAP-S, BHEAP-S) by ADS, CBG e CBE.

The total activity recovery yield obtained for immobilized systems by covalent binding using glutaraldehyde varied very little between the biocatalysts (considering pure - PS and modified silicas - 2HEAP-S, m-2HEAP-S, and BHEAP-S) which suggests that treatment with glutaraldehyde did not produce significant improvements in the enzymatic activity. Barbosa et al. (2012) reported a similar effect when employed glutaraldehyde as activating agent, without significant improvement on the activity of the enzyme, concluding that the immobilization protocol can affect the final effect of chemical modification on the properties of the enzyme. According to Mendes et al. (2011a) the high glutaraldehyde reactivity cause distortion in the enzyme three dimensional structure, leading to an inactive conformation of many enzyme molecules, or to the poor orientation of the immobilized protein, preventing substrate access to the catalytic site.

On the other hand, the values of total activity recovery yield obtained for immobilized systems by covalent binding using epichlorohydrin (CBE) were similar for the immobilized biocatalysts with modified silicas (2HEAP-S, m-2HEAP-S and BHEAP-S) in comparison with pure silica (PS), ranging 182.2 % to 235.61 % (Figure 4.11). The total activity recovery yield of immobilized systems using epichlorohydrin was markedly superior to that of all others and 7-folds as much as that of immobilized systems using glutaraldehyde. Among activating agents tested the epichlorohydrin had the best performance, according Santos *et al*. (2008) this agent require only one hydroxyl group to the reaction, compared to the two groups required by glutaraldehyde and, as consequence, it is possible that a higher number of active sites for enzyme binding are available in supports activated with epichlorohydrin.

The immobilized systems were incubated with 0.5% Triton X-100. The Triton X-100 acts as the opposite, improving desorption because of the competition with lipases for hydrophobic regions of silica gel (SUGAHARA and VARÉA, 2014). According to Palomo et al (2005) only those lipases not covalently immobilized on supports via lipase–lipase interactions could be desorbed by this very mild treatment.

Although the covalente binding is a method reported for promove a strong linkage and physical adsorption for promove a weak linkage, the values of desorption of lipase were high for immobilized biocatalysts by covalente binding than for immobilized biocatalysts for physical adsorption. The presence of 0.5 % Triton X-100 facilitated the desorption of lipase of 90 % for immobilized biocatalyst by covalent binding using

epichlorohydrin (Table S4.5), 50 % for immobilized biocatalyst by covalent binding using glutaraldehyde. These results suggest that the activating agent was founded to have an influence on immobilized lipase. Mendes et al. (2011b) reported that when a short spacer arm activating agent (as epichlorohydrin) is used, the lipase molecules are very close to the support surface, and, consequently, a steric hindrance on lipase molecules may occur. Thus, immobilized systems with glutaraldehyde (activating agent with spacer arm) allowed obtaining derivatives with higher immobilized protein amount in comparison with immobilized systems with epichlorohydrin.

Immobilized biocatalyst by physical adsorption presented desorption of 31 % and 42 % (Table S4.5) for immobilized preparations on modified silicas and pure silica, respectively, indicating that the physical adsorption method fixed the lipase on the silica supports strongly in comparison to other immobilization protocols (CBE and CBG). This context suggests that the nature of adsorption of the *Burkholderia cepacia* lipase on silicas support was hydrophobic probably by interfacial activation. According to Fernandes-Lorente et al. (2008) this protocol fixes the open form of lipases via interactions between the hydrophobic surroundings of their active centre – the internal face of the lid and the area of the lipase around the active center that interacts with it – and the hydrophobic surface of the support becoming these immobilized enzyme preparations very stable under different experimental conditions.

3.8. Optimal pH and Temperature on activity

Figure 4.12 showed the effect of pH on the activity of the biocatalysts immobilized by physical adsorption and covalent binding using glutaraldehyde and epichlorohydrin. The pH is one of the most influential parameters for enzymatic activity in an aqueous medium and pH shift depends mainly on the method of immobilization and the interaction between the enzyme and support (Cabrera-Padilla et al., 2012).

For immobilized systems using the pure (PS) and modified silicas (2HEAP-S, m-2HEAP-S and BHEAP-S) by physical adsorption and covalent binding with glutaraldehyde the maximum relative activity occurred at pH more acidic, equal to 3,

while free lipase from *Burkholderia cepacia* the optimum pH of 7 (Figure 4.12 (a) and (b)).

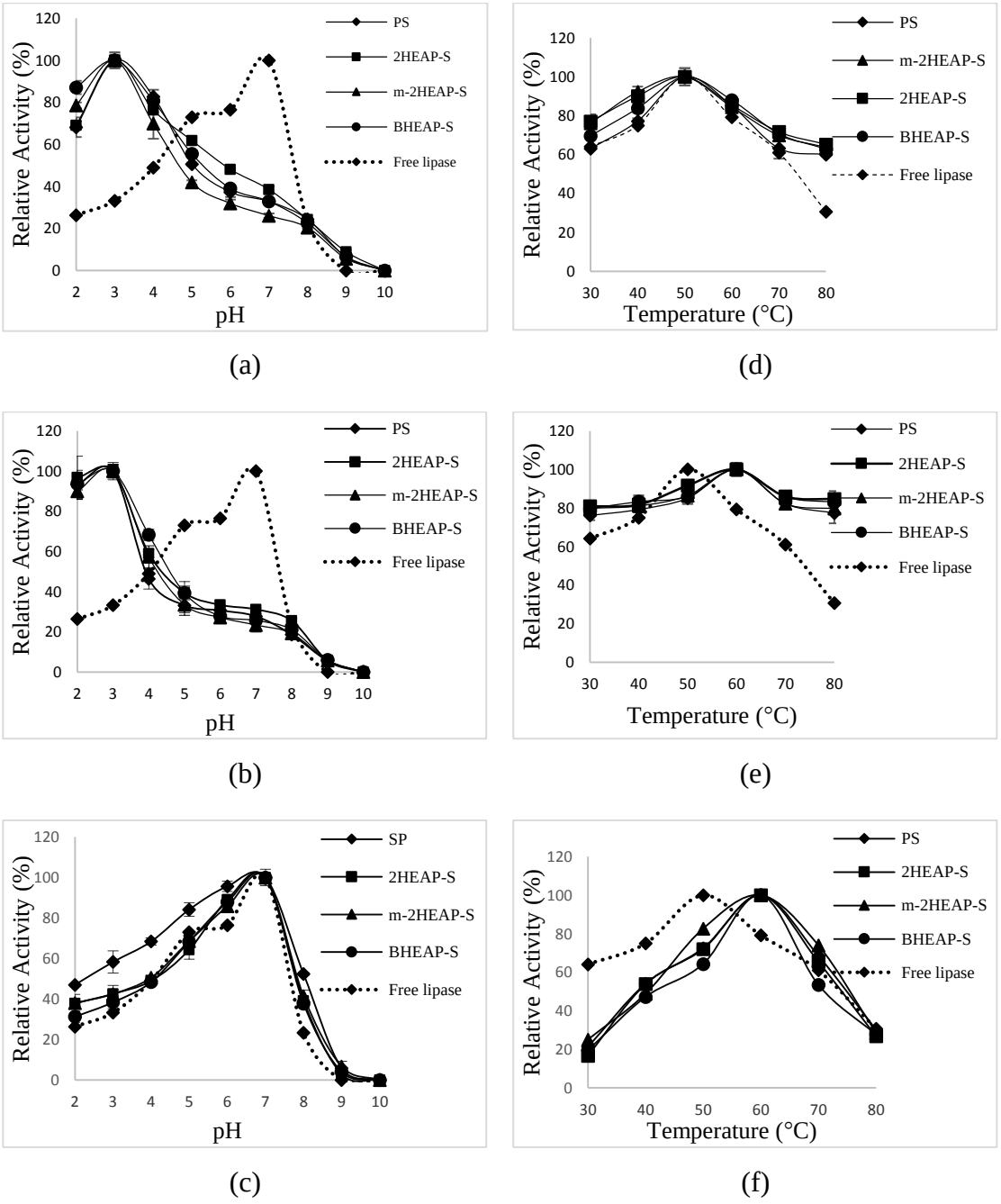


Figure 4.12: Effect of pH and temperature on the activity of the free lipase and immobilized biocatalyst by physical adsorption (a) (d), covalent binding with glutaraldehyde (b) (e) and covalent binding with epichlorohydrin (c) (f).

The shift of optimal pH of the immobilized lipase has been reported for different lipase sources and support types (Yi et al., 2009, Da Rós et al., 2010, Cabrera-Padilla et

al., 2013). Carvalho et al. (2013) have suggested that the shift of the optimum pH from neutral to more acid, comparing free versus immobilized enzyme, is presumably due to variations in the ionization state of the system's components as the pH changes.

On the other hand, the immobilized silica by covalent binding with epichlorohydrin, Figure 4.12 (c), showed maximum relative activity in the optimum pH of the free lipase (pH 7), indicating, in this case, that the immobilization protocol employed in the preparation of biocatalysts did not affect the enzyme charge. Similar behavior was also observed in the literature (Jegannathan et al., 2009; Christopher et al., 2012; Abdulla and Ravindra 2013).

Reaction temperature has an important influence on the activity, stability of a biocatalyst, and the thermodynamic equilibrium of a reaction (Chen et al., 2013). The influence of temperature on the relative activity of free and immobilized lipase on silica was conducted in the temperature range between 30 and 80 °C and the maximum activities of the immobilized biocatalysts were defined as 100 % relative activity.

The Figure 4.12 showed that the maximum relative activity occurred at 50 °C for immobilized biocatalysts by physical adsorption and free lipase and 60 °C for immobilized biocatalysts by covalent binding using glutaraldehyde and epichlorohydrin. The optimal temperature of the immobilized biocatalysts by covalent binding showed greater tolerance to heat compared with free lipase and immobilized biocatalysts by physical adsorption. These results could be attributed to an improved physical and mechanical stability of the lipase arising from the covalent binding of lipase on the supports (Liu et al., 2009). Covalent binding between lipase and supports may increase the rigidity of three-dimensional structure, which protects the enzyme from inappropriate changes at high temperatures (Ranjbakhsh et al., 2012).

3.9. Thermal and Operational stability

Thermal stability indicates that the immobilization process tends to stabilize the enzyme. Thus, the half-life time ($t_{1/2}$) of the enzyme, that is the time which takes for the activity to reduce to a half of the original activity, is shown to be inversely proportional to the rate of denaturation (Da Rós et al., 2010). Immobilized biocatalysts revealed higher

half-life time for the lipase immobilized by physical adsorption (from 231.1 to 309 h) while the half-life ($t_{1/2}$) of the free lipase was 4.66 h (Table 4.6).

The half-life for biocatalysts immobilized by physical adsorption was higher than the values obtained by Hu et al. (2012) and Cabrera-Padilha et al. (2012). The first authors founded more than 70% of the initial activity after being incubated for 6 h at 70 °C to the *Burkholderia cepacia* lipase immobilized on modified mesoporous material SBA-15 by physical adsorption and the second study found 77 h to *Candida rugosa* lipase immobilized by physical adsorption on a natural biopolymer poly(3-hydroxybutyrate-co-hydroxyvalerate) - PHBV.

Table 4.6: Half-life time ($t_{1/2}$) and operational stability of immobilized biocatalysts on pure (PS) and modified silicas (2HEAP-S, m-2HEAP-S, and BHEAP-S) by ADS, CBG e CBE.

Lipase form	Half-life time ($t_{1/2}$, h)	Recycle	Relative activity (%)
Free lipase	4.6	-	-
Immobilized on PS - ADS	231.1	40	73.85
Immobilized on 2HEAP-S - ADS	301.5	40	74.09
Immobilized on m-2HEAP-S - ADS	309.0	40	74.89
Immobilized on BHEAP-S - ADS	303.7	40	71.20
Immobilized on PS – CBG	5.7	1	9.27
Immobilized on 2HEAP-S – CBG	6.8	1	18.58
Immobilized on m-2HEAP-S – CBG	6.8	1	20.47
Immobilized on BHEAP-S - CBG	6.2	1	16.28
Immobilized on PS – CBE	4.8	1	55.73
Immobilized on 2HEAP-S – CBE	6.8	1	52.38
Immobilized on m-2HEAP-S – CBE	6.3	1	55.62
Immobilized on BHEAP-S - CBE	6.2	1	52.43

ADS - Physical adsorption,
CBG - covalent binding with glutaraldehyde,
CBE - covalent binding with epichlorohydrin.

The immobilized biocatalysts by covalent binding presented half-life time in the range of 6.2 h. Similar value half-life time (6.2 h) was found by Da Rós et al. (2010) for

Burkholderia cepacia lipase immobilized on SiO_2–PVA by the same immobilization method (covalent binding). However, the half-life time for the biocatalyst immobilized on pure silica (PS) was less (5.7 h for biocatalyst immobilized on pure silica by CBG and 4.8 h for biocatalyst immobilized on pure silica by CBE). Thus, was found that the modified silicas with PILs (2HEAP-S, m-2HEAP-S, and BHEAP-S) showed improvement of thermal stability provide better microenvironment to lipase immobilization.

It is noteworthy that all the immobilized lipases showed better thermal stability in comparison with free lipase and the immobilization on modified silicas by physical adsorption increased significantly the thermostability of the lipase. According to Zivkovic et al. (2015) localized electrostatic attractions together with hydrophobic interactions, which govern the adsorption of the lipase to supports, led to restrict movement of lipase and, therefore, an increase in thermal stability.

Higher relative activity was observed after 40 recycles for biocatalysts immobilized by physical adsorption (Table 4.6), despite this being considered a method with a poor reuse potential. Souza et al. (2012) reported reuse up to three times keeping 50 % of its initial activity for lipase from *Bacillus* sp. ITP-001 immobilized by physical adsorption on sol–gel silica. Barbosa et al. (2014) reported that the BC lipase immobilized on airgel matrix in the presence of additive (PIL) presented reuse of 23 cycles. Based on the results obtained in this study, the biocatalysts immobilized by physical adsorption is very suitable for commercial applications because of its easy total activity recovery from the reaction system and efficient reuse.

On the other hand, the activity decreased after first reuse for biocatalysts immobilized by CBE and no reuse was identified for biocatalysts immobilized by CBG, because the relative activity fell more than 50%. These results were in accordance with the Triton test, which demonstrated ease of desorption to immobilized biocatalyst by covalent binding. Probably the immobilized biocatalysts by ADS increased the enzyme rigidity, improving its stability.

The enzyme leakage, rigidity of the immobilized enzyme structure, size of the spacer arm of the support, degree of the support activation and chemical modification of immobilized may affect the properties of the enzyme, altering the enzyme selectivity and stability (Abdulla and Ravindra, 2013; Rios et al., 2016). One of the most important

advantages of the immobilized enzyme is its reusability because can effectively reduce the cost in industrial applications (Zivkovic et al., 2015; Shahrestani et al., 2016). Thus, the immobilized systems by ADS deserve to be highlighted because has high half-life and reuse values.

3.12. Kinetic parameters

To better illustrate the differences in the catalytic properties observed between immobilized biocatalysts, they were used in hydrolysis reactions and kinetic properties of free and immobilized lipases on silica are summarized in Table 4.7. The kinetic parameter V_{max} defines the highest specific activity when the enzyme is saturated with substrate and K_m corresponds to the substrate concentration that is sufficient to reach a reaction velocity of ½ V_{max}. Large K_m indicates that substrate and enzyme do not prefer to be close for a long time. In general, the K_m of an enzyme in the immobilized state is not same as that of the free enzyme because of the diffusion limitations (Yang et al., 2013; Singh and Ahmad 2014).

In this study, the biocatalysts immobilized by covalent binding using glutaraldehyde (CBG) presented higher K_m values in comparison the free lipase, indicating that the affinity towards the substrate was reduced. The increased K_m values may be attributed to either structural changes in the enzyme upon immobilization or changes in the accessibility of the active site to the substrate. In addition, the high reactivity of the activating reactant, like glutaraldehyde, might have led to excessive cross-linking in the support and, as a consequence, an increase in the K_m value for the immobilized systems occurred (Da Rós et al. 2010).

However, the K_m value of the lipase decreased upon immobilization by physical adsorption (ADS) and covalent binding using epichlorohydrin (CBE) allowed increased substrate access in the support. From all immobilized systems, the biocatalysts immobilized by physical adsorption were markedly inferior to that all others. These data suggest that the immobilized biocatalysts by ADS allowed a more compatible environment for lipase, resulting in increased affinity for the substrate and better accessibility to the active site.

Table 4.7: Kinetic parameter for free lipase and immobilized biocatalysts on pure (PS) and modified silicas (2HEAP-S, m-2HEAP-S and BHEAP-S) by ADS, CBG e CBE.

	V_{max} (U.g^{-1})	K_m (mM)
Free Lipase	2509.67	543.68
Immobilized on PS - ADS	1308.53	15.12
Immobilized on 2HEAP-S - ADS	1415.66	10.23
Immobilized on m-2HEAP-S - ADS	1602.29	9.38
Immobilized on BHEAP-S - ADS	1476.52	9.71
Immobilized on PS – CBG	1429,22	684.71
Immobilized on 2HEAP-S – CBG	1418.45	683.80
Immobilized on m-2HEAP-S – CBG	1433.76	683.45
Immobilized on BHEAP-S - CBG	1407.98	684.01
Immobilized on PS – CBE	4593.12	437.69
Immobilized on 2HEAP-S – CBE	4780.38	372.16
Immobilized on m-2HEAP-S – CBE	4874.17	363.34
Immobilized on BHEAP-S - CBE	4710.43	379.66

ADS - Physical adsorption,
CBG - covalent binding with glutaraldehyde,
CBE - covalent binding with epichlorohydrin.

3.13. Chemical characterisation of the biocatalysts - FTIR analysis

All immobilized biocatalysts and free *Burkholderia cepacia* lipase were characterized by FTIR (Figure 4.13). The FTIR spectrum of free lipase shows the characteristic band at 3400 cm^{-1} (-NH_2), 3000 (C-H) cm^{-1}, 1650 cm^{-1} (C=O), 1500-1400 cm^{-1} (-NH). According to Andrade et al. (2010), the amide I band is observed at 1650 cm^{-1}, the amide II band around 1500 cm^{-1}, and the amide III and IV bands at 1285 and 726 cm^{-1}. The vibrational bands around 1100–1000 cm^{-1} are associated with the C–C and C–N composite vibrations of the protein chain.

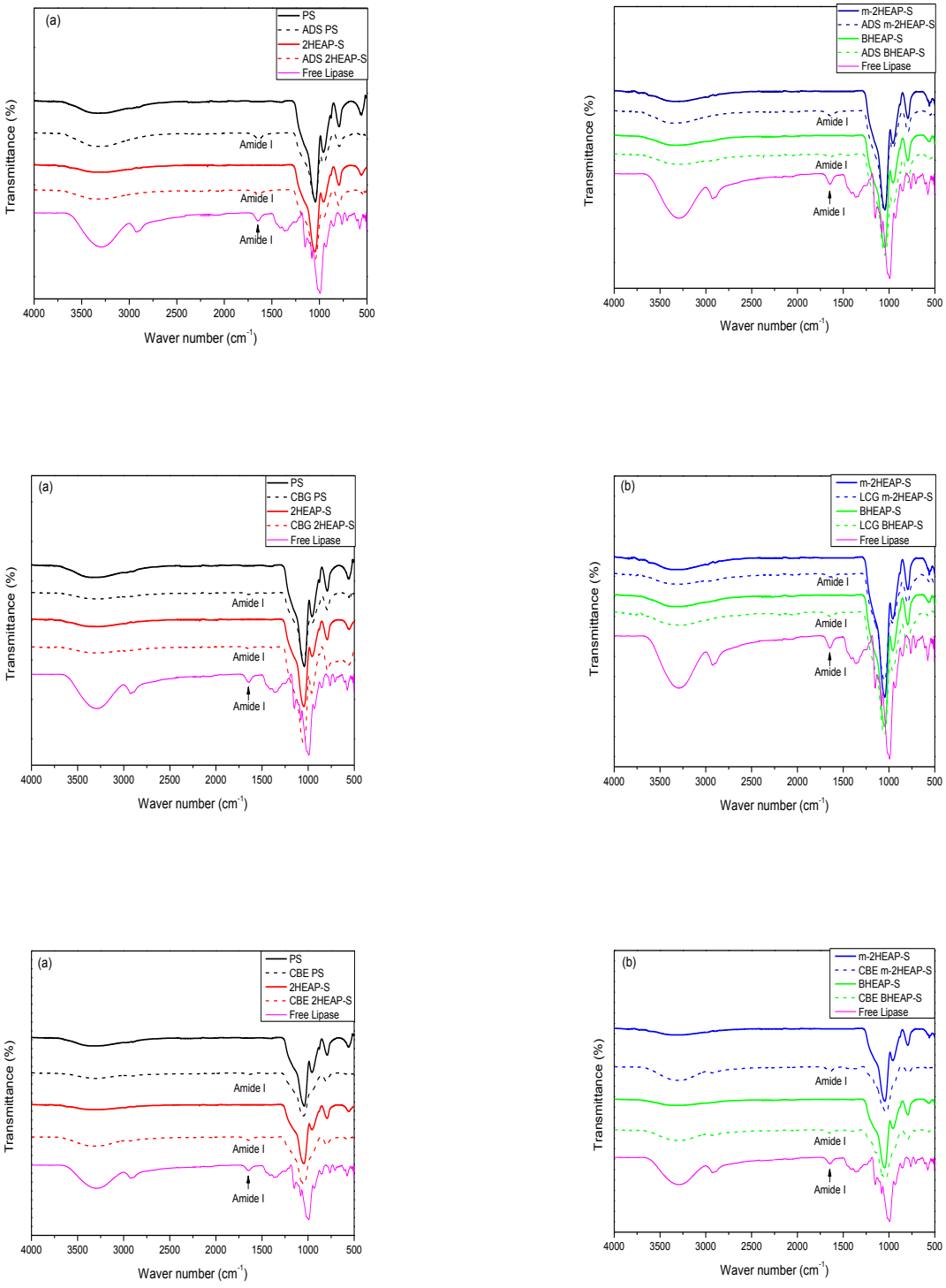


Figure 4.13: FTIR of free lipase and immobilized biocatalysts on pure (PS) and modified silica by ADS (a)(b), by CGB (c)(d) and by CBE (e)(f).

The band corresponding to amide I originates from the C=O stretching vibration of the peptide group, whose frequency depends on the hydrogen binding and coupling along the protein chain and is therefore sensitive to the protein conformation (Natalello et al., 2005). In all spectra, this band was visible, confirming the presence of the enzyme in the immobilized systems. Compared with other immobilized systems, the spectrum of the immobilized silica by covalent binding, in which epichlorohydrin was used as activating agent, exhibits a greater similarity with the spectrum of free lipase.

The amide II position is sensitive to the environment around the protein. Its shift seems to reflect the immobilization and possible structural changes, however a detailed assignment is not possible at the present time because of the complexity involved (Andrade et al., 2010).

3.14. Ethyl Esters Synthesis

The biocatalysts immobilized on PS – ADS, m-2HEAP-S – ADS, PS – CBE and m-2HEAP-S – CBE were applied in ethyl esters synthesis using waste coconut oil and ethanol as sources of raw material, molar ratio of 1:12 and 40°C of temperature. In order to compare the role and effect of heating form in enzyme-catalysed ethyl esters synthesis, transesterification reactions were carried out in Dubnoff Shaking Baths (conventional heating), microwave and ultrasound (non-conventional heating).

The Table 4.8 presents the values of the ethyl esters yields obtained from ethyl esters synthesis. The waste coconut oil ethyl ester yield ranged from 6 % to 92 %. Among the biocatalysts tested, immobilized lipase on m-2HEAP-S – CBE exhibited the biggest conversion efficiency showed the maximum yield (65 %, 36 % and 92 %) for all heating form. Ethyl esters yield of 92 % is favorable data considering that coconut oil is residual and has not undergone refining or degumming process. In addition, these results confirm that the interfacial activation of the immobilized biocatalyst by CBE was strongly favored by the characteristics of immobilization and experimental conditions, highlighting the biocatalyst (m-2HEAP-S-CBE) as the most active and the most promising in synthesis of ethyl esters from waste coconut oil and ethanol.

Table 4.8: Maximum ethyl esters yields obtained from transesterification reactions with waste coconut oil and ethanol (Molar Ratio: 1:12 and Temperature: 40°C).

Immobilized Biocatalysts	Ethyl esters yields (%)			
	Dubnoff Shaking Baths		Microwave	Ultrasound
	24h	96 h	8 h	8 h
PS – ADS	23	36	10	6
m-2HEAP-S – ADS	28	37	14	7
PS – CBE	40	60	25	71
m-2HEAP-S – CBE	42	65	36	92

Although great characteristics under aquoso conditions, immobilized biocatalysts by ADS presented low values of ethyl ester yield with all heating form. However, some researchers have suggested that the conformations of the enzymes are dependent on the solution conditions, in other words, some lipases show open conformation under aqueous conditions and closed conformation under organic conditions (SCHRAG et al., 1997; BON et al., 2008).

Reactions with ultrasound presented higher yields in less time with biocatalysts immobilized by CBE. The reaction time decreased from 96 to 8 h and the yield increased from 65 to 92 % using m-2HEAP-S – CBE in comparison with conventional heating, translating a 17-fold conversion rate enhancement. Other authors (Ribeiro et al. 2012) obtained 80.5% conversion of waste coconut oil in ethyl esters in 24 h of reaction with Novozym 435 at 60 °C and molar ratio of 1:10, using conventional heating. Thus, heating form was found to be determinant for the ethyl esters yield.

Furthermore, the cavitation collapse from the ultrasound produces extreme conditions locally such as high temperature, high pressure, turbulence, and high shear forces that can help to generate fine emulsions between immiscible fluids to enhance mass transfer and essentially increase the efficiency of the catalyst for achieving optimal yield (Gole and Gogate, 2013; Bhangu et al., 2016).

The high ethyl esters yield values attained in the transesterification of waste coconut oil made possible to obtain samples having viscosity values of 3.4 $mm^2.s^{-1}$, which meet the standards of the National Agency for Petroleum, Natural Gas and Biofuels ANP

07/2008, American National Standard ASTM D6751 and European standard EN 14214. Other characteristics of coconut oil and ethyl esters sample are shown in Table S4.9.

3.15. Thermal stability on microwave and ultrasound (non-conventional heating)

The thermal stability of *Burkholderia cepacia* lipase immobilized on m-2HEAP-S – CBE was tested under microwave and ultrasound, whereas this was the biocatalyst most active and promising and that the heating form was determinant for the ethyl esters yield. This is an important step because all the processes thermally deactivate enzymes. In this context, the evaluation of thermal stability under non-conventional heating was carried out by incubating of the lipase (free and immobilized) at 60 °C, under microwave (about 100 W) and ultrasound (about 220 W) and the respective half-life times for the biocatalyst were calculated.

Table 4.10 depicts values of half-life times according to time of exposure to microwave and ultrasound. The half-life times for lipase immobilized on m-2HEAP-S by CBE were higher when compared with free lipase for both heating form, demonstrating that the immobilized system is thermally more stable than free lipase.

The half-life times for the free and immobilized lipase under microwave irradiation were from 6.2 and 8.2 h, respectively. Microwave irradiation leads to direct coupling of molecules by selective absorption of radiation by polar compounds. This way, microwave irradiation seems to improve thermal stability of the enzyme as a function of enzyme hydration which can be used efficiently to improve the process or modify the selectivity (Yadav and Pawar, 2012). However, under ultrasound the values of half-life times were higher (14.9 h for free lipase and 21.1 h for immobilized lipase). These results are in agreement with the literature, which indicates intensification in the enzymatic activity under effects of the ultrasonic cavitation (Yu et al. 2010; Veljkovic et al. 2012; Subhedar and Gogate, 2016).

Table 4.10: Half-life time ($t_{1/2}$) of free lipase and immobilized biocatalysts on modified silica (m-2HEAP-S) by CBE under microwave and ultrasound.

Heating form	Lipase form	$t_{1/2}$ (h)
Microwave	Free	6.2
	Immobilized	8.2
Ultrasound	Free	14.9
	Immobilized	21.1

4. CONCLUSIONS

Modified silicas by protic ionic liquids with differents cations were successfully prepared and used as support to immobilize *Burkholderia cepacia* lipase by physical adsorption and covalent binding. However, the biocatalysts exhibited different features, regarding substrate specificity (hydrolysis reactions and ethyl esters synthesis) and stability under different conditions. As a result, it was found that the PILs excess was efficiently removed of the modified silicas by soxhlet extraction. The characterization revealed positive changes in the structural properties and the modified silica with the more hydrophobic PIL (m-2HEAP) showed higher pore diameter, pore volume, surface area and total activity recovery yield (64.78 %) when immobilized by physical adsorption, making it an important modification agent of silica. The biocatalysts immobilized by covalent binding using epichlorohydrin (CBE) present greater total activity recovery yield. Of another hand, the biocatalysts immobilized by physical adsorption showed better stabilities including thermal stability ($t_{1/2}$ from 231 to 309 h) operational stability (higher relative activity after 40 recycles), smaller K_m values and smaller lipase desorption. The yield of ethyl esters reached 92% assisted by ultrasound, 40 °C and 1:12 of the molar ratio (waste coconut oil:ethanol) when catalyzed by biocatalysts immobilized on m-2HEAP-S by CBE and 21.1 h of half-life time ($t_{1/2}$) was foundes to it assisted by ultrasound, indicating it as a promising alternative. These results clearly indicated that modified silicas were ideal candidates for *Burkholderia cepacia* lipase immobilization.

ACKNOWLEDGEMENTS

This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, POCI-01-0145-FEDER-007679 (FCT Ref. UID /CTM /50011/2013), financed by national funds through the FCT/MEC and when appropriate co-financed by FEDER under the PT2020 Partnership Agreement, CNPq finantial support is also acknowledged (Grant 306560/2013-5).

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SUPPLEMENTARY MATERIAL

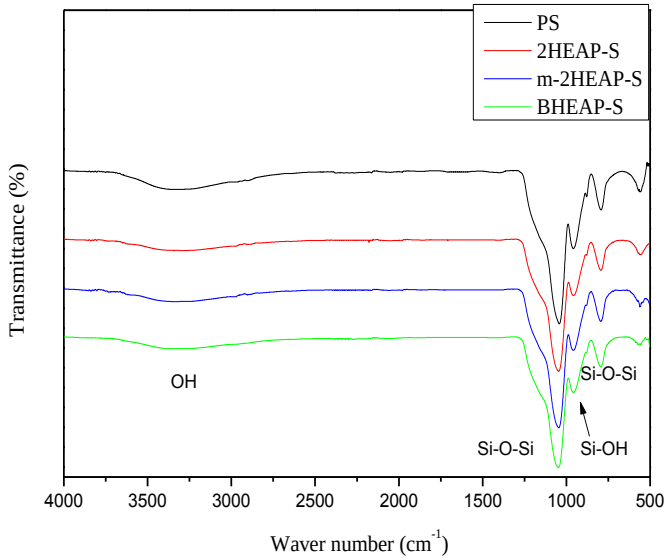

Figure S4.4: FTIR spectra for the silica (pure - PS and modified – 2HEAP-S, m-2HEAP-S, BHEAP-S).

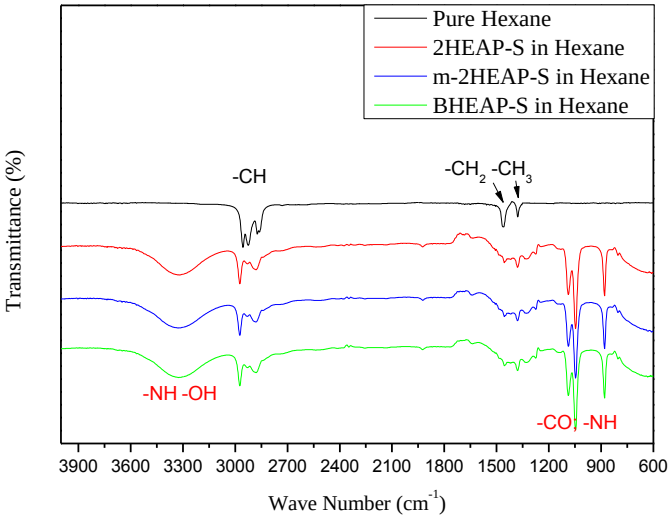

Figure S4.5: FTIR spectra for the hexane used in the soxhlet extraction (2HEAP-S, m-2HEAP-S, BHEAP-S in hexane) and pure hexane.

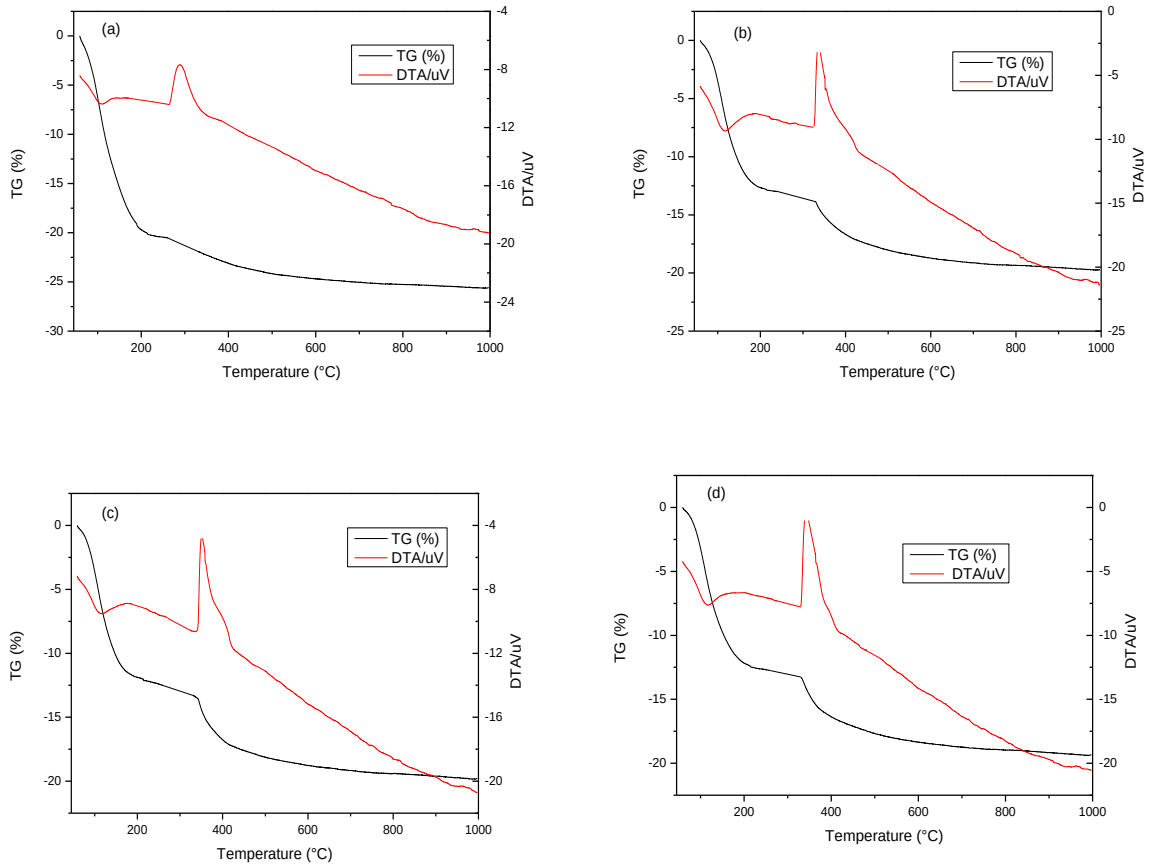

Figure S4.6: TG/DTA curves of pure silica PS (a) and modified silicas: 2HEAP-S (b), m- 2HEAP-S (c), BHEAP-S (d).

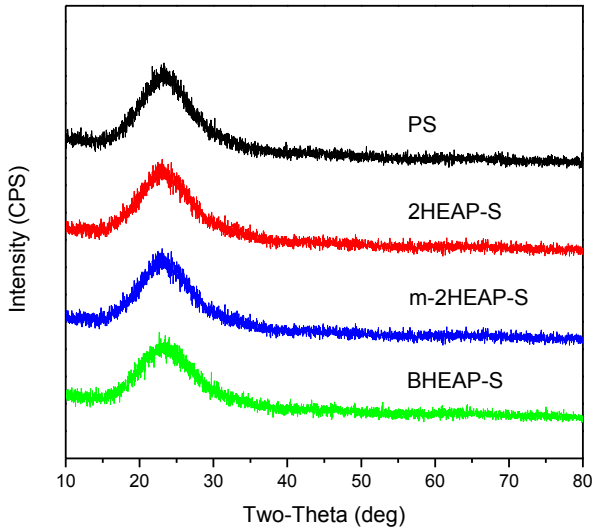

Figure S4.7: XRD pattern of the silica obtained by sol-gel technique (standard – PS and modified -2HEAP-s, m-2HEAP-S, BHEAP-S).

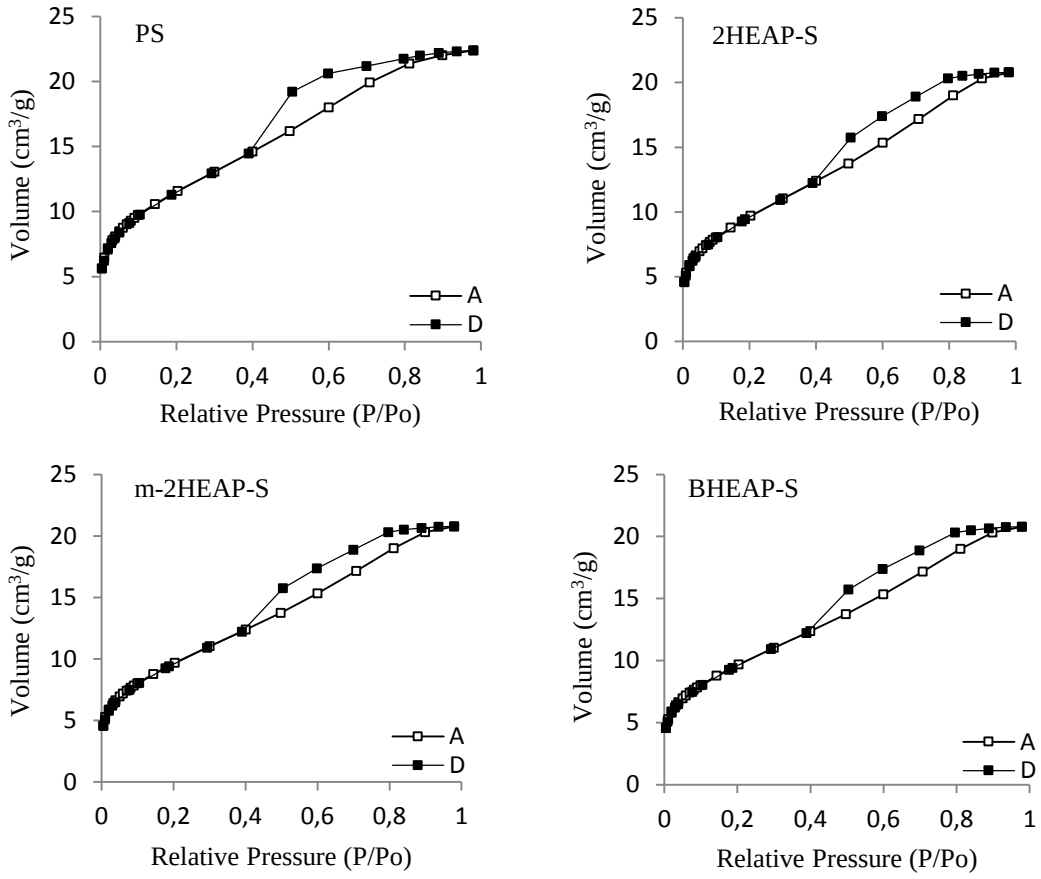

Figure S4.8: Nitrogen adsorption–desorption isotherms of the standard silica (PS) and modified silicas: 2HEAP-S, m-2HEAP-S, BHEAP-S.

Table S4.5: Effect of the environment on desorption of *Burkholderia cepacia* lipase immobilized on standard and modified silica.

Lipase form	Lipase desorption (%)
Immobilized on PS - ADS	42.60
Immobilized on 2HEAP-S - ADS	29.45
Immobilized on m-2HEAP-S - ADS	31.78
Immobilized on BHEAP-S - ADS	33.18
Immobilized on PS – CBG	50.84
Immobilized on 2HEAP-S – CBG	49.55
Immobilized on m-2HEAP-S – CBG	51.19
Immobilized on BHEAP-S - CBG	48.16
Immobilized on PS – CBE	89.14
Immobilized on 2HEAP-S – CBE	91.03
Immobilized on m-2HEAP-S – CBE	90.56
Immobilized on BHEAP-S - CBE	91.14

ADS - Physical adsorption,
CBG - covalent binding with glutaraldehyde,
CBE - covalent binding with epichlorohydrin.

Table S4.9: Characterization of coconut oil and ethyl esters sample (92%).

Properties	Unit	Coconut oil	Ethyl Esters	ANP 07/2008*	ASTMD6751**	EN 14214**
Density at 25°C	kg/m³	901	862	850-900	-	860–900
Kinematic viscosity at 40 °C	mm²/s	32	3.4	3.0-6.0	1.9-6.0	3.5-5.0
Saponification value	mg_{KOH}/g	274.44	163.35	-	-	-
Iodine value	$gI_2/100gsample$	19.97	19.65	-	≤120	≤120
Acid value	mg_{KOH}/g	2.64	8.72	0.5 max	0.5 max	0.5 max
Peroxide value	meq/Kg	1.25	0.45	-	-	-
Water	%	1.08	2.85	0,05	-	0,05

* Lobo et al. (2009)

** Kakati et al. (2017)

4.3. ARTIGO III

SURFACE FUNCIONALIZATION OF MULTI-WALL CARBON NANOTUBES WITH DIFFERENT DIAMETERS FOR GENERATION OF EFFICIENT IMMOBILIZED SYSTEM WITH *BURKHOLDERIA CEPACIA* LIPASE

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Abstract

Carbon nanotubes (CNTs) possess unique features for enzyme immobilization. Therefore, this work studied the immobilization of lipase from *Burkholderia cepacia* on funcionalized multi-wall carbon nanotubes (MWCNTs), with different diameters, by physical adsorption. For this, carbon nanotubes were funcionalized by preliminary treatments (carboxylation and amination) to attain carboxyl and amino groups on their surfaces before enzyme immobilization and the samples were characterized. Standard, surface-funcionalized and immobilized CNTs were characterized. Elemental analysis, thermogravimetric analysis and SEM showed differences in purity, surface area and diameter between in purity, surface area and diameter between the CNTs used. FTIR revealed differences in CNTs after funcionalization. The biocatalyst immobilized on CNTs with smaller diameter and funcionalized (CNT NC 7000 – NH_2) showed great total activity recovery yield (65.98 %) and 7 cycles of reuse. In this work, the diameter of the CNTs as well as their surface- funcionalization were founded to be determinant for the total activity recovery yield and Reusability.

Keywords: Carbon nanotubes, diameter, surface funcionalization, *Burkholderia cepacia* lipase immobilization.

1. INTRODUCTION

Enzyme immobilization on carbon nanotubes (CNTs) is rapidly emerging as a new research area, since these materials provide the upper limits in balancing the key factors that determine the efficiency of biocatalysts, including surface area, mass transfer resistance, effective enzyme loading and a curve-shaped surface which allows an ideal disposition of these globular biomolecules (Tan et al., 2012; Fan et al., 2016).

Moreover, the conjugation of enzymes with CNTs can increase both enzyme activity and stability, even when subjected to strongly denaturing environments (Deep et al., 2015; Tavares et al., 2015). Among enzymes, lipases (EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of triacylclycerols to glycerols and free fatty acids at the lipid water interface and are the most widely used enzymes in organic synthesis and in more than 20% of biotransformation reactions (Prlainovic et al., 2016).

Despite this, the investigation of different methods to immobilize enzymes on CNTs is of great relevance. These include the physical adsorption of the enzyme onto CNTs, which is a method mainly based on supramolecular chemistry using various adsorption forces, such as hydrogen bonding, van der Waals forces, π-π interactions and hydrophobic interactions (Cang-Rong e Pastorin, 2009; Pavlidis et al., 2010; Chung et al., 2015). The noncovalent method is considered to be a more promising technique for preserving the conformational structure of the immobilized enzymes (Feng and Ji, 2011).

On the other hand, CNTs presented high hydrophobicity, which leads to processing difficulties associated with the low dispersion of entangled CNTs and are extremely resistant to wetting. In this sense, different funcionalization strategies of CNTs have been considered to overcoming these barriers, like covalent functionalization method that include sidewall and tip-end reactions, which provide covalent linkages between CNTs and functional groups (Raghavendra et al., 2014; Chung et al., 2015). Covalent funcionalization of CNTs requires the formation and generation of the oxygen-containing functional groups, more specifically the hydroxyl and carboxylic groups on the surfaces. For this purpose, carboxylic acid groups are considered as one of the best choices because they can undergo a variety of reactions for further modification and are easily formed through the various oxidizing treatments (Majeed et al., 2013). Moreover, surface-

functionalization of CNTs may present a possible solution to create an enzyme-preferable microenvironment (Rastian et al., 2014).

Thus, the proposal of this work was to study the effect of CNTs surface funcionalization, with two different diameters, on the total activity recovery yield of biocatalysts immobilized through physical adsorption.

2. MATERIALS AND METHODS

2.1. Generals

Lipase from *Burkholderia cepacia* (Amano Lipase) with enzymatic activity of the 2521.15 U/mL was purchased from SIGMA – ALDRICH (Japan). Multi-Wall carbon nanotubes CNT NC 7000 was obtained from Nanocyl (Surface Area: 250-300 m^2/g and 10 % of metal oxide) and CNT PR-24-PS was obtained from Pyrograf Products (Surface Area: 45 m^2/g). All other chemicals and reagents were purchased from Sigma and used as received.

2.2. Surface funcionalization of CNTs

The CNTs were subjected to two types of preliminary treatments (carboxylation and amination). Carboxylated CNTs were prepared according to Pavlidis et al. (2010). CNTs (CNT NC 7000 and CNT PR-24-PS) were suspended in 40 mL of a concentrated H_2SO_4/HNO_3 mixture (3:1 v/v) and sonicated for 3 h. The suspension was then centrifuged, washed with distilled water and dried at 50 °C in vacuum overnight (denoted as CNT NC 7000–COOH and CNT PR-24-PS-COOH). For amination, the carboxylated CNTs were suspended in 10% APTS (diluted in ethanol) and sonicated for 4 h followed by overnight stirring for funcionalization of side walls (Raghavendra et al. 2014). The APTS funcionalized CNTs were recovered by centrifugation with ethanol and deionized water to remove excess APTS and ethanol, respectively. This method of funcionalization results in production of amine groups on the CNT surface (denoted as CNT NC 7000–NH_2 and CNT PR-24-PS-NH_2).

2.3. Characterization techniques

Carbon, Hydrogen and Nitrogen elements were quantified to standard CNTs by Truspec 630-200-200 with the following detection method: C and H infrared absorption and N thermal conductivity.

Thermogravimetric analysis (Shimadzu DTG-60H/DTA-TG simultaneous apparatus) were performed to standard CNTs under a nitrogen atmosphere that started from room temperature and went up to 1000 °C, increasing at a heating rate of 20 °C.min^{-1}.

The images from scanning electron microscopy (SEM) were performed to standard CNTs in a Hitachi SU-70 microscope operated in secondary electron mode at 15 kV.

Transmission electron microscopy (TEM) measurements were realized to standard and immobilized CNTs on a JEOL JEM-2200FS microscope operated at 200 kV. Samples for TEM measurements were prepared by dry adhesion of the samples to a holey carbon grid.

Fourier Transform Infrared Spectroscopy (FTIR) analysis (spectrophotometer FTIR BOMEMMB-100) were performed to standard, funcionalized and immobilized CNTs. Spectra were obtained in the wavelength range from 400 to 4000 cm^{-1}. The samples of immobilized biocatalysts also were submitted to FTIR analysis.

2.4.Lipase immobilization onto CNTs

The lipase from *Burkholderia cepacia* was immobilized by physical adsorption on CNTs using the procedure from Cabrera-Padilla et al. (2013). Briefly, hexane was added to support with vigorous agitation at room temperature for 2 h, then enzymatic solution (enzyme solubilised in 0.1 M sodium phosphate buffer, pH 7.0) was added to the hexane and support suspension and agitated for another 2 h. The enzyme-support system was incubated for 24 h at 4 °C. The immobilized lipase was recovered by centrifugation with repeated hexane wash and dried under vacuum at room temperature for 72 h. Filtrates and washes were collected and used for activity determination. The immobilized lipase was then stored at 4 °C.

2.5.Lipase activity

Enzymatic activities of free and immobilized biocatalysts samples were assayed by olive oil emulsion method according to modifications by Soares et al. (1999). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 µmol of free fatty acid per min under assay conditions (37 °C; pH 7.0; 10 min incubation). Analysis of enzymatic activities performed on the free and immobilized lipases were used to determine the total activity recovery yield (Ya) according to Eq. (1).

$$Ya\ (\%)=\frac{Us}{Uo}\ x\ 100 \qquad (1)$$

Where U_S corresponds to units of total enzymatic activity present in the support and Uo represents the units of activity offered for immobilization.

3. RESULTS AND DISCUSSION

3.1. CNTs' Characterization

Elemental analyses were conducted to investigate the C-H-N-content in the standard CNTs (CNT NC 7000 and CNT PR-24-PS). This technique demonstrates the presence of quantifiable amounts of C, H, and N in the tubes (NXUMALO and COVILLE, 2010). No hydrogen and nitrogen were detected for CNT NC7000 (Table 4.11), however the other elements were detected for CNT PR-24-PS. Noticeably small differences between the CNTs elemental content may indicate different purities.

Table 4.11: Elemental composition of the standard CNTs.

Sample	C (%)	H(%)	N(%)
CNT NC 7000	87.58	0	0
CNT PR-24-PS	95.28	0.75	0.54

The TG curve of carbon nanotubes showed loss weight 0.07 mg and 0.28 mg to CNT NC 7000 and CNT PR-24-PS, respectively (Figure 4.14). The CNT NC 7000 sample exhibited high thermal stability. The mass loss for CNT PR-24-PS sample can be attributed to side-wall functional groups, amorphous carbon as well as oxygen-containing group loss (Brković et al., 2015). According to Morsy et al. (2014) the mass loss during ramp heating can be due to the burning of amorphous carbon and carbon nanotubes, leaving only the catalyst as the residue. The presence of Fe residual catalyst contained in the tubes difficult to oxidize protected metallic particles and graphite nanoparticles because their graphite structure is highly resistant to oxidation. These argument is in accordance with the elemental analysis results and purity of the samples, considering presence of the metal oxide to CNT NC 7000.

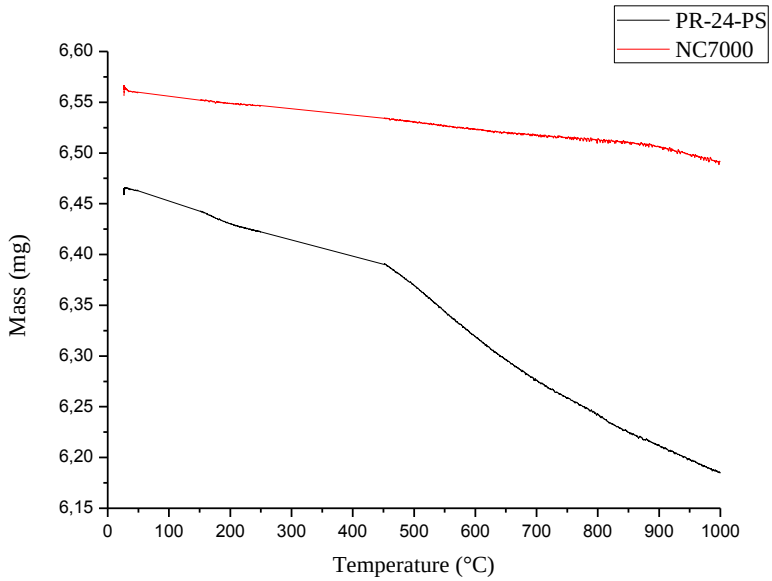
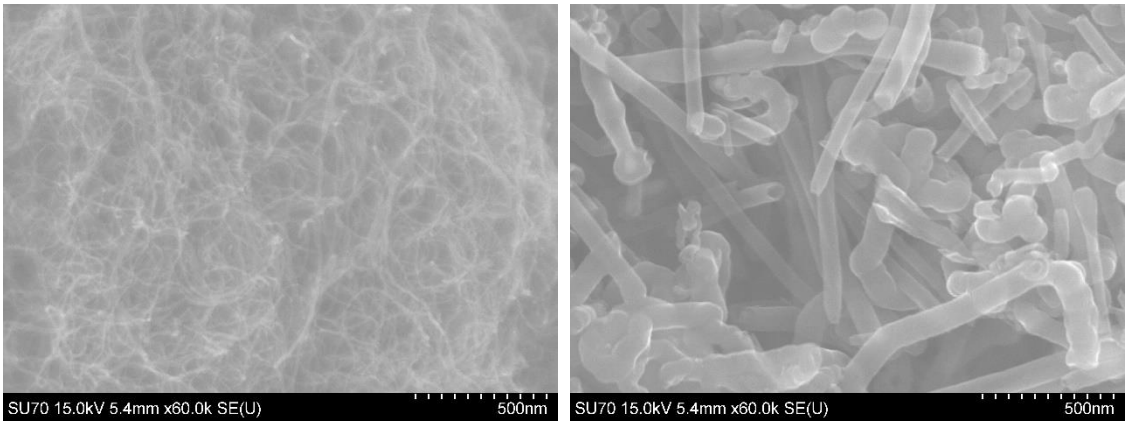


Figure 4.14: TG curve of standard CNTs (NC 7000 and PR-24-PS).

The SEM images of CNTs were performed to further investigate the morphology and structure of the carbon nanotubes (Figure 4.15). From the SEM images it was observed bundles of tangled tubes with typical tube lengths in an order of micrometer; and presence of some impurities on the surface of CNTs. In comparison, the CNT NC 7000 have a smaller diameter and consequently higher surface area (surface area 250-300 m^2/g and average diameter 9.5 nm) than CNT PR-24-PS (surface area 45 m^2/g and average diameter 100 nm).



CNT NC 7000 CNT PR-24-PS

Figure 4.15: SEM images of standard CNTs (NC 7000 and PR-24-PS).

3.2. CNTs' Funcionalization

In order to facilitate the formation of the enzyme–CNT complex, the CNTs were submitted to surface funcionalization to modify the surface chemistry (-COOH and - NH_2). During the acid oxidation of CNTs by a mixture of HNO_3 and H_2SO_4 at a ratio 1:3 (v/v), the carbon bonded network of the nanotube layers is broken by the ultrasonic wave emitted from the sonicator, allowing the induction of oxygen units in the form of carboxylic (from acid mixture) onto the surface of nanotubes. Furthermore, in acid oxidation, the diffusion of the oxidizing agent from acid mixture involve the removal of metallic impurities and amorphous carbon (Mubarak et al., 2014, Yan et al., 2015).

Avilés et al. (2011) suggesting that functional groups of the amination are better bonded to the CNT surface when an oxidative treatment is conducted before, because disappearance of the metal impurities. Therefore, this method has been used for attachment of a variety of biomolecules (Raghavendra et al., 2014). The effectiveness of surface-funcionalization of CNTs may influence the surface adsorption of lipase.

In this way, the FTIR spectra were obtained to indicate and identify the existence of functional group attached on the surface CNTs. Figure 4.16 shows comparative FTIR spectra for CNTs – Standard and CNTs - surface-funcionalized (CNTs - COOH and CNTs – NH_2).

The FTIR analysis from the standard CNTs (NC 7000 and PR-24-PS) showed appearance of the same bands. The band at 3435 cm^{-1} refers to the OH stretching, band at 2920 cm^{-1} corresponding to asymmetric stretching of CH_2, band at 1635 cm^{-1} can be associated with the stretching of the carbon nanotube backbone. It is usually assumed that these groups are located at defect sites on the sidewall surface. The band at 1050-1030 cm^{-1} was ascribed to the C–O stretching (Kumar et al., 2009; Morsy et al., 2014).

A comparison of the peak intensities of standard CNTs and funcionalized CNTs clearly shows appearance of new bands. The funcionalized CNTs – COOH showed a broad bands at 3435 cm^{-1}, which refers to the OH stretching of the hydroxyl group which can be ascribed to the oscillation of carboxyl groups (O=C-OH and C-OH). In addition, the vibration bands characterizing the C‑O of the carboxyl group appeared at around 1210‑1320 cm^{-1} (Chang et al., 2011; Vanyorek et al., 2014). The funcionalized CNTs – NH_2 presented bands at 3430 cm^{-1} probably due to the stretching vibration of ‑NH‑ group, 2900 cm^{-1} attributed to CH bonds, 1635 and 1500 cm^{-1} correspond to amides (- CO‑NH‑) I and II (Zhang et al., 2010), and 1180 cm^{-1} correspond to the N–H in-plane deformation vibrations coupled with the valence C–N bond stretching vibrations (Brković et al., 2015). The presence of these functional groups implies that the as received CNTs presented several functional groups that were introduced during the surface-funcionalization process.

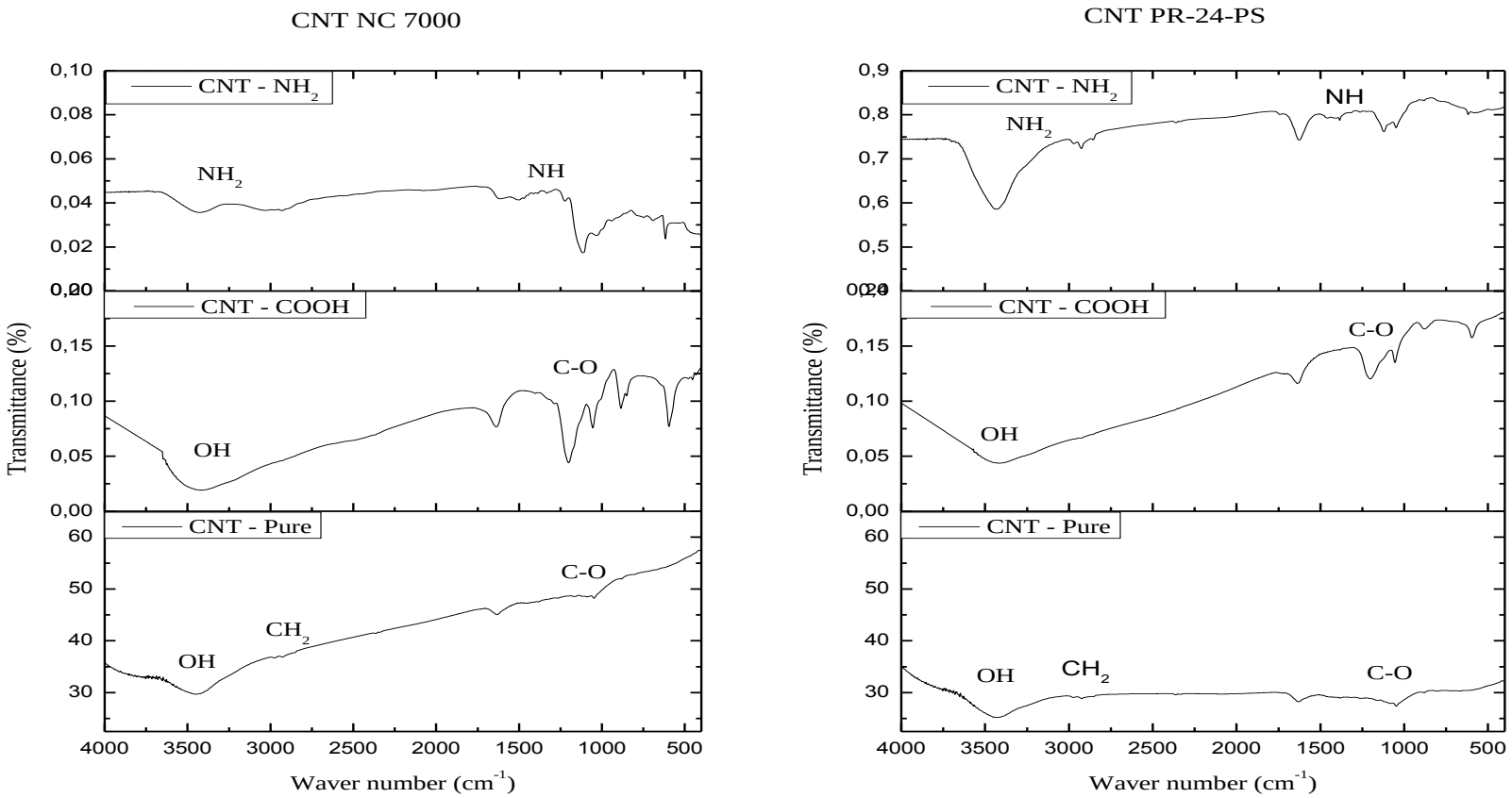


Figure 4.16: FTIR spectra of CNTs (standard and surface-funcionalized).

3.3. Lipase Immobilization

The enzyme loading was investigated in the range of 0.1 to 0.6 g *Burkholderia cepacia* lipase. As shown in Table 4.12, the maximum total activity recovery yield occurred at 0.15 g of the loading to CNT NC 7000 and 0.45 g to CNT PR-24-PS and fixed activity increased until 0.3 g of the loading to CNT NC 7000 and 0.45 g to CNT PR-24-PS, respectively. After that, the total activity recovery yield and fixed activity declined. Higher loadings did not result in significant increase in fixed activity on support, probably due to multilayer adsorption of enzyme and formation of enzyme aggregates because the adsorption is primarily on the surface, diffusion restrictions to reactants would be negligible (Yadav and Jadhav, 2005). Thus, considering the total activity recovery yield and fixed activity, the enzymatic loading selected for immobilization of *Burkholderia cepacia* lipase in the funcionalized carbon nanotubes was 0.15 and 0.3 g to CNT NC 7000 and 0.45 g to CNT PR-24-PS.

Table 4.12: Lipase loading to immobilization on standard CNTs (NC 7000 and PR-24-PS).

CNTs	Loading ($g_{lipase}/g_{suporte}$)	Ya (%)	OA (U/g)	FA (U/g)
NC 7000	0.10	13.72 (±0.47)	184.67	25.34
	0.15	34.89 (±0.00)	153.00	53.39
	0.3	30.89 (±0.29)	237.08	73.24
	0.45	16.59 (±0.45)	223.19	37.02
	0.6	6.17 (±1.52)	93.72	5.78
PR-24-PS	0.10	4.05 (±0.34)	53.90	1.90
	0.15	10.04 (±0.26)	68.06	6.83
	0.3	16.19 (±0.00)	133.04	21.54
	0.45	17.05 (±0.07)	260.50	44.43
	0.6	9.66 (±3.35)	124.33	12.01

Ya – Total Activity Recovery Yield
OA – Offered Activity
FA – Fixed Activity

In order to examine the effects of the surface property changes resulted from surface-funcionalization on immobilization of the *Burkholderia cepacia* lipase, total activity recovery yield was evaluated. From the Table 4.13, no significant increase in total

activity recovery yield to CNT PR-24-PS was observed, despite the funcionalization of this carbon nanotube. Larger diameter of CNTs (case PR-24-PS) is expected to drastically reduce nonspecific binding of molecules by hydrophobic interactions (Raghavendra et al., 2014). Silva et al. (2014) reported that activity of immobilized laccase was influenced by the CNT diameter. The authors founded maximum total activity recovery yield for the CNT with smaller diameter (range 10 to 20 nm). In this work, the diameter of carbon nanotubes was an important factor for the lipase immobilization.

Table 4.13: Lipase loading to immobilization on surface-funcionalized CNTs.

CNTs	Loading ($g_{lipase}/g_{suporte}$)	Ya (%)	OA (U/g)	FA (U/g)
NC 7000	0.15	34.89 (±0.00)	153.00	53.38
	0.30	30.89 (±0.29)	237.08	73.24
NC 7000 - COOH	0.15	49.53 (±0.47)	212.84	105.41
	0.30	25.90 (±0.00)	224.38	58.11
NC 7000 – NH_2	0.15	65.98 (±2.16)	283.54	187.08
PR-24-PS	0.45	17.05 (±0.15)	260.50	44.43
PR-24-PS – COOH	0.45	19.04 (±0.34)	245.43	46.72
PR-24-PS – NH_2	0.45	19.89 (±0.00)	256.37	50.98

Ya – Total Activity Recovery Yield
OA – Offered Activity
FA – Fixed Activity

On the other hand, the total activity recovery yield increased for CNTs NC 7000 funcionalized. The results obtained indicated that *Burkholderia cepacia* lipase was preferentially adsorbed on the CNT NC 7000–NH_2 (total activity recovery yield – 65.98 % and fixed activity – 187.08 U/g). Besides, there were more enzymes adsorbed on the funcionalized CNTs than the standard CNTs, possibly owing to that the funcionalization of CNTs enhanced the interfacial adhesion between the nanotubes and the enzymes (Yu et al., 2015). In the present case, the high surface area presented by the CNT NC 7000 with the lowest diameter and presence of functional groups may play a role in the efficient loading of the enzyme along the CNT surface.

Yu et al. (2015) and Zniszczoł et al. (2016) also reported results indicating that the enzymes were preferentially adsorbed on CNT–NH_2 than CNT–OH and CNT–COOH. The total activity recovery yield this work (66 %) is over than value obtained by Yadav

and Jadhav (2005). The authors founded maximum of 48.5 % of percent immobilization to immobilized system with *Pseudomonas cepacia* lipase on silica HMS.

3.4. Biocatalysts Characterization

Immobilized biocatalysts on surface-funcionalized CNTs of diametrs different with maximum values of total activity recovery yield (CNTs NC 7000 – NH_2 and CNTs PR-24-PS – NH_2) were submitted to analyze of operational stability (Figure 4.17) and TEM (Figure 4.18).

The operational stability of immobilized *Burkholderia cepacia* lipase on different CNTs was evaluated and subsequent cycles of hydrolysis were conducted, all under the same conditions of reaction. The study of the stability in consecutive reaction cycles provides essential information about the biocatalyst resistance and effectiveness in repeated uses (Tavares et al., 2015).

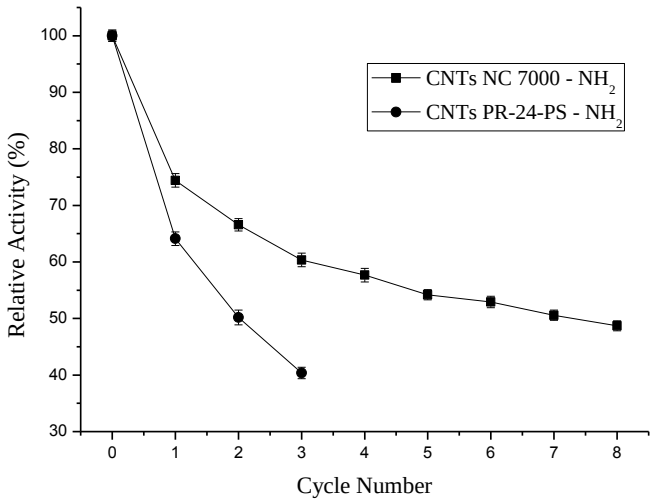


Figure 4.17: Operational stability tests for *Burkholderia cepacia* lipase immobilized on CNTs NC 7000 – NH_2 and CNTs PR-24PS – NH_2.

The results in Figure 4.17 show more stability for immobilized CNTs NC 7000 - NH_2, which presented reuse for 7 cycles in the reaction of hydrolysis of olive oil, with 50.6 % relative activity. Immobilized CNTs PR-24-PS – NH_2 furnished a less stable system, showed reuse lower compared to immobilized CNTs NC 7000 - NH_2, with

50.2 % relative activity with 2 cycles of reuse. The system formed with CNTs of smaller diameter (immobilized CNTs NC 7000 – NH_2) probably increased of enzyme rigidity improving its stability. The decrease in the activity observed can be probably related to *Burkholderia cepacia* lipase leaching from the support since it is not covalently attached, once the catalytic properties of biocatalysts are strongly dependent on the immobilization protocol (Palomo et al., 2003).

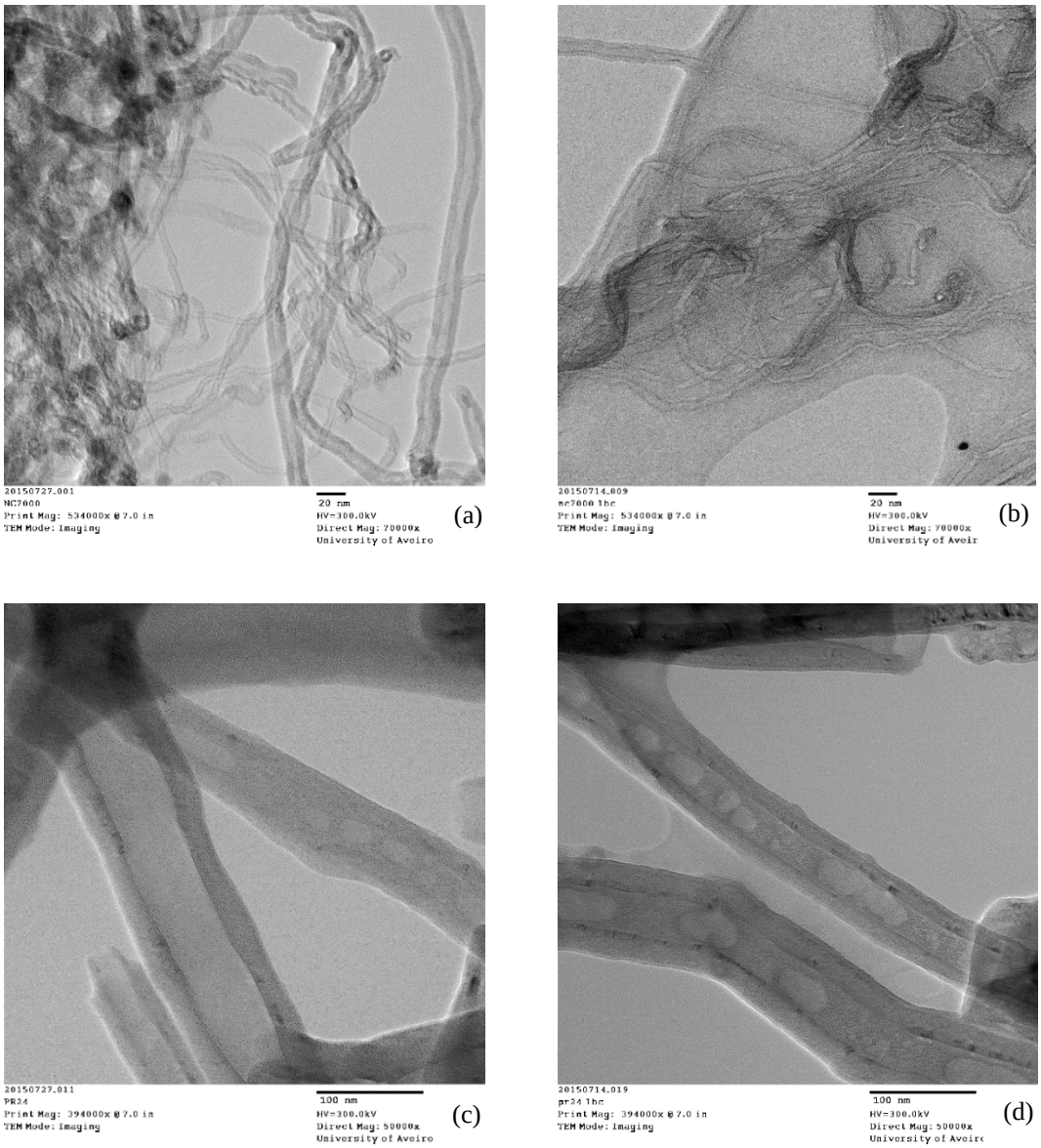


Figure 4.18: TEM images of standard CNTs NC 7000 (a), immobilized CNTs NC 7000 (b), standard CNTs PR-24-PS (c) and immobilized CNTs PR-24-PS (d).

TEM analysis were applied in order to collect information about structure changes of CNTs after lipase immobilization. Figure 4.18 showed the presence of a visible deposition of the *Burkholderia cepacia* lipase molecules (as network structure) upon carbon nanotubes. Thus, *Burkholderia cepacia* lipase was spontaneously immobilized by simply contacting the CNTs, in the absence of any surface funcionalization or coupling reagents.

4. CONCLUSION

Successful funcionalization and *Burkholderia cepacia* lipase immobilization by physical adsorption method. Among the immobilized systems the immobilized biocatalyst on CNTs with smaller diameter and funcionalized (CNT NC 7000 – NH_2) showed higher total activity recovery yield, ranging from 34.89 to 65.98 % and more stability with 7 cycle reuse. Therefore, the smallest diameter of the carbon nanotubes (CNTs) as well as the surface funcionlization with COOH groups followed NH_2 groups were founded to be determinant in total activity recovery yield.

ACKNOWLEDGMENTS

The financial supports from Coordination for the Improvement of Higher Education Personnel (CAPES), Doctoral Sandwich Program Abroad (PDSE), Brazilian National Council for Scientific and Technological Development (CNPq), FCT/MEC (Ref. FCT UID/CTM /50011/2013) and when appropriate co-financed by FEDER under the PT2020 Partnership Agreement.

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CAPÍTULO V

5. CONCLUSÕES

Diante dos resultados obtidos no decorrer deste trabalho, pode-se concluir que os objetivos inicialmente propostos foram alcançados e abaixo estão apresentadas as conclusões principais:

- O alongamento da cadeia alquílica dos LIPs testados aumentou o impacto negativo destes compostos químicos em vários micro-organismos testados neste trabalho.

- Os líquidos iônicos m-2HEAPr e m-2HEAP representam os menos tóxicos.

- As análises de FTIR mostraram que a lavagem por meio do soxleht foi eficiente na remoção dos líquidos iônicos próticos dos suportes de sílica sintetizados.

- A caracterização dos suportes sintetizados revelou mudanças positivas nas propriedades estruturais dos suportes. A análise termogravimétrica mostrou que o suporte puro apresentou mais componenetes orgânicos em compração aos suportes modificados. A análise de BET demonstrou que o suporte modificado com o LIP mais hidrofóbico apresentou maior tamanho e volume de poros em comparação com os outros suportes. As imagens obtidas por meio da Microscopia Eletrônica de Transmissão sugeriram que os suportes são formados por aglomerados e que o suporte puro apresentou partículas de sílica individuais menores.

- Os maiores valores de rendimento de recuperação de atividade total foram obtidos para os suportes imobilizados por ligação covalente utilizando epicloridrina. No entanto, para os biocatalisadores imobilizados por adsorção física o rendimento de recuperação de atividade total dobrou para o suporte modificado com o LIP mais hidrofóbico (m-2HEAP-S) em comparação com os outros suportes utilizados para imobilização por esta técnica. Os biocatalisadores imobilizados por ligação covalente utilizando glutaraldeído não demosntraram diferenças significativas no rendimento de recuperação de atividade total.

- A caracterização dos suportes após imobilização revelaram que, dentre os biocatalizadores, os que foram imobilizados por adsorção física apresentaram melhor

estabilidade térmica ($t_{1/2}$ 231-309 h), estabilidade operacional (maior atividade em relação aos 40 reciclagens) e menores valores de K_m. As análises de FTIR apresentaram a banda referente a amida I para todos os sistemas imobilizados.

- 92 % de rendimento em ésteres etílicos foi obtido utilizando óleo de coco residual e etanol como fontes de matéria prima e biocatalisador imobilizado em suporte modificado m-2HEAP-S por LCE a partir da reação de transesterificação assistida por ultrassom.

- Tempo de meia-vida foi intensificado com o uso do ultrassom (21,1 h) quando usado o biocatalisador imobilizado em suporte modificado m-2HEAP-S por LCE.

- As análises de FTIR demosntraram modificações na superfície dos CNTs após carboxilação seguida de aminação.

- As imagens obtidas por meio da Microscopia Eletrônica de Transmissão sugeriram que a lipase foi imobilizada nos CNTs.

- O biocatalisador imobilizado em nanotubos de carbono com menor diâmetro e funcionalizado (CNT NC 7000 - NH2) apresentou o melhor valor de rendimento de recuperação de atividade total (65,98 %) após a funcionalização da superfície.

Desta forma, a preocupação com o desenvolvimento de novos materiais capazes de gerar biocatalisadores com ótimo desempenho biocatalítico e ótima estabilidade térmica e opracional foi atingida.

CAPÍTULO VI

6. SUGESTÕES PARA TRABALHOS FUTUROS

Deste modo a partir dos resultados promissores obtidos neste estudo sugere-se a realização das seguintes etapas futuras:

- Obter a caracterização bioquímica dos biocatalisadores imobilizados em nanotubos de carbono;

- Aplicar os biocatalisadores imobilizados em nanotubos de carbono em reações de transesterificação, entre outros;

- Estabelecer condições favoráveis para um melhor rendimento em ésteres etílicos utilizando o biocatalisadores imobilizados por LCE em sílica modificada com o LI mais hidrofóbico;

- Após o estabelecimento da eficiência dos biocatalisadores imobilizados em reações de biotransformação faz-se necessário a aplicação em biorreatores em regime batelada e contínuo;

- Estudar a viabilidade econômica dos métodos desenvolvidos neste estudo.

CAPÍTULO VII

7. LISTA DE DIVULGAÇÃO DOS RESULTADOS

Durante este período de estudo os resultados foram publicados em diferentes meios de divulgação, como listados a seguir:

Artigo publicado na Chemosphere:

Maria V.S. Oliveira, Bruna T. Vidal, Claudia M. Melo, Rita de C.M. de Miranda, Cleide M.F. Soares, João A.P. Coutinho, Sónia P.M. Ventura, Silvana Mattedi, Alvaro S. Lima. (Eco)toxicity and biodegradability of protic ionic liquids. *Chemosphere 147 (2016) 460 e 466*.

Resumo em Evento Nacional:

- Paulo Alexandre Almeida Rodrigues, Maria Vanessa Souza Oliveira, Matheus Mendonça Pereira, Silvana Mattedi, Cleide Mara Faria Soares, Álvaro Silva Lima. Uso de sílica modificada por líquidos iônicos baseados em aminas e imobilização de lipase por ligação covalente - *Semana de Pesquisa da Unit (16ª SEMPESq),* 2014.

- Paulo Alexandre Almeida Rodrigues, Maria Vanessa Souza Oliveira, Silvana Mattedi, Filipe José Oliveira, Rui Silva, Cleide Mara Faria Soares, Álvaro Silva Lima. Imobilização de lipase por adsorção em suportes de sílica modificada: preparação e caracterização - *Semana de Pesquisa da Unit (17ª SEMPESq),* 2015.

- Maria Vanessa Souza Oliveira, Paulo Alexandre Almeida Rodrigues, Cleide Mara Faria Soares, Álvaro Silva Lima. Imobilização de lipase de burkholderia cepacia por ligação covalente em sílica modificada com líquido iônico prótico - *Semana de Pesquisa da Unit (17ª SEMPESq),* 2015.

- Aroni Campos Professor, Nayara Bezerra Carvalho, Maria Vanessa Souza Oliveira, Acenini Lima Balieiro, Adriana de Jesus Santos (ITP), Cleide Mara Faria Soares, Álvaro Silva Lima. Transesterificação enzimática de óleo de coco bruto em ultrassom de sonda e aquecimento convencional - *Semana de Pesquisa da Unit (17ª SEMPESq),* 2015.

Resumo em Evento Internacional:

- Maria Vanessa Souza Oliveira, Paulo Alexandre Almeida Rodrigues, Silvana Mattedi, Filipe José Oliveira, Rui Ferreira e Silva, Cleide Mara Faria Soares, Álvaro Silva Lima. Protic ionic liquid modified mesoporous silica obtained by the sol-gel technique: synthesis and characterization - *VII International Materials Symposium and 17th Conference of Sociedade Portuguesa de Materiais - Materiais 2015,* 2015.

- Maria V. S. Oliveira, Paulo A. A. Rodrigues, Silvana Mattedi, Filipe J. Oliveira, Rui F. Silva, Cleide M. F. Soares, Álvaro S. Lima. Application of protic ionic liquids as modifiers for sílica - *Jornadas CICECO 2015, do Laboratório Associado CICECO - Aveiro Institute of Materials,* 2015.

Trabalho Completo em Evento Nacional:

- Maria Vanessa Souza Oliveira1, Paulo Alexandre Almeida Rodrigues1, Luana Carvalho Garcia1, Silvana Mattedi2, Filipe José Oliveira3, Rui Silva3, Cleide Mara Faria Soares1,4 e Álvaro Silva Lima. Síntese e imobilização de sílica obtida pela técnica sol-gel modificada por líquidos iônicos próticos - *XX Simpósio Nacional de Bioprocessos (SINAFERM) e o XI Simpósio de Hidrólise Enzimática de Biomassas (SHEB*), 2015.

- Maria Vanessa Souza Oliveira, Paulo Alexandre Almeida Rodrigues, Silvana Mattedi, Rui Silva, Cleide Mara Faria Soares, Álvaro Silva Lima. Efeito da substituição da amina do líquido iônico prótico utilizado como agente de modificação de silica na imobilização de lipase de burkholderia cepacia - *XXI Congresso Brasileiro de Engenharia Química, COBEQ 2016 - XVI Encontro Brasileiro sobre o Ensino de Engenharia Química, ENBEQ* 2016.

- Maria Vanessa Souza Oliveira, Aroni Campos Professor, Nayara Bezerra Carvalho, Adriana de Jesus Santos, Cleide Mara Faria Soares, Álvaro Silva Lima. Transesterificação enzimática de óleo de coco bruto usando ultrassom e micro-ondas - *XXI Congresso Brasileiro de Engenharia Química, COBEQ 2016 - XVI Encontro Brasileiro sobre o Ensino de Engenharia Química, ENBEQ* 2016.

- Maria Vanessa Souza Oliveira, Aroni Campos Professor, Acenini Balieiro Lima, Adriana de Jesus Santos, Juliana Lisboa Santana, Silvana Mattedi, Cleide Mara Faria Soares, Álvaro Silva Lima. Transesterificação enzimática de óleo de coco bruto assistida por ultrassom - *6º Congresso da Rede Brasileira de Tecnologia de Biodiesel, 9º Congresso Brasileiro de Plantas Oleaginosas, Óleos, Gorduras e Biodiesel, 2016.*

Trabalho Completo em Evento Internacional:

- Maria Vanessa Souza Oliveira, Paulo Alexandre Almeida Rodrigues, Matheus Mendonça Pereira, Silvana Mattedi, Cleide Mara Faria Soares, Álvaro Silva Lima. Evaluation of the Effect of Protic Ionic Liquids in Synthesis of Silica Sol-gel and Immobilization of *Burkholderia cepacia* Lipase. *12th International Chemical and Biological Engineering Conference (CHEMPOR 2014),* 2014.

CAPÍTULO VIII

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