

UNIVERSIDADE TIRADENTES – UNIT  
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE PROCESSOS - PEP

## **EMPREGO DE ADITIVOS NA IMOBILIZAÇÃO SOL-GEL DE LIPASES**

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# EMPREGO DE ADITIVOS NA IMOBILIZAÇÃO SOL-GEL DE LIPASES

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*“Se não deu certo, é porque ainda não chegou ao fim”.*

*(Fernando Sabino)*



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## EMPREGO DE ADITIVOS NA IMOBILIZAÇÃO SOL-GEL DE LIPASES

Ranyere Lucena de Souza

O uso de lipases como catalisadores de reações típicas da indústria química é cada vez mais aplicado, devido à sua capacidade em modificar a velocidade de uma reação. No entanto, as enzimas na sua forma livre não possibilitam a sua reutilização, o que do ponto vista econômico, tornam sua aplicação inviável. Diversos métodos de imobilização de biomoléculas são propostos na literatura, tais como se destaca a técnica sol-gel, por ser bastante promissora. Contudo, este processo apresenta alguns inconvenientes, pois durante a etapa de formação do gel, a enzima pode perder seu potencial catalítico e estabilidade. Desta forma, é comum a utilização de aditivos a fim de minimizar estes efeitos negativos. O emprego de macromoléculas como o PEG (polietilenoglicol), e mais recentemente o uso de líquidos iônicos, apresentam potencialidade em processos de imobilização. Portanto, o objetivo deste trabalho foi utilizar aditivos (Líquidos Iônicos Próticos - LIP, Polietilenoglicol e sais quaternários de amônia – Aliquat 336) durante o processo de imobilização sol-gel das lipases de *Burkholderia cepacia* e de *Bacillus* sp. ITP-001, caracterizar a estrutura dos imobilizados por meio das isotermas de adsorção e dessorção de nitrogênio, Microscopia Eletrônica de Varredura – MEV, Espectroscopia no Infravermelho por Transformada de Fourier - FTIR, Análise Termogravimétrica - TGA e Análise de Calorimetria Diferencial de Varredura - DSC e, avaliar o potencial na produção de ésteres e emulsificantes e verificar rendimento de recuperação total da atividade lipolítica. Os imobilizados da lipase de *Burkholderia cepacia* apresentaram rendimento total da atividade de 1526% enquanto que os de *Bacillus* sp. ITP-001 apresentaram 305% nas amostras com 1,0 e 0,5% respectivamente de LIP de caráter mais hidrofóbico. O uso do LI de caráter mais hidrofóbico no imobilizado de *Burkholderia cepacia*, favoreceu ainda, a conversão de 45,6% na reação de transesterificação de óleo de soja e etanol. Nas análises de adsorção e dessorção de nitrogênio, MEV e FTIR, foi possível verificar a alteração na estrutura porosa dos biocatalisadores e a presença dos grupamentos amina. O emprego de LIP no processo de imobilização conferiu maior resistência térmica as matrizes produzidas, verificadas pelas curvas TGA auxiliadas das curvas DSC.

**Palavras-Chave:** lipase, sol-gel, aditivos, líquidos iônicos, aliquat.

Abstract of Dissertation presented to the Process Engineering Graduate Program of University Tiradentes as a partial fulfillment of the requirements for the degree of Master of Science (M.Sc.)

## USE OF ADDITIVES IN DETENTION SOL-GEL LIPASES

Ranyere Lucena de Souza

The use of lipases as catalysts for reactions typical of the chemical industry is increasingly used because of its ability to modify the rate of reaction. However, the enzymes in the free form does not allow its reuse, economic view point to make application feasible. Several methods of immobilization of biomolecules are proposed in the literature, such as highlights the sol-gel technique, to be very promising. However, this process has some drawbacks, since during the step of forming the gel, the enzyme may lose its catalytic potential and stability. Thus, it is common to use additives to minimize these negative effects. The uses of macromolecules as PEG (polyethylene glycol), and more recently the use of ionic liquids, have potential in the process of immobilization. Therefore, the objective of this study was to use additives (protic ionic liquids - PIL, polyethylene glycol and quaternary ammonium salts - Aliquat 336) during the sol-gel immobilized lipase from *Burkholderia cepacia* and *Bacillus* sp. ITP-001, to characterize the structure of immobilized through adsorption and desorption isotherms of nitrogen, Scanning Electron Microscopy - SEM, Spectroscopy Fourier Transform Infrared - FTIR, Thermogravimetric Analysis – TGA, and Differential Scanning Calorimetry - DSC and evaluate the potential for the production of esters and emulsifiers and check recovery yield of total lipolytic activity. The immobilized lipase from *Burkholderia cepacia* showed total income from the activity of 1526% while those of *Bacillus* sp. ITP-001 showed 305% in the samples with 1.0 and 0.5% respectively PIL more hydrophobic character. The use of IL in more hydrophobic character of immobilized *Burkholderia cepacia*, has favored the conversion of 45.6% in the transesterification reaction of soybean oil and ethanol. In the analysis of nitrogen adsorption and desorption, SEM and FTIR, it was possible to verify the change in the porous structure of the biocatalysts and the presence of amine groups. The use of PIL in the process of immobilization produced greater thermal resistance matrices produced, verified by TGA curves of DSC helped.

**Key words:** lipase, sol-gel, additives, ionic liquids, aliquat.

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## INTRODUÇÃO

Melhorar a qualidade de vida é um dos princípios da sustentabilidade, contudo, o maior desafio dá-se na continuidade dos avanços em pesquisas, diminuindo principalmente os danos ao meio ambiente. Algumas terminologias são utilizadas atualmente, como por exemplo, “Química Verde”, cujo termo demonstra a preocupação do desenvolvimento de tecnologias e processos capazes de reduzir ou não gerar poluição, ou seja, reduzir o impacto que processos químicos provocam ao meio ambiente, gerando o desenvolvimento econômico e social de forma limpa e sustentável. Neste enfoque, o estudo de biocatalisadores e seus processos são considerados como aplicação da "Química Verde" estrategicamente promissora e o produto obtido via biocatálise pode ser denominado “produto verde”. A aplicação de enzimas como catalisadores exemplifica muito bem os avanços na área, que apesar das limitações na aplicação industrial, vem sendo cada vez mais estimulada, pois as vantagens do ponto de vista ambiental são bem maiores do que a dificuldade encontrada na biotransformação utilizando enzimas livres ou imobilizadas para catalisar as diversas reações de interesse industrial.

O mercado mundial de enzimas apresenta perspectiva no faturamento em 2014 de US\$ 4 bilhões em 2014 (Bon *et al.*, 2008). Um dos grandes desafios nas últimas décadas foi contornar a problemática do uso de enzimas na forma livre, que não são passíveis de reutilização por serem solúveis nos meios reacionais. Neste sentido, o uso de técnicas que possibilitem à estabilidade dos biocatalisadores, são desejáveis na produção de biocatalisadores para aplicações industriais.

O processo de imobilização de enzimas em suportes insolúveis ao meio reacional tem sido objeto de pesquisa há mais de 50 anos, por isso vários métodos e suportes foram avaliados e assim muitas aplicações sugeridas. O principal objetivo desta técnica é reutilizar a enzima, mantendo-a estável, especialmente se são difíceis de serem obtidas e apresentem custo elevado. Além disso, seu emprego conduz a produtos com melhor qualidade, reduzindo os custos de purificação dos produtos.

Dentre as técnicas de imobilização utilizadas, destaca-se a imobilização de enzimas em matrizes sol-gel, que consiste em reter a molécula alvo no interior da matriz ou em sua superfície, o que confere estabilidade mecânica e bioquímica superior as técnicas convencionais. No entanto, a técnica sol-gel apresenta algumas desvantagens decorrentes do processo de encapsulamento, uma destas, trata-se do acesso do substrato ao sítio ativo da enzima imobilizada, que pode ser atribuído a estrutura porosa, bem como a inativação ou

desnaturação da enzima. Uma alternativa para se contornar estas desvantagens durante o processo de imobilização é a utilização de aditivos.

O uso de aditivos no processo de imobilização sol-gel é relatado na literatura como agentes que influenciam positivamente o aumento da atividade e da estabilidade de enzimas imobilizadas. Essa influência está diretamente associada à proteção da enzima contra a inativação durante a etapa de encapsulamento, à retenção da camada de água ao redor do biocatalisador e aos efeitos dispersantes das moléculas da enzima. Dentre os principais aditivos utilizados na imobilização de enzimas, destacam-se a caseína, gelatina, albumina, álcool polivinílico, polietilenoglicol e, recentemente, o uso de líquidos iônicos, que são sais fundidos a baixas temperaturas, e que, apesar de serem documentados principalmente para substituir solventes orgânicos voláteis, apresentam enorme potencial em aplicações podendo como aditivos durante o processo de encapsulamento sol-gel de enzimas.

As lipases imobilizadas obtidas por técnicas tradicionais ou novas técnicas de imobilização são chamados de biocatalisadores verdes, e são capazes de catalisar de várias reações químicas, dentre elas, a transesterificação, esterificação e hidrólise de ácidos graxos. Estas reações são responsáveis pela síntese de vários produtos de interesse industrial, como por exemplo, o biodiesel e emulsificantes.

Nos últimos anos, a Pós-Graduação da Engenharia Química da Universidade Estadual de Maringá (UEM), a Escola de Engenharia Química de Lorena (EEL-USP), a Pós-Graduação da Engenharia de Processos da Universidade Tiradentes (UNIT) e com a colaboração da Universidade de Aveiro (UA) e da Universidade Federal da Bahia (UFBA), têm investigado o potencial catalítico de lipases imobilizadas em matrizes hidrofóbicas, tendo como objetivo desenvolver a tecnologia de obtenção de biocatalisadores ativos e estáveis para a aplicação em reações típicas das lipases.

A realização deste trabalho tem como objetivo aprimorar a tecnologia de preparação das lipases de *Bacillus* sp. ITP-001 e de *Burkholderia cepacia* imobilizadas com a presença de diferentes aditivos como o Aliquat 336, o polietilenoglicol, e o líquido iônico prótico. O uso de aditivos tem como propósito aumentar a eficiência catalítica dos biocatalisadores e minimizar os efeitos negativos ocorridos durante o processo de imobilização. O estudo consistiu em avaliar o efeito propiciado pela variação da concentração dos aditivos, como também, para o caso do líquido iônico prótico, a influência do aumento da cadeia alquílica do LIP, utilizado no processo de imobilização.

# Capítulo I

## 1. OBJETIVOS

### 1.1. Objetivo Geral

Imobilizar lipase em matrizes sol-gel empregando aditivos e avaliar o potencial nas reações de transesterificação e esterificação.

### 1.2. Objetivos Específicos

- Avaliar o efeito da concentração (0,5 a 3,0%, m/v) do Aliquat 336 no processo de imobilização sol-gel da lipase de *Bacillus* sp. ITP-001;
- Avaliar o efeito da cadeia alquílica e da concentração do líquido iônico prótico no processo de imobilização sol-gel da lipase de *Bacillus* sp. ITP-001 e *Burkholderia cepacia*;
- Avaliar o efeito da concentração (0,5 a 3,0%, m/v) do polietilenoglicol de massa molar 1500 no processo de imobilização sol-gel da lipase de *Burkholderia cepacia*;
- Estudar reações de hidrólise do azeite de oliva, esterificação do ácido láurico e transesterificação do óleo de soja para obtenção de ácidos graxos, emulsificantes e biodiesel;
- Caracterização físico-química dos biocatalisadores imobilizados pelas técnicas de: Adsorção e dessorção de nitrogênio; Microscopia Eletrônica de Varredura – MEV; Espectroscopia no Infravermelho com Transformada de Fourier – FTIR; Termogravimetria – TG e Calorimetria Exploratória de Varredura – DSC.

# Capítulo II

## 1. REVISÃO BIBLIOGRÁFICA

Neste capítulo serão brevemente enfocados os principais temas relacionados ao trabalho desenvolvido. Inicia-se pela conceituação de enzimas, imobilização de enzimas por meio da encapsulação empregando a técnica sol-gel, o uso de aditivos no processo de imobilização e potencial de produção de biodiesel e emulsificantes.

### 1.1. Enzimas

Enzimas são catalisadores biológicos que aumentam a velocidade das reações químicas que ocorrem nos organismos e células, sem sofrer alteração durante o processo. (Cabral *et al.*, 2003).

A reação catalítica ocorre no sítio ativo da enzima e, normalmente, em várias etapas. Desconsiderando a transferência de massa, a primeira etapa é a ligação do substrato à enzima, a qual ocorre devido às interações altamente específicas entre o substrato e as cadeias laterais dos aminoácidos que constituem o sítio ativo. Dois modelos importantes foram desenvolvidos para descrever o processo de ligação. O primeiro deles, o modelo chave-fechadura, no qual assume um alto grau de complementaridade entre a forma do substrato e a geometria do sítio de ligação da enzima. Este modelo, atualmente é de interesse histórico, uma vez que não leva em conta uma propriedade importante das proteínas, ou seja, sua flexibilidade conformacional. O segundo modelo leva em consideração a flexibilidade tridimensional da enzima. O modelo sugere um encaixe induzido, ou seja, o sítio ativo tem uma forma tridimensional diferente antes da ligação ao substrato, ocasionado pela ligação do substrato a enzima, o que resulta em um encaixe complementar ao substrato (Bon *et al.*, 2008).

Como qualquer outro catalisador, a enzima modifica a velocidade uma reação química. A velocidade desta reação corresponde à variação do número de moles de substrato, por unidade de massa ou volume do biocatalisador (Nelson *et al.*, 2006). Pode admitir-se que, em dadas condições, uma molécula de enzima possui uma atividade característica. A medição dessa atividade é um aspecto essencial na caracterização de um sistema de reação enzimática. As enzimas diminuem a energia de ativação durante uma reação, propiciando menor tempo de reação (Cabral *et al.*, 2003). A parte significativa da energia usada para aumentar a velocidade enzimática é derivada das interações (pontes de hidrogênio, interações iônicas e hidrofóbicas) entre o substrato e a enzima (Nelson *et al.*, 2006).

Atualmente, em geral, as enzimas são caracterizadas como catalisadores biológicos ou biocatalisadores, em sistemas *in vivo* e *in vitro*, de natureza protéica, altamente específica e com grande poder catalítico (Wiseman, 1995). Podem ser de origem microbiana, vegetal ou animal, obtidas por meio de processos fermentativos e trituração de tecidos vegetais e animais (Fuciños *et al.*, 2005; José *et al.*, 2004; Bacha *et al.*, 2005).

As enzimas microbianas podem ser extracelulares, dispersas no meio de cultivo, ou intracelulares, localizadas no interior celular, e, portanto obtidas por meio da ruptura da célula (Fellows, 1994).

Atualmente são conhecidas milhares de enzimas diferentes e não é viável o uso exclusivo de nomes triviais para a sua identificação. Em 1961, a Comissão para Enzimas (*Enzyme Commission* – EC) da União Internacional de Bioquímica (IUB), normatizou uma classificação e nomenclatura de enzimas e de co-enzimas (Cabral *et al.*, 2003). A EC classificou as enzimas por meio das suas propriedades catalíticas, podendo ser: oxidorreduções, transferases, hidrolases, liases, isomerases e ligases, como mostra a Tabela 1 (Nelson *et al.*, 2006). Essas classes são posteriormente divididas em subclasses, para especificar o tipo de reação e a natureza química dos reagentes.

**Tabela 1:** Classificação internacional de enzimas.

Nº	Classe	Tipo de reação catalisada
1	Oxidorreduções	Transferência de elétrons (íons hidretos ou átomos de H)
2	Transferases	Reações de transferência de grupos
3	Hidrolases	Reações de hidrólise (transferência de grupos funcionais da água)
4	Liasas	Adição de grupos a ligações duplas, ou formação de duplas ligações pela remoção de grupos
5	Isomerases	Transferência de grupos dentro de moléculas para produzir formas isoméricas
6	Ligases	Formação de ligações C–C, C–S, C–O e C–N pelas reações de consideração acopladas à clivagem do ATP

Fonte: Nelson *et al.*, 2006.

As enzimas, como catalisadores biológicos, apresentam diversas vantagens relativamente aos seus congêneres químicos, em particular a:

*Regiosseletividade*, devido à sua estrutura tridimensional complexa, as enzimas conseguem distinguir entre grupos funcionais quimicamente iguais, situados em diferentes regiões da mesma molécula-sustrato (Ghanem e Aboul-Enein, 2005);

*Enantiosseletividade*, as enzimas são quase todas formadas por L-amino ácidos, sendo, portanto, catalisadores quirais. Em conseqüência, qualquer tipo de quiralidade ou pró-quiralidade presente no substrato é reconhecida durante a formação do complexo enzima-substrato. Desta forma, na presença de uma enzima, substratos pró-quirais podem ser transformados em produtos opticamente ativos, e ambos os enantiômeros de um substrato racêmico podem interagir com velocidades diferentes, possibilitando a resolução racêmica (Guisan, 2006);

*Quimiosseletividade*, uma vez que o propósito de uma enzima é atuar em um único tipo de grupo funcional, outras funcionalizações sensíveis são preservadas. Permitindo uma grande importância na síntese orgânica, pois evita etapas de proteção e desproteção em reações que apresentam alta quimiosseletividade (Ghanem e Aboul-Enein 2005; Krishna, 2002; Schmid *et al.*, 2002).

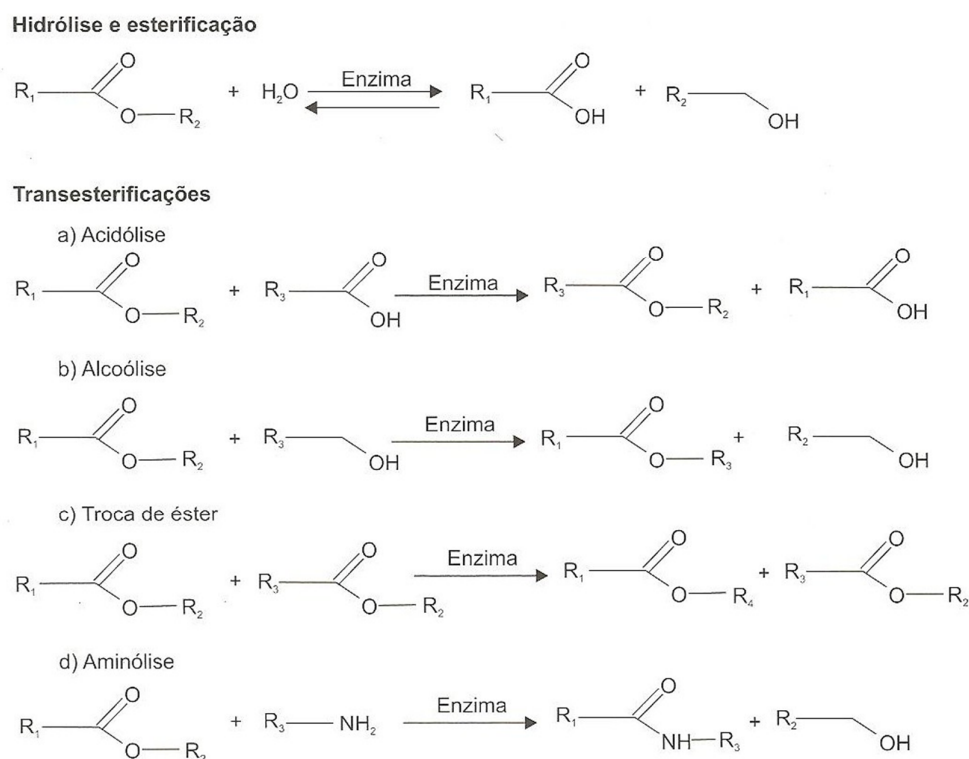
Os biocatalisadores são também eficientes do ponto de vista energético, dado operarem a temperatura e pressões moderadas, bem como em faixas moderadas de valores de pH (Cabral, *et al.*, 2003). Efetivamente a biocatálise é uma das técnicas químicas que reduzem e/ou eliminam o uso de solventes e reagentes ou geração de subprodutos tóxicos, que são nocivos à saúde humana ou ao ambiente, sendo, considerado como “catalisadores ecologicamente corretos”, atendendo aos princípios da química verde - *green chemistry* (Bommarius e Riebel, 2004).

O uso de enzimas como catalisadores de processos industriais está atualmente dividido em três grandes segmentos: enzimas técnicas, destinadas principalmente aos setores de produtos de limpeza, têxtil, de couros, de álcool como combustível e de papel e celulose; enzimas para alimentos e bebidas; e, enzimas para ração animal. As principais enzimas industriais são: protease, amilase, celulases, xilanases, fitase e as lipases (Bon *et al.*, 2008).

As lipases destacam-se cada vez mais no cenário da biotecnologia, impulsionado principalmente pelos avanços em biocatálise (síntese orgânica e quiral), produtos farmacêuticos, biodiesel e óleos e gorduras. A evolução das técnicas relacionada com a biotecnologia ajudará na redução dos custos de produção de enzimas, estimulando ainda mais o consumo das lipases (Guncheva e Zhiryakova, 2011).

### 1.1.1. Enzimas Lipolíticas

As lipases (glicerol éster hidrolases, E.C. 3.1.1.3) são enzimas que catalisam reações de hidrólise. Estas enzimas hidrolíticas são capazes de atuar em diversas reações como, a hidrólise de ligações éster-carboxilas liberando ácidos graxos e alcoóis orgânicos, além de catalisar reações de esterificação, interesterificação e transesterificação (Figura 1). Considerando que apresentam quimio, régio e enantiosseletividade para uma variedade de substratos (Singh *et al.*, 2010), versatilidade quanto às características do meio reacional (aquoso, ou restrito de água, orgânico ou supercrítico) e atividade elevada em meio reacional livre de solventes, as caracterizam como “enzima promiscua” (Guisan, 2006; Hult *et al.*, 2010).



**Figura 1:** Reações típicas catalisadas por lipases. Fonte: Bon *et al.*, 2008.

A estrutura tridimensional das enzimas e as propriedades de ativação passaram a ser fatores determinantes para a caracterização das lipases. As estruturas das lipases, de uma forma geral foram caracterizadas por um padrão conformacional comum, denominado  $\alpha/\beta$  hidrolase, onde está situada a tríade catalítica (Ser-His-Asp). Elas possuem uma estrutura comum composta de uma sequência de  $\alpha$ -hélice e  $\beta$ -pregueada.

As lipases são comumente encontradas na natureza, podendo ser obtidas a partir de fontes animais vegetais e microbianas. Do ponto de vista industrial as lipases microbianas são consideradas de maior importância, porque além de apresentarem procedimentos mais simples de obtenção, a partir do caldo fermentativo, são geralmente mais estáveis e com propriedades mais diversificadas (Gandra *et al.*, 2008 e Barbosa *et al.*, 2011).

Estas enzimas de diferentes origens encontram-se disponíveis hoje em dia no mercado (Tabela 2) e desde 1979 são empregadas em larga escala como catalisadores em diferentes setores industriais como pode ser visto na Tabela 3 (Bon *et al.*, 2008).

**Tabela 2:** Exemplos de lipases comerciais disponíveis no mercado.

<b>Lipase</b>	<b>Aplicação principal</b>	<b>Empresas que comercializam</b>
<i>Candida rugosa</i>	Síntese orgânica	Amano, Biocatalysts, Boehringer Mannheim, Fluka, Genzyme, Sigma
<i>Candida antártica A/B</i>	Síntese orgânica	Boehringer Mannheim, Novozymes
<i>Thermomyces lanuginosus</i>	Aditivo para detergentes	Boehringer Mannheim, Novozymes
<i>Rhizomucor miehei</i>	Processamento de alimentos	Amano, Biocatalysts, Novozymes
<i>Burkholderia cepacia</i>	Síntese orgânica	Amano, Boehringer Mannheim, Fluka
<i>Pseudomonas alcaligenes</i>	Aditivo para detergentes	Genencor
<i>Pseudomonas mendocina</i>	Aditivo para detergentes	Genencor
<i>Chromobacterium viscosum</i>	Síntese orgânica	Biocatalysts, Asahi

Fonte: Bon *et al.*, 2008.

**Tabela 3:** Exemplos de lipases em processos industriais.

<b>Processo</b>	<b>Início da operação</b>	<b>Empresa</b>
Interesterificação de gorduras	1979, 1983	Fugi Oil, Unilever
Hidrólise de ésteres	1988	Sumitomo
Transesterificação	1990	Unilever
Acilação	1996	BASF

Fonte: Bon *et al.*, 2008.

As lipases de uma forma geral são caracterizadas por um padrão conformacional comum, formada por resíduos de aminoácidos. Em particular, as lipases de *Burkholderia*



*cepacia*, denominadas anteriormente *Pseudomonas cepacia*, é uma das lipases mais populares usadas em síntese orgânica sendo bastante utilizadas nas reações de hidrólise e transesterificação além de apresentar capacidade em catalisar reações em meios não aquosos (Noureddini *et al.*, 2005). É uma proteína com 320 aminoácidos e 33 kDa de massa molar. O sítio ativo é coberto por uma tampa que sofre rearranjos conformacionais e muda seu estado entre a enzima inativa (tampa fechada) e ativo (tampa aberta). Quando ocorre a ligação do substrato na superfície da enzima, esta tampa move-se, alterando a forma fechada da enzima para a forma aberta, expondo o sítio ativo, agora acessível ao substrato e ao mesmo tempo, expondo uma larga superfície hidrofóbica que facilita a ligação da lipase à interface (Bommarius e Riebel, 2004; Cyler e Schrag, 1999). Possui alta atividade em uma ampla faixa de pH (3,5-11), no entanto, apresenta pH ideal entre 7,0-8,0 (Schrg *et al.*, 1997; Kim *et al.*, 1997). Outro exemplo de lipase são as obtidas a partir de *Bacillus*. Estas também apresentam significativa importância na biocatálise com relação ao potencial de síntese reacional, e demonstra especificidade, estabilidade, tolerância a inúmeros solventes, sais e detergentes. Podem ser aplicadas em diversos ramos da indústria, no entanto, seu uso em transformações sintéticas (esterificação, transesterificação, acidólise) é amplamente inexplorado (Nawani *et al.*, 2006; Ghori *et al.*, 2011; Guncheva e Zhiryakova, 2011).

Um inconveniente ao uso das enzimas nos setores industriais está atrelado ao fato de que, fatores químicos, físicos ou biológicos podem desnaturar as enzimas, o que impossibilita sua reutilização. Uma estratégia para superar este inconveniente é imobilizar as enzimas em suportes, possibilitando estabilidade catalítica, reutilização e fácil recuperação.

## **1.2. Imobilização de Enzimas**

Conforme anteriormente abordado, as enzimas apresentam excelentes propriedades como atividade, seletividade e especificidade. Apesar do elevado potencial de aplicação das enzimas, as pressões comerciais imprimem a necessidade de melhorias e adequações das condições de reação, de modo que as enzimas cumpram suas funções biológicas em tempos menores de conversão e com eficácia.

Deste modo, nos últimos 50 anos, a tecnologia de imobilização desenvolveu-se rapidamente e o seu uso tem se tornado uma questão de concepção racional. É possível relacionar o desenvolvimento de biocatalisadores imobilizados em três etapas (Tabela 4). No início do século 19, micro-organismos imobilizados foram empregados industrialmente em uma base empírica. O principal fato foi no desenvolvimento de filtro biológico e na percolação no processo de clarificação de águas residuais (Guisan, 2006).

A história moderna da imobilização de enzimas remonta dos anos 1940, mas a maior parte do trabalho inicial foi ignorada por bioquímicos, sendo primeiramente publicado em revistas de outras áreas. A base da tecnologia atual foi desenvolvida na década de 1960 e houve um aumento expressivo de publicações. Na segunda etapa, apenas as enzimas simples eram imobilizadas, e a partir da década de 70, sistemas mais complexos incluindo reações de duas enzimas imobilizadas e envolvendo a regeneração do co-fator foram desenvolvidos (Brodelius *et al.*, 1987).

**Tabela 4:** Etapas no desenvolvimento de enzimas imobilizadas.

<b>Etapa</b>	<b>Data</b>	<b>Uso</b>
Primeira	1815	Uso empírico de processos tais como o ácido acético e tratamento de águas residuais.
Segunda	1960	Única imobilização de enzimas: a produção de L-aminoácidos, isomerização da glicose.
Terceira	1985-1995	Imobilização de varias enzimas, incluindo o co-fator de regeneração e de imobilização das células. Produção de L-aminoácidos a partir de ceto-ácidos em reatores de membrana.

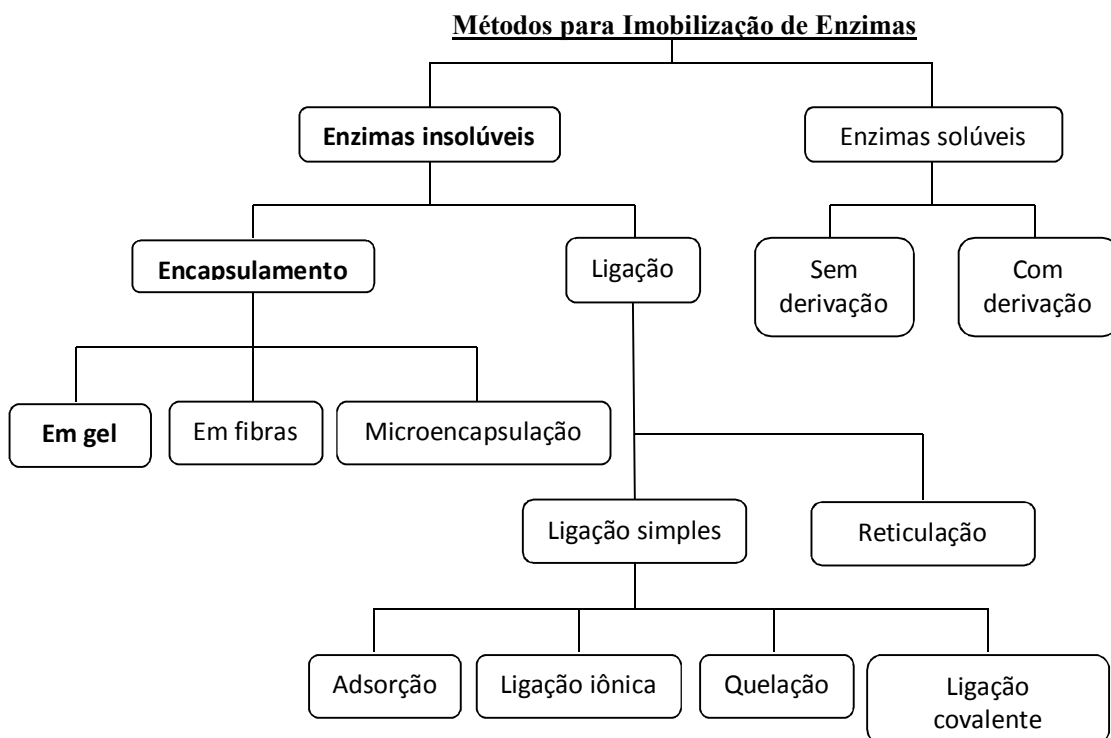
**Fonte:** Guisan, 2006.

O termo enzima imobilizada inclui: (I) a modificação das enzimas de forma a torná-las insolúveis em água; (II) a utilização de enzimas na forma solúvel em reatores equipados com membranas de ultrafiltração, que permite o escoamento dos produtos da reação, porém retendo a enzima no interior do reator; e (III) a restrição da mobilidade da enzima pela ligação a outra molécula que torna o sistema insolúvel no meio reacional (Kennedy e Cabral, 1983; Malcata *et al.*, 1990). Neste sentido, o aumento da aplicação de enzimas imobilizadas, fisicamente confinadas ou localizadas na superfície de um material que é utilizado como suporte (Desimone *et al.*, 2008) mantendo suas propriedades catalíticas e incluindo a possibilidade de ser reutilizada, implica na aceitação comercial destes catalisadores biológicos (Bon *et al.*, 2008).

O processo de imobilização pode inibir ou aumentar a atividade e estabilidade da enzima, porém não existe uma regra que prediga a manutenção destes parâmetros após o processo de imobilização (Gandhi *et al.*, 1997), que pode ocorrer por meio da adsorção ou ligação da enzima em um material insolúvel, pelo uso de um reagente multifuncional por ligações cruzadas, confinamento em matrizes formadas por géis poliméricos ou encapsulação por meio de uma membrana polimérica (Zanin e Moraes, 2004).

A Figura 2 apresenta a classificação dos métodos de imobilização proposta por Kennedy e Cabral (1987) e esquematizada por Bon *et al.* (2008), a qual procura combinar a natureza da

interação responsável pela imobilização e o tipo de suporte utilizado: (i) a modificação da enzima, por meio de técnicas apropriadas, para torná-las imóveis no meio reacional (ligação em suportes) e ligação cruzada intermolecular ou reticulação (crosslinking); (ii) enzimas solúveis, utilizadas em reatores equipados com membranas semipermeáveis de ultrafiltração e fibras ocas que retêm a enzima no interior do reator (enzimas solúveis sem derivatização); (iii) enzimas que tiveram sua mobilidade restringida pela ligação com outra macromolécula, porém permanecendo o complexo solúvel em água (enzimas solúveis com derivatização); (iv) enzimas que se encontram no interior de uma matriz ou microcápsulas (encapsulação).



**Figura 2:** Classificação dos métodos utilizados para imobilização de enzimas. Fonte: Bon *et al.*, 2008.

Porém, alguns fatores devem ser apontados, não como uma desvantagem do processo, mas sim como pontos a serem evitados ou prevenidos. Dentre estes fatores podem ser citados (Rosevear *et al.*, 1987; Zanin e Moraes, 2004):

*Perda da atividade durante o processo de imobilização:* a imobilização da enzima envolve o manuseio do biocatalisador (enzima-suporte), o que adiciona custos ao processo e invariavelmente resulta em inativação parcial da enzima. Como os métodos de imobilização, de modo geral, envolvem interações fracas (forças de van der Waals), ou fortes (ligação covalente), entre a estrutura da proteína enzimática e o suporte, invariavelmente há uma

alteração da estrutura tridimensional da proteína, resultando em menor atividade. Além disso, também podem ocorrer alterações de orientação e acesso do substrato ao sítio ativo, reduzindo a atividade da enzima, ou ainda levando à redução aparente da especificidade ao substrato (Bon *et al.*, 2008).

*Efeitos difusionais (transferência de massa):* a enzima estando com sua mobilidade restringida pelo fato de estar ligada a um suporte, perde parte de sua acessibilidade ao substrato, o que resulta novamente numa aparente redução da atividade, neste caso provocada por restrições difusionais, ou seja, limitações de acesso do substrato ao sítio ativo devido à presença da matriz sólida. Também pode haver um acúmulo de produto próximo ao sítio ativo, o que pode afetar a cinética da reação, pela redução da velocidade de reação, ou ainda provocando alteração do pH no microambiente da enzima (Fogler *et al.*, 2002).

*Características físicas do biocatalisador e do fluido:* normalmente as enzimas imobilizadas devem ser utilizadas quando o substrato é solúvel. Quando as enzimas estão retidas no interior de matrizes porosas, os poros devem facilitar o livre acesso do substrato ao mesmo tempo em que retêm a molécula de enzima no seu interior (Fogler *et al.*, 2002).

*Estabilidade do biocatalisador:* o custo da imobilização deve ser compensado pelo tempo de meia vida do biocatalisador. O suporte deve manter suas propriedades físicas (resistência mecânica, não ser susceptível ao ataque por reagentes químicos) durante o tempo de meia-vida estimado. Normalmente os substratos utilizados nas reações enzimáticas contêm substâncias em suspensão, lipídeos, que podem se adsorver ao suporte e bloquear os poros, diminuindo a acessibilidade do substrato à enzima, promovendo uma redução aparente do tempo de meia-vida da enzima imobilizada (Fogler *et al.*, 2002).

Além disso, as técnicas de imobilização de enzima necessitam de suportes para a ligação ou encapsulamento da enzima. Portanto, para a escolha do suporte, que podem ser de origem orgânico (naturais e sintéticos) ou inorgânico (minerais e fabricados), vários parâmetros são importantes e devem ser considerados como, por exemplo, resistência mecânica, estabilidade química e física, de caráter hidrofóbico/hidrofílico, capacidade de carregamento da enzima e custo, entre outros (Carvalho *et al.*, 2006).

Portanto, as enzimas podem ser imobilizadas em diferentes suportes para diversos processos. Em geral, pode-se supor que a simplicidade e o custo da imobilização, não sejam relevantes quando se trabalha em escala de laboratório, mas seria crítico para aplicações industriais (Guisan, 2006), fato este, evidenciado por Pandey *et al.* (1999), especialmente na indústria farmacêutica, de detergentes, couros e panificação, entre outras.

### ***1.2.1. Encapsulamento Sol-Gel***

Diferentes técnicas de imobilização são utilizadas para conservar a natureza catalítica do biocatalisador, dentre elas, destaca-se o método de encapsulação sol-gel baseado em métodos químicos e físicos, que consiste em proteger às biomoléculas em um volume definido e criar um único compartimento, representando um microambiente separado do ambiente externo (Kato *et al.*, 2011). Este processo mostra-se versátil por imobilizar uma variedade de biomoléculas, incluindo enzimas, anticorpos, micro-organismos, células vegetais e animais ter sido imobilizada pela técnica sol-gel (Sglavo *et al.*, 1999; Soares *et al.*, 2006; Pinheiro *et al.*, 2008; Cohen *et al.*, 2010; Meunier *et al.*, 2010; Zarcuła *et al.*, 2010). O encapsulamento sol-gel atraiu uma considerável atenção em áreas como biotecnologia, medicina, farmacêutica, catálise, nutrição e ecologia (Kato *et al.*, 2011).

Atualmente, dispõe-se de um grande número de matrizes, englobando materiais orgânicos e inorgânicos, naturais ou sintéticos (Zanin *et al.*, 2004).

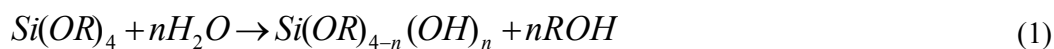
Apesar da existência de suportes baratos, como polímeros e minerais naturais, a utilização de enzimas imobilizadas em processos tem exigido a elaboração de matrizes específicas para essa finalidade, que resultem em preparados imobilizados com elevadas atividades e características hidrodinâmicas adequadas ao uso em diversos reatores (Chiou *et al.*, 2004), possibilitando ainda, o emprego dos materiais sol-gel como suporte para a imobilização de enzimas tanto por encapsulação (Soares *et al.*, 2004; Pinheiro *et al.*, 2008.) como por ligação covalente (Reetz *et al.*, 1996; Bruno *et al.*, 2004).

As mais recentes tecnologias requerem materiais com combinação de propriedades que não são encontradas nos materiais convencionais. Tais materiais como, os híbridos orgânico-inorgânico são preparados pela combinação de componentes orgânicos e inorgânicos e constituem uma alternativa para a produção de novos materiais multifuncionais, com uma larga faixa de aplicações (Desimone *et al.*, 2008).

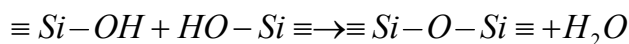
Independentemente da estratégia utilizada para preparar uma matriz híbrida, o processo sol-gel é, indiscutivelmente, o mais empregado. O processo envolve diversas variáveis, como tempo e temperatura da reação, natureza do catalisador, concentração de reagentes, entre outros. Estas variáveis determinam as características finais dos materiais, incluindo a porcentagem de hidrólise e condensação de grupos reativos, densidade de reticulação e homogeneidade do produto. Além disso, aditivos químicos podem ser usados para melhorar o processo e obter materiais com melhores propriedades, o que possibilita modificações nas propriedades mecânicas, controle de porosidade e ajuste no balanço hidrofílico e hidrofóbico (Jose *et al.*, 2005).

A produção de matrizes sol-gel é iniciada pela hidrólise parcial dos precursores (silicatos de alquilo ou alcóxidos), resultando em um oligômero que pode ser transesterificado com glicerol. O oligômero presente é totalmente hidrolisado, formando-se uma dispersão coloidal (sol), sendo adicionada uma solução contendo o biocatalisador, dando início ao processo de policondensação. Deste resulta a formação de uma fase distinta (o hidrogel ou xerogel) contendo o biocatalisador. A matriz é então envelhecida, resultando, no final do processo, nano ou micromatrizes (Cabral *et al.*, 2003).

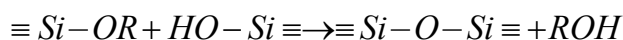
A reação química envolvida no processo sol-gel convencional pode ser dividida em duas etapas básicas. A etapa (1) corresponde à hidrólise do grupo alcóxido com a formação de grupos reativos do tipo silanol.



A etapa (2) a condensação do grupo silanol, a qual leva inicialmente à formação do sol e, eventualmente, ao gel (Kubota *et al.*, 2001)



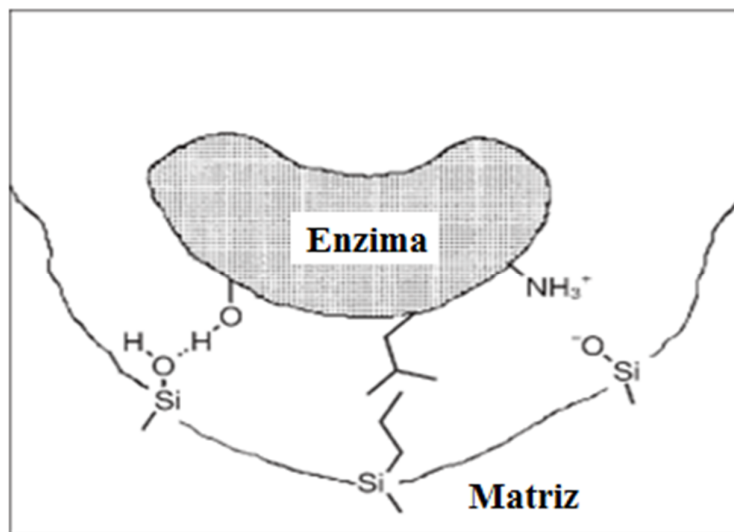
ou, (2)



As reações de hidrólise e condensação ocorrem via substituição nucleofílica biomolecular no átomo de silício (SN<sub>2</sub>-Si). Como os alcóxidos de silício possuem uma baixa reatividade (relativa aos alcóxidos metálicos), catalisadores ácidos (H<sub>3</sub>O<sup>+</sup>), básicos (OH<sup>-</sup>) e/ou nucleofílicos (F<sup>-</sup>, N-metilimidazol, hexametilfosforamida - HMPA) são adicionados ao processo para promover um aumento na velocidade das reações de hidrólise e condensação.

No processo sol-gel a hidrofobicidade reforçada na matriz de óxido de silício se correlaciona com o aumento da atividade da enzima. Maior estabilidade térmica e atividade enzimática parecem resultar de interações multipontos por meio de ligações de hidrogênio, bem como interações iônicas e hidrofóbicas (van der Waals), que pode ser esquematizada na Figura 3. As interações hidrofóbicas podem resultar em um tipo de ativação interfacial, a lipase pode ter sua forma conformacional presa na matriz com a "tampa-aberta", ou seja, na

sua forma ativa (Guisan, 2006). Conferindo assim, uma estabilidade mecânica e química superiores aos polímeros convencionais, permitindo uma retenção eficiente do biocatalisador, além de uma estabilidade operacional e de armazenamento elevadas (Cabral *et al.*, 2003).



**Figura 3:** Visão esquemática das interações não covalentes entre a matriz de gel e lipase.  
Fonte: Guisan 2006.

Recentemente foi testada com sucesso uma matriz híbrida constituída de polissiloxano-álcool polivinílico (POS-PVA) para imobilização de diferentes fontes de lipase: *Mucor miehei* (Bruno *et al.*, 2004; Bruno *et al.*, 2005), pancreática (Paula *et al.*, 2005; Paula *et al.*, 2008) e *Candida antarctica*. Essa matriz combina as características físico-químicas de materiais inorgânicos e orgânicos, permitindo a manipulação da hidrofobicidade, condutividade elétrica, carga iônica, porosidade e propriedades mecânicas em geral (Bruno *et al.*, 2004), bem como elevada atividade e estabilidade.

Na imobilização da  $\alpha$ -amilase pelo processo sol-gel, a fase inorgânica foi obtida com o tetraetoxissilano e 3-trimetoxissilopropilmetacrilato, verificando-se que a enzima imobilizada apresentou estabilidade por 30 dias, numa temperatura de 25°C em água (Arakaki e Airoidi, 1999). As lipases de *Aspergillus niger* e *Rhizopus arrhizus* também foram imobilizadas pelo processo sol-gel e apresentaram uma excelente retenção da atividade enzimática; 5,2 e 0,5  $\mu\text{mol/h.mg}$  para a enzima imobilizada e 0,33 e 0,17  $\mu\text{mol/h.mg}$  para a enzima livre, respectivamente (Reetz *et al.*, 1996).

### **1.3. Aditivos no Processo de Imobilização**

Um significativo melhoramento na atividade e estabilidade de enzimas imobilizadas, quando o procedimento de imobilização é realizado em presença de aditivos é descrito na literatura (Gonçalves *et al.*, 1999; Reetz *et al.*, 1996; Soares *et al.*, 2006; Hara *et al.*, 2010).

O estudo destes aditivos está associado com sua co-imobilização em processo de encapsulamento, sendo alguns de seus efeitos atribuídos a: (i) proteção da inativação da enzima durante a etapa de imobilização; (ii) retenção da camada de água ao redor do biocatalisador e (iii) efeitos dispersantes das moléculas da enzima e facilitadores de transporte de massa quando aditivos são usados como matrizes de imobilização, podendo ele estar presente ou ausente no meio de dispersão (Soares *et al.*, 2005). Diferentes tipos de aditivos são utilizados nas técnicas de imobilização, tais como: albumina, ciclodiatrina, polietilenoglicol (PEG), líquido iônico, entre outros. Nas recentes técnicas de imobilização, enfatiza-se o uso de diferentes aditivos, entre eles, o Aliquat 336, o polietilenoglicol e os líquidos iônicos serão discutidos nos próximos tópicos.

#### **1.3.1. Polietilenoglicol - PEG**

O uso de polietilenoglicol como aditivo na imobilização enzimática propicia melhor distribuição da lipase na superfície do suporte, permitindo um melhor contato entre a interface água/óleo, favorecendo uma condição necessária para a expressão da atividade hidrolítica da lipase imobilizada (Soares *et al.*, 1999). Segundo Rocha *et al.*, (1998) e Villeneuve *et al.*, (2000) no caso específico das lipases, o uso de aditivos macromoleculares mostram efeitos estabilizantes significativos na atividade enzimática por meio do revestimento da interface, impedindo desta forma, uma mudança de sua estrutura proteica. Rocha *et al.*, (1998) propôs ainda, que a presença de PEG durante o processo de imobilização de lipase, provavelmente, afeta o nível de hidratação da enzima, modificando a hidroflicidade do microambiente.

Nesta abordagem, Villeneuve *et al.* (2000) sugeriu que as lipases são covalentemente ligadas a um material insolúvel em água, por meio os grupos funcionais da proteína (grupamento amina) e os grupos sobre o material ativado. Experimentalmente a utilização do PEG com menor massa molar aumenta significativamente o rendimento na recuperação da atividade enzimática da lipase de *Candida rugosa*, atingindo valores de 60% (Soares *et al.*, 2006). A influencia positiva na estrutura porosa dos imobilizados também foi associado ao uso destas macromoléculas (Soares *et al.*, 2004).



### **1.3.2. Aliquat 336**

O Aliquat 336 é um sal quaternário de amônio sintetizado a partir da metilação de aminas, capaz de formar sais solúveis em óleo de espécies aniônicas em pH neutro ou ligeiramente alcalino. Seu uso é bastante aplicado em recuperar ou purificar complexos iônicos como o Zinco, Cádmio, Urânio, Ferro, como também ácidos. No tratamento de resíduos, é usado com sucesso para recuperar ácidos/sais ou remover certos metais pesados de águas residuais. Também é utilizado para controlar a formação de espuma durante o tratamento de águas residuais contendo tensoativos aniônicos.

O Aliquat, recentemente tem sido descrito como agente manipulador de permeabilidade atuando como um plastificante em membranas poliméricas, sobretudo carboxilado PVC e poliuretano, formando uma camada externa de proteção aos biosensores, desta forma podendo auxiliar na formação porosa da matriz hidrofóbica sol-gel (Pauliukaite *et al.*, 2007).

### **1.3.3. Líquido Iônico - LI**

Os líquidos iônicos (LIs) são compostos que apresentam pressão de vapor baixa, são pouco voláteis a temperatura ambiente e estão sendo utilizados como solventes alternativos em sínteses orgânicas em substituição aos solventes orgânicos inflamáveis, reduzindo a possibilidade e os riscos de acidentes (Silva, 2005). Desta forma, na última década tem crescido o interesse por esta classe de compostos, dado que entraram na elite de compostos da chamada Química Verde (Jiang *et al.*, 2009). Do ponto de vista prático, a versatilidade estrutural dos LIs é de suma importância, podendo sintetizar um número ilimitado de compostos, visto que suas propriedades são determinadas pela estrutura e interação entre os íons. É a grande assimetria de tamanho entre o cátion e ânion e o número de combinações que lhes conferem propriedades únicas que podem ser sintetizados especificamente para um determinado fim. Espera-se, que num futuro próximo, novas classes de líquidos iônicos com propriedades eletroquímicas ainda melhores que as dos sistemas atuais estarão disponíveis (Zarcula *et al.*, 2010).

Os LIs apresentam uma série de vantagens, comparado aos solventes orgânicos convencionais: densidade superior à da água para a maioria dos LIs variando entre 1 e 1,6 g/cm<sup>3</sup> (Consorti *et al.*, 2001; Gardas *et al.*, 2007; Marsh *et al.*, 2004); baixo ponto de fusão (<30 °C) permanecendo no estado líquido a temperaturas superiores a 300°C, sob uma atmosfera inerte; pressão de vapor desprezível (Earle *et al.*, 2006), já que os íons interagem por forças de Coulomb extremamente fortes (Abraham *et al.*, 2003) o que possibilita a não volatilização de compostos orgânicos durante a manipulação e/ou operações industriais; além

disto, possui acidez de Brønsted-Lewis variável podendo ser ajustada variando o ânion ou variando as cadeias alquílicas do cátion; boa condutividade elétrica, grande mobilidade iônica, larga janela de estabilidade eletroquímica, e excelente estabilidade térmica (Álvarez *et al.*, 2010a).

Nos solventes orgânicos convencionais existem interações por pontes de hidrogênio, dipolo-dipolo e de van der Waals; os líquidos iônicos possuem além destas interações, interações iônicas (atrações ou repulsões eletrostáticas mútuas de partículas carregadas), que os tornam miscíveis numa larga gama de concentrações com substâncias polares (Gardas *et al.*, 2007). Outra propriedade interessante dos LIs é a separação de misturas de reações, como catalisadores e produtos obtidos, o que não ocorre facilmente em presença de solventes orgânicos (Consorti *et al.*, 2001). Simultaneamente, as cadeias alquílicas dos cátions ou dos ânions determinam a sua solubilidade em fluidos menos polares, atribuindo um caráter mais hidrofóbico ou hidrofílico ao líquido iônico. Estes fatores induzem a aplicação dos LIs como aditivos em processos de encapsulamento sol-gel de enzimas (Vila-Real *et al.*, 2011).

A utilização de LIs em biocatálise tem sido recentemente relatada, os estudos baseiam-se no fato de que os biocatalisadores imobilizados apresentarem perdas na sua atividade e estabilidade enzimática (Lee *et al.*, 2007; Zarcuła *et al.*, 2010; Vila-Real *et al.*, 2010). O uso do líquido iônico como aditivo em métodos de imobilização de enzimas, em especial suportes sol-gel, tem sido sugerido, como o agente capaz de estabilizar enzimas, protegendo a camada de hidratação ao redor da enzima e/ou pela alteração conformacional ocasionando ativação permanente da enzima (Hara *et al.*, 2010).

Lee *et al.* (2007) relatou que no processo de imobilização de *Candida rugosa*, utilizando a técnica sol-gel, as enzimas imobilizadas apresentaram alta estabilidade, aumento na atividade enzimática cerca de 10 vezes maior que na lipase em sua forma livre e 16 vezes maior em reações de esterificação comparado aos imobilizados ausente de LI.

Zhou (2005) observou, que particularmente, os LIs formados a partir do ânion tetrafluoroborato [BF<sub>4</sub>] têm sido relatados como bons na formação da sílica mesoporosa. Propôs ainda, que a formação de pontes de hidrogênio entre [BF<sub>4</sub>] - grupo silanol, desempenha uma função crucial para a formação da estrutura de mesoporos.

A influência positiva do uso do LI também foi verificada por Zarcuła *et al.* (2010) utilizando LIs de base imidazólio no processo de imobilização da lipase de *Pseudomonas fluorescens* em matrizes híbridas sol-gel como aditivo, os resultados obtidos com os biocatalisadores imobilizados apresentaram rendimento de recuperação total de atividade geralmente superior a 100%.

Lee *et al.* (2007) verificou maior estabilidade de lipase imobilizada sol-gel, utilizando LI de caráter hidrofóbico como o 1-octil-3-metilimidazólio bis[(trifluorometil)sulfonil]amida ( $[C_8mim][Tf_2N]$ ), alcançando 80% de sua atividade inicial após 5 dias de incubação em comparação com lipase imobilizada sem presença de LI que foi totalmente desativada. Além disto, a presença de líquidos iônicos que possuem caráter hidrofóbico dentro das matrizes sol-gel conduz a uma formação da estrutura mesoporosa, favorecendo a reação enzimática e reduzindo as limitações de difusão. Há, no entanto, líquidos iônicos com natureza hidrofílica que podem inativar as lipases em meio não aquoso por retirar a água que é essencial para manter ativa a conformação da enzima (Vila-Real *et al.*, 2011). A constatação de que muitas enzimas em seus ambientes naturais (*in vivo*) atuam em ambientes ricos em lipídeos hidrófobos, mostrou que estes meios predominantemente não-aquosos são igualmente apropriados para a expressão da atividade biocatalítica (Aires-Barros, 2002).

Os estudos realizados relatam a utilização de LIs constituídos principalmente dos cátions orgânicos a base do imidazólio, piridino, tetraalquilamônio e tetraalquilfosfônio, são os chamados líquidos iônicos apróticos (Álvarez *et al.*, 2010). O uso destes compostos ainda apresenta elevados custos de síntese, dificultando sua aplicação industrial (Zarcula *et al.*, 2010). Entretanto, Álvarez *et al.*, (2010b) relata que os LIs obtidos a partir de aminas, ácidos orgânicos e inorgânicos, os chamados líquidos iônicos próticos (LIPs), apresentam baixo custo, simplicidade de síntese, e diferentes aplicações o que favorece o interesse industrial.

Kenedy *et al.*, (2011) e Álvarez *et al.*, (2008), descrevem os LIPs como uma sub-classe dos LIs e que ao contrário dos líquidos iônicos apróticos, a ionicidade destes, depende da posição de equilíbrio da neutralização ácido-base de Bronsted (Graveas e Drummond, 2008).

Aplicações em potencial foram identificadas para o uso de LIP na biocatálise, como a dissolução de ligantes hidrofóbicas (por exemplo, ferroceno) para incorporá-las em um cristal de proteína. Foi atribuída ao uso do LIP em proteínas: aumento na solubilidade; melhor distribuição; agente de precipitação; e como aditivo (Graveas e Drummond, 2008).

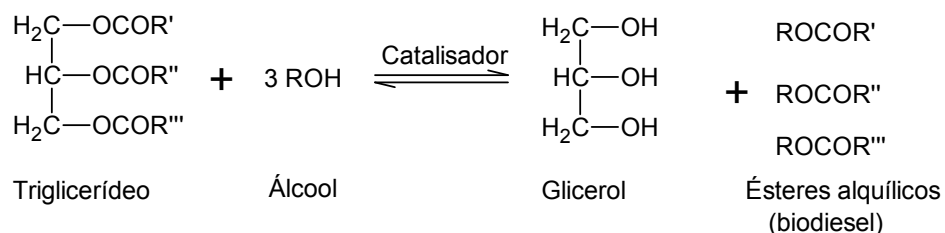
No entanto, as enzimas imobilizadas na presença de LIs mostram dificuldade em encontrar uma regra simples para prever o comportamento do biocatalisador. Estudos complementares sobre a característica estrutural dos imobilizados precisam ser realizados a fim de avaliar a influência destes aditivos na atividade enzimática, na enantiosseletividade e na estabilidade das matrizes imobilizadas (Diego *et al.*, 2009). O mesmo se faz para o uso dos LIP, que neste caso, não há relatos na literatura de seu uso envolvido na imobilização de biomoléculas.

O interesse industrial de biocatalisadores imobilizados é cada vez mais alto, visto os avanços das técnicas de imobilização que propiciam biocatalisadores mais eficientes e possíveis de catalisar diversas reações como a transesterificação e esterificação.

#### 1.4. Reação de Transesterificação

A reação de transesterificação é o processo químico mais viável para obtenção de biodiesel em todo o mundo. Consiste em reagir um triglicerídeo com um álcool inferior, na presença de um catalisador, resultando na produção de uma mistura de ésteres alquílicos de ácidos graxos (denominado de biodiesel) e glicerol. Esta transformação ocorre em três etapas sequenciais: inicialmente, as moléculas de triglicerídeos são convertidas em diglicerídeos, depois em monoglicerídeos e, finalmente, obtém-se um éster metílico ou etílico (biodiesel) de ácido graxo e glicerina como subproduto, como mostra a Figura 4 (Zhang *et al.*, 2003).

Diversos álcoois podem ser empregados, e são considerados como o agente de transesterificação, deve conter até no máximo oito átomos de carbono em sua cadeia. No entanto, devido às propriedades conferidas ao produto, os álcoois metílico e etílico, são os mais empregados no processo (Darnoko *et al.*, 2000). Observam-se, em decorrência da estequiometria da reação, que teoricamente a partir de um mol de triglicerídeo são obtidos três moles de ésteres.



**Figura 4:** Esquema da reação geral de transesterificação do triglicerídeo. Fonte: Geris *et al.*, 2007.

A alcoólise de óleos vegetais ou de gordura animal pode ser conduzida por uma variedade de rotas tecnológicas em que diferentes tipos de catalisadores podem ser empregados, como bases inorgânicas (hidróxidos de sódio e potássio), ácidos minerais (ácido sulfúrico), resinas de troca iônica (resinas catiônicas fortemente ácidas), argilominerais ativados, hidróxidos duplos lamelares e enzimas lipolíticas (lipases) (Vargas *et al.*, 1998; Ramos *et al.*, 2003).

A utilização da catálise homogênea em meio alcalino é a rota tecnológica predominante no meio industrial para a produção do biodiesel. Portanto, pode-se perfeitamente afirmar que

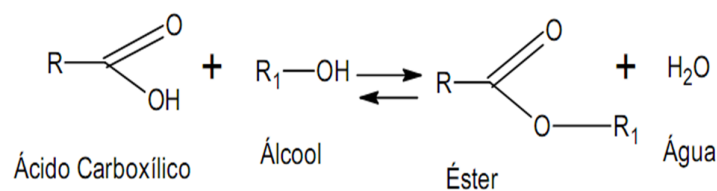
esta rota tecnológica, por sua maior rapidez e simplicidade, ainda prevalece como a opção mais imediata e economicamente viável para a transesterificação de óleos vegetais e gorduras animais (Ma *et al.*, 1999; Zagonel e Ramos, 2001). No entanto, o uso de biocatalisadores em aplicações industriais é objeto de estudo de diversas pesquisas. A principal razão do uso da via química clássica para a produção de biodiesel é a alta produtividade. Entretanto, os catalisadores químicos mostram várias desvantagens, dentre os quais podem-se citar a formação de uma grande variedade de subprodutos e efluentes tóxicos. Portanto, a utilização de células ou enzimas é uma das estratégias para substituição dos catalisadores químicos, devido à sua grande seletividade para o substrato, minimizando a produção de produtos tóxicos, além disso, biocatalisadores são biodegradáveis e agem sob condições ambientalmente amigáveis, requerendo menos energia para realizar a catalise, atingindo normalmente uma faixa de temperatura entre 20 e 60°C, para a síntese do biodiesel (Antczak *et al.*, 2009; Constesini *et al.*, 2010).

Lipases são caracterizadas por serem aplicáveis à produção de biodiesel e, portanto, a maioria das lipases utilizadas para essa finalidade apresentam tanto alta enantioseletividade quanto regioseletividade ao substrato. São exemplos as lipases *Pseudomonas fluorescens*, *Burkholderia cepacia*, *Candida rugosa*, *Candida antarctica* e *Cylindracea Candida* (Antczak, *et al.*, 2009).

Recentemente Dizge *et al.* (2009) verificou com sucesso a produção de biodiesel utilizando óleo de girassol, soja e óleo residual de cozinha utilizando a lipase *T. lanuginosus* imobilizada polímeros microporosos com rendimentos superiores a 80%. Abrindo assim, a oportunidade do desenvolvimento de catalisadores heterogêneos (Toda *et al.*, 2005; Silva Neto *et al.*, 2007), com bases orgânicas (Schuchardt *et al.*, 1997), sob condições supercríticas e enzimática (Fukuda *et al.*, 2001) a fim de minimizar os problemas associados com a utilização de catalisadores químicos.

### **1.5. Reação de Esterificação**

A síntese de emulsificantes é termo empregado para descrever a formação de ésteres. Esta reação chamada de esterificação, como descreve Markley (1961) ocorre entre um ácido carboxílico e um álcool, produzindo éster e água (Figura 5). Neste caso, como na reação de transesterificação, o produto final da reação pode ser obtido por meio de rotas químicas ou bioquímicas. Desta forma o uso de catalisadores biológicos como as enzimas, ganha destaque por apresentarem potencial em catalisar estas reações.



**Figura 5:** Esquema da esterificação (Markley 1961).

Os emulsificantes são utilizados em formulações de diferentes produtos de interesse comercial. Na indústria farmacêutica, os emulsificantes são utilizados como agentes aglutinantes em comprimidos e em medicamentos de liberação lenta (Guisan, 2008). Na indústria alimentícia e de cosméticos os emulsificantes são usados para estabilizar emulsões, e na indústria de cosméticos são ingredientes empregados na formulação de cremes e outros produtos. Portanto, é grande o interesse industrial por estes produtos, permitindo o incentivo a pesquisas mais aprofundadas nas reações de esterificação para a produção de diferentes emulsificantes (Paula *et al.*, 2005; Freitas *et al.*, 2007; Guisan, 2008). Diferentes estudos, tais como realizados por Reetz *et al.* (1996), Paula *et al.* (2005); Freitas *et al.* (2007), entre outros, relatam a produção de emulsificantes.

Reetz *et al.* (1996) observaram que o desempenho de lipase da *P. cepacia* encapsulada em gel, preparada pela hidrólise do silano alquil-substituído MTMS/PDMS (xerogel) utilizando o aditivo PVA, na esterificação do ácido láurico a 30 °C com octanol em isoctano, foi cerca de 88 vezes superior ao obtido pela lipase Lipozyme (preparação comercial de lipase imobilizada produzida pela NOVO-NORDISK).

Sekeroglu *et al.* (2004) utilizaram a lipase de *Candida antarctica* imobilizada em resina de troca iônica (Novozyme 435) para a síntese de laurato de isopropila a partir de álcool isopropílico e ácido láurico. A peneira molecular foi usada para deslocamento da reação para a síntese. A atividade máxima da enzima foi obtida em 60°C. A quantidade de produto pareceu ser linearmente proporcional à concentração da enzima até 50 mg. Foi observado o linear na taxa de produção de laurato isopropila no tempo de 50 min de reação.

Paula *et al.* (2005) estudaram a reação de síntese de ésteres utilizando o solvente terc-butanol, pois permitiu a solubilização parcial dos materiais de partida (carboidratos) sendo, ao mesmo tempo, atóxico e compatível com a atividade enzimática. O sistema constituído de frutose, ácido oléico, terc-butanol e lipase imobilizada em POS-PVA (polisiloxano-álcool polivinílico) foi o mais efetivo na obtenção de ésteres de açúcares.

Freitas *et al.* (2007) avaliaram a influência de fatores que afetam a síntese de ésteres glicéricos, tais como: razão molar entre os reagentes, fonte de lipase e tipo de agente de

ativação do suporte obtido pela técnica sol-gel. O meio reacional contendo excesso de glicerol favoreceu a síntese de ésteres glicéricos e a Lipozyme IM20 foi a preparação de lipase imobilizada mais adequada para a síntese, atingindo conversões molares superiores a 94%. O uso da lipase CALB L imobilizada em POS-PVA também alcançou resultados satisfatórios (cerca de 80% de conversão) possibilitando a formação de 36% de 2,3-diidroxipropil dodecanoato (monolaurina).

## 2. FUNDAMENTAÇÃO TEÓRICA

Neste tópico serão brevemente descritos as principais técnicas de caracterização desenvolvidas neste trabalho.

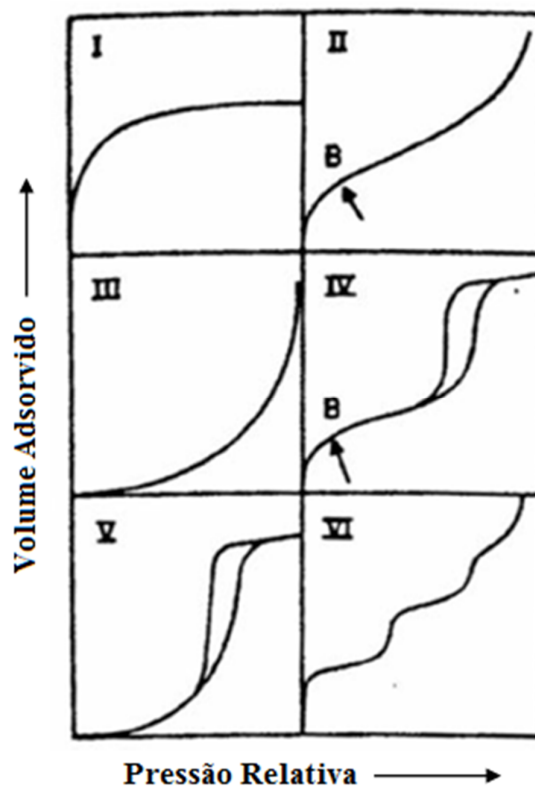
### 2.1. Isotermas de Adsorção e Dessorção

Adsorção de gás é uma técnica muito utilizada para avaliar a área de superficial, tamanho de poro, volume de poros e a distribuição de tamanho de poro de materiais sólidos porosos (Jaroniec *et al.*, 1999). Nesta técnica, a quantidade de gás adsorvido por um sólido é medida, que por sua vez está diretamente relacionado com as propriedades e a estrutura do material poroso (Groen *et al.*, 2003). Dependendo da natureza do sólido adsorvente e da informação requerida, vários tipos de gases podem ser utilizados como o nitrogênio ( $N_2$ ), dióxido de carbono ( $CO_2$ ) e argônio (Ar).

O volume de gás adsorvido pelo sólido é medido em uma ampla gama de pressões relativas, as curvas formadas entre o volume adsorvido com variação da pressão relativa ( $p/p_0$ ) é chamado a isotérmica de adsorção.

Conforme mostrado na Figura 6, as isotermas de adsorção dividem-se em seis tipos básicos (Sing *et al.*, 1985). As isotermas do tipo I são características de sólidos microporosos com superfícies externas relativamente pequenas. A quantidade adsorvida tende para um valor limite quando a pressão relativa tende a 1. Esta isoterma representa também a adsorção química, caso em que o valor limite corresponde à formação de uma monocamada molecular adsorvida. No caso das isotermas II e III, a quantidade adsorvida tende para valores elevados quando a pressão relativa tende a 1. Correspondem à adsorção em camadas múltiplas sobrepostas e ocorrem em sólidos não-porosos ou macroporosos. As isotermas dos tipos IV e V correspondem respectivamente às isotermas II e III quando o sólido apresenta mesoporos (diâmetro médio de 20 a 500 Å), nos quais ocorre o fenômeno de condensação capilar. A quantidade adsorvida tende a um valor máximo finito e corresponde ao enchimento completo

dos capilares. A isoterma do tipo VI (em degraus) ocorre em superfícies uniformes não-porosas e representa uma adsorção camada a camada. A altura do degrau equivale à capacidade de cada monocamada (Sing *et al.*, 1985).

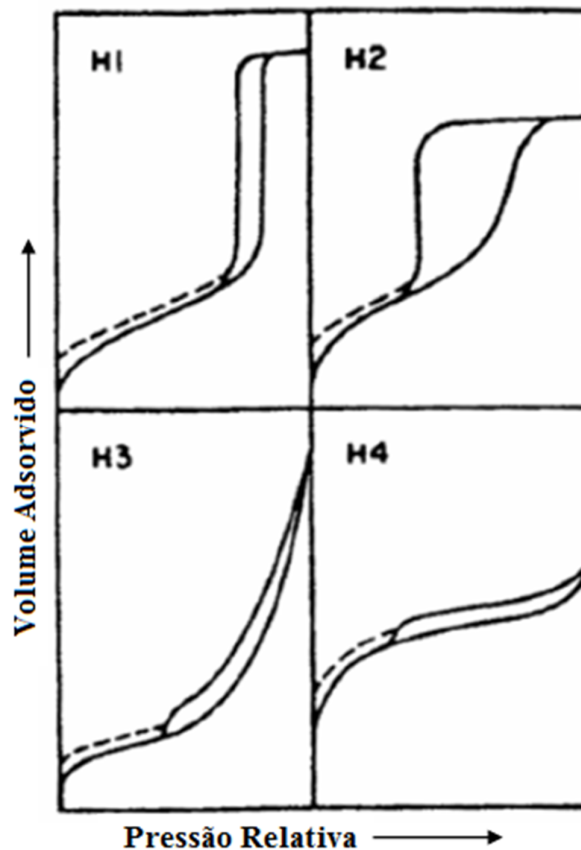


**Figura 6:** Isothermas de adsorção e dessorção classificados pela IUPAC. Fonte: Sing *et al.*, 1985.

A histerese é a não-simultaneidade ou atraso do fenômeno de dessorção em relação à adsorção física e está sempre associada à condensação capilar em estruturas mesoporosas. Há quatro tipos de histerese, conforme pode ser visto na Figura 7. A histerese do tipo H1, caracterizada por dois ramos da isoterma quase verticais e paralelos durante uma extensa gama de valores da ordenada, representa materiais porosos constituídos por aglomerados rígidos de partículas esféricas de tamanho uniforme ordenadas regularmente. Disso resulta uma distribuição estreita dos tamanhos de poros. A histerese do tipo H2, em que só o ramo de dessorção é praticamente vertical, corresponde a uma distribuição definida de tamanhos e formas de poros. Geralmente associa-se este tipo de histerese aos diferentes mecanismos de condensação e evaporação em poros com um gargalo estreito e corpo largo (poros em forma de tinteiro). A histerese do tipo H3 caracteriza-se por dois ramos da isoterma em relação à



vertical  $p/p_0 = 1$  e está associada a agregados não-rígidos de partículas em forma de placa, originando poros em fenda. A histerese do tipo H4 apresenta os dois ramos da isoterma quase horizontais e paralelos durante uma extensa gama de valores da abscissa. Este tipo também está associado a poros estreitos em fenda e sua forma semelhante à isoterma do tipo I é sugestiva de microporosidade (Sing *et al.*, 1985).



**Figura 7:** Classificação dos loops de histereses. Fonte: Sing *et al.*, 1985.

## 2.2. Termogravimetria (TG)

Baseando-se nos estudos da variação da massa de uma amostra, a termogravimetria é o resultado de uma transformação física (sublimação, evaporação, condensação) ou química (degradação, decomposição, oxidação) em função do tempo ou da temperatura.

Os fornos, de um modo geral, são capazes de operar na faixa de 1000 - 1200°C, existindo também fornos que podem operar até 1600 - 2400°C. A temperatura do forno e da amostra é determinada através de um par termoeletrico e o sensor deve estar localizado próximo da amostra ( $\cong 1$  a 2 mm).

É possível controlar a atmosfera que envolve a amostra, podendo viabilizar o trabalho com atmosfera estática ou dinâmica à pressão ambiente, sob pressão ou a vácuo. Podem ser utilizados gases inertes (nitrogênio, argônio), oxidantes (oxigênio) ou corrosivos.

Como resultados desses experimentos termogravimétricos, tem-se curvas nas quais pode-se observar variações de massa, de modo que possa originar produtos voláteis (Ionashiro *et al.*, 1980).

As curvas termogravimétricas podem ser utilizadas para análises em que se observa mudança de massa. Caso essa mudança de massa não seja observada, pode-se utilizar técnicas térmicas como: DSC, DTA e TMA. Se essa mudança de massa for muito pequena (<1%), pode-se empregar a análise de gás desprendido (EGA) (Conceição, 2000).

Nos estudos termogravimétricos, as principais aplicações são:

- Decomposição e estabilidade térmica das substâncias orgânicas e inorgânicas e dos mais variados materiais, tais como: minerais, carvão, madeira, petróleo, polímeros, alimentos, fármacos e outros;
- Corrosão de metais em atmosferas constituídas por diferentes gases e em faixas muito amplas de temperatura;
- Velocidade de destilação e evaporação de líquidos e de sublimação de sólidos;
- Desidratação, higroscopicidade, absorção, adsorção, dessorção, determinação do teor de umidade, fração volátil e teor de cinzas de vários materiais;
- Cinética das reações, inclusive de reações no estado sólido e, também, em possíveis descobertas de novos compostos químicos;
- Determinação da pureza e da estabilidade térmica de reagentes analíticos, inclusive padrões primários e secundários;
- Estudo sistemático das propriedades térmicas dos precipitados, de acordo com os processos de precipitação utilizados;
- Desenvolvimento de processos analíticos gravimétricos;
- Curva de ignição dos meios de filtração e da conveniência de se secar ou calcinar um precipitado;
- Determinação de um único componente ou da composição de misturas com dois ou três componentes;
- Caracterização funcional de compostos orgânicos, TG-CG/MS;
- Definição da estequiometria;
- Estabelecimento da composição e estabilidade térmica de compostos intermediários;

- Composição do resíduo e decomposição térmica em várias condições de atmosfera e temperatura;
- Sensibilidade do mecanismo e do registro.

### **2.3. Calorimetria Exploratória Diferencial (DSC)**

Calorimetria Exploratória Diferencial (DSC) é uma técnica que mede as temperaturas e o fluxo de calor associado com as transições dos materiais em função da temperatura e do tempo. Essas medidas informam, qualitativamente e quantitativamente sobre mudanças físicas e químicas que envolvem processos endotérmicos (absorção de calor), exotérmicos (liberação de calor) ou mudanças na capacidade calorífica (Mothé e Azevedo, 2002).

Dentre as aplicações da DSC, pode-se citar:

- Estudo de eventos térmicos: calor específico, pureza, polimorfismo, transição vítrea, gelatinização, cinética de reações, comportamento de fusão e cristalização;
- Identificação de substâncias: através da forma, posição e número de picos endotérmicos ou exotérmicos em função da temperatura;
- Determinação quantitativa de substâncias - pois o calor da reação é proporcional à quantidade de substância;
- Identificação, composição quantitativa de materiais e estabilidade térmica e oxidativa: sendo utilizada na área de polímeros, metalúrgica, geologia, cerâmica, alimentos;
- Determinação da estabilidade térmica, oxidação e transição vítrea de fármacos.

### **2.4. Espectros na Região do Infravermelho**

A análise dos espectros na região do infravermelho é uma importante técnica de caracterização, pois fornece uma avaliação qualitativa da presença de grupos funcionais próprias da sílica, ou na identificação de grupos funcionais.

### **2.5. Espectroscopia Eletrônica de Varredura – MEV**

O microscópio eletrônico de varredura (MEV) é um equipamento capaz de produzir imagens de alta ampliação (até 300.000x) e resolução. As imagens fornecidas pelo MEV possuem um caráter virtual, pois o que é visualizado no monitor do aparelho é a transcodificação da energia emitida pelos elétrons, ao contrário da radiação de luz a qual estamos habitualmente acostumados.

O princípio de funcionamento do MEV consiste na emissão de feixes de elétrons por um filamento capilar de tungstênio (eletrodo negativo), mediante a aplicação de uma diferença de

potencial que pode variar de 0,5 a 30 kV. Essa variação de voltagem permite a variação da aceleração dos elétrons, e também provoca o aquecimento do filamento. A parte positiva em relação ao filamento do microscópio (eletrodo positivo) atrai fortemente os elétrons gerados, resultando numa aceleração em direção ao eletrodo positivo. A correção do percurso dos feixes é realizada pelas lentes condensadoras que alinham os feixes em direção à abertura da objetiva. A objetiva ajusta o foco dos feixes de elétrons antes dos elétrons atingirem a amostra analisada.

A Microscópio Eletrônico de Varredura (MEV) é um instrumento muito versátil e usado rotineiramente para a análise microestrutural de materiais sólidos. Apesar da complexidade dos mecanismos para a obtenção da imagem, o resultado é uma imagem de muito fácil interpretação.

### 3. INTRODUÇÃO AO CAPÍTULO III

O capítulo III apresenta em forma de artigos científicos (ARTIGO I, II, III e IV), e estão organizados conforme as normas propostas pelo periódico de publicação. Este capítulo traz uma pequena introdução, os materiais e métodos utilizados no desenvolvimento de cada artigo, os resultados obtidos e sua discussão, além das conclusões de cada etapa.

No primeiro artigo (Capítulo III – ARTIGO I - “Influence of the use of Aliquat 336 in the immobilization procedure in sol-gel of lipase from *Bacillus* sp. ITP – 001”) verifica a melhor concentração de Aliquat 336 no rendimento de imobilização da lipase de *Bacillus* sp. ITP – 001 imobilizada pela técnica sol-gel. Artigo submetido ao periódico Journal Molecular Catalysis B: Enzymatic.

O segundo artigo (Capítulo III – ARTIGO II – “Protic ionic liquid as additive on immobilization of lipase on silica sol-gel”) verifica a influência da cadeia alquílica e a concentração dos líquidos iônicos próticos utilizados como aditivo no processo de imobilização da enzima de *Burkholderia cepacia*. O artigo será submetido ao periódico Journal Molecular Catalysis B: Enzymatic.

O terceiro artigo desta dissertação (Capítulo III – ARTIGO III – “Protic ionic liquid applied to enhance the immobilization of lipase from *Bacillus* sp. ITP-001 in sol-gel matrices”) verifica a influência da cadeia alquílica e a concentração dos líquidos iônicos próticos utilizados como aditivo no processo de imobilização da lipase de *Bacillus* sp. ITP-001. Este artigo será submetido ao periódico Colloids and Surfaces B: Biointerfaces.

O quarto artigo desta dissertação (Capítulo III – ARTIGO IV – “Employment of polyethylene glycol in the process of sol-gel encapsulation of *Burkholderia cepacia* lipase”) verifica a influência da concentração de PEG, utilizado como aditivo, no processo de imobilização sol-gel da lipase de *Burkholderia cepacia*. O artigo será submetido ao periódico Journal of Thermal Analysis and Calorimetry.

### Artigo I

#### **Influence of the use of Aliquat 336 in the immobilization procedure in sol-gel of lipase from *Bacillus* sp. ITP – 001**

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## Abstract

Aliquat 336, a liquid hydrophobic material, was used at different concentrations (0.5 to 3.0%, w/v) as an additive in the preparation of encapsulated lipase from *Bacillus* sp. ITP-001 on sol-gel silica matrices using tetraethoxysilane (TEOS) as the precursor. The resulting hydrophobic matrices and immobilized lipases were characterized with regard to specific surface area (the BET method), adsorption-desorption isotherms, pore volume ( $V_p$ ) and size ( $d_p$ ) by nitrogen adsorption (the BJH method) and scanning electron microscopy (SEM). The catalytic activities and the corresponding encapsulation yields were assayed in the hydrolysis of olive oil. In comparison with pure silica matrices, the immobilization process in the presence of Aliquat336 decreased the values for specific surface area and increased the values for pore specific volume ( $V_p$ ) and mean pore diameter ( $d_p$ ). This behavior may be related to the partial adsorption of the enzyme on the external surface of the hydrophobic matrix as indicated by scanning electron microscopy. Aliquat336 concentrations in the range from 0.5-1.5% (w/v) provided immobilized derivatives with higher activity and better substrate affinity. The total activity recovery yield (71%) was obtained for the immobilized enzyme prepared in the presence of 1.5% Aliquat which gave the following characteristics: specific surface area = 183 m<sup>2</sup>/g, pore specific volume ( $V_p$ ) = 0.36 cc/g and mean pore diameter ( $d_p$ ) = 91 Å.

Keywords: lipase, immobilization, sol-gel, Aliquat 336

## 1. Introduction

Lipase (EC 3.1.1.3) is an important enzyme with a broad variety of applications in the food industry, fine chemistry, and in the pharmaceutical industry due to the multiplicity of reactions it catalyzes [1,2]. Applications of lipase can be achieved more economically and efficiently by immobilization to enhance its activity, selectivity, and operational stability. Therefore, numerous efforts have been focused on the preparation of lipases in immobilized forms, which involve a variety of both support materials and immobilization methods [3,4].

Compared with chemical methods for the immobilization of enzymes [5], physical methods, especially adsorption, may have a higher commercial potential because they are simpler, less expensive and can retain high catalytic activity. The adsorption of lipase onto a porous support may be one of the most widely employed methods used in continuously operating packed beds and stirred tank reactors, especially in large-scale operations [6].

A very large number of enzymes have been immobilized by the sol-gel technique. Although sol-gel immobilized enzymes usually exhibit better activity and stability than free enzymes [7], there are some drawbacks in the sol-gel immobilization process. One is the shrinkage of the gel during the condensation and drying process, which may cause denaturation of enzymes. The released alcohols during the hydrolysis of silicon alkoxide can also inactivate enzymes. The slow rate of diffusion of substrate molecules to the enzyme within the sol-gel matrix hinders the catalytic activity of the immobilized enzyme in materials with a pore diameter smaller than 20 Å [8]. One way to overcome these drawbacks could be the use of additives to stabilize enzymes within sol-gel matrices. Sugars, amino acids, polyols and surfactants have been used to increase the activity and stability of various enzymes. These additives can increase the activity and stability of immobilized enzymes by altering the hydration of the enzyme and reducing shrinkage of the gel. They can also affect the gel's physical properties by participating in condensation reactions with free silanol groups [9].

The study of different additives, such as polyethylene glycol (PEG-1500) and albumin, in the immobilization procedure of microbial lipase from *Candida rugosa* showed higher yields (59.6% and 32.2%, respectively) when compared to the same immobilized lipase without additives. New additives emphasizing better yields in the immobilization process may be well-regarded by the industry due to the high market values of the enzyme that could reach US\$ 4 billion in 2014 [10]. Aliquat 336 is a versatile and affordable cation source for an entirely new family of hydrophobic ionic liquids [14] and has been successfully used to recover acids/acid salts or to remove certain heavy metals from wastewater. It also has



properties as an anti-static agent for textile fabrics and carpeting, in the decolorization and deodorization of fermentation broths, as an adhesion promoter and as a surface curing aid for fluorocarbon elastomers [11]. In addition, Aliquat 336 has been used as agent handler permeability, acted as a plasticizer in polymeric membranes exhibiting mainly carboxylated PVC and polyurethane, and also been used to form an outer layer of protection for biosensors produced by the sol-gel technique [12]. Gill *et al.* [13] have also reported that plasticizers are generally used to improve the physical properties of a polymer material.

The behavior of hydrophobic/hydrophilic particles in a water–oil system has been studied by many researchers. Takahara *et al.* [15] prepared spherical silica particles by partial modification with an alkylsilylation agent having both hydrophobic and hydrophilic surfaces. Their findings showed that the particles could assemble at the phase boundary of a dual-phase mixture to form a micellar-like structure in water in the presence of an organic solution of a toluene/polystyrene mixture, which indicated that the hydrophobic/hydrophilic particles could make contact with both liquid phases in the emulsion. Hannisdal *et al.* [16] found that sol-gel matrices with hydrophobic groups could stabilize the model emulsion of water and oil. The stabilization efficiency was greatly affected by the particles' wetting properties, which were quantified in terms of contact angle ( $\theta$ ) measurements. This phenomenon was explained by Kralchevsky *et al.* [17] by means of interfacial bending energy and dilatation energy. Therefore, methyl-modified silica gels with residual hydroxyl groups may play an important role in the micro-structure of a liquid-liquid system, and its amount may have an effect on the mass transfer surface of the reaction emulsion [18].

In this work, Aliquat 336 was used to protect the lipase from *Bacillus* sp. ITP-001 from inactivation during the immobilization procedure by the sol-gel technique. This lipase producer was an isolated strain from petroleum-contaminated soil in our institution which has already shown high activity when used in typical lipase reactions [19]. To enhance its applicability, immobilization was envisaged and the concentration of Aliquat 336 used here as an additive was evaluated in terms of the immobilized morphological structure and lipase activity recovery.

## **2. Experimental procedures**

### *2.1. Enzyme*

Lipase was obtained by fermentation of a newly isolated *Bacillus* sp. ITP-001 strain from petroleum-contaminated soil (ITP, Aracaju, Sergipe, Brazil) [19]. The strain was cultivated in

500 ml Erlenmeyer flasks containing 200 ml medium with the following composition (% w/v):  $\text{KH}_2\text{PO}_4$  (0.1),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.05),  $\text{NaNO}_3$  (0.3), yeast extract (0.6), peptone (0.13), and starch (2%) as the carbon source. The fermentation conditions were: initial pH 5.0; incubation temperature 32°C; and stirring speed 200 rpm. After 72 h of cultivation, coconut oil (4% w/v) was added as the lipolytic enzyme inductor as described by Feitosa *et al.* [19]. Lipase obtained from the fermented medium was purified using the aqueous two-phase system containing polyethylene glycol (PEG,  $M_w$  8000, Merck) and potassium phosphate buffer solution (30%, w/v). In the first step, the protein content was precipitated with ammonium sulfate; however only the protein contaminant was precipitated with 80% saturation. The enzyme, which remained at the aqueous solution, was dialyzed against distilled water for 18 h. The dialyzed enzymatic solution was used to prepare an aqueous two-phase system from stock solutions of PEG (50%, w/v) in phosphate buffer (30%, w/v). 6% NaCl was added to increase the hydrophobicity between the phases. The system was kept at 4°C for 12 h at pH 6.0 after the phases were separated; the enzymes in solution were freeze-dried. [20].

## 2.2. Chemicals

Triethylmethylammonium chloride (Aliquat 336®) was used as the additive in the immobilization procedure. The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (New Jersey, United States) and used without further purification. Ethanol (minimum 99%), ammonia (minimum 28%), hydrochloric acid (minimum 36%) and gum Arabic were obtained from Synth (São Paulo, Brazil). Olive oil was purchased at a local market. Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. Other chemicals were of analytical grade and used as received.

## 2.3. Encapsulation of lipase from *Bacillus sp. ITP-001* in sol-gel matrices

The methodology previously established by Patent PI0306829-3 [21] was used and is briefly described as follows: 30 mL of TEOS were dissolved in 36 mL of absolute ethanol under an inert nitrogen atmosphere. To this, 0.22 mL of hydrochloric acid dissolved in 5.0 mL of ultra-pure water which was slowly added and the mixture was agitated (200 rpm) for 90 min at 35°C. The enzyme (2.7 g) and Aliquat 336 (at concentrations from 0.5 to 3.0%, w/v) were added to 10 mL of ultra-pure water; at the same time, 1.0 mL of ammonium hydroxide

dissolved in 6.0 mL of ethanol was added (hydrolysis solution) and the mixture was kept under static conditions for 24 hours to complete polycondensation. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 72 h. For comparison, the encapsulated lipase from *Bacillus* sp. ITP-001 was prepared similarly in the absence of Aliquat 336 (EN-Bacillus) and pure silica sol-gel (PS) was prepared in the absence of the enzyme and additive. This yielded samples designated as PS, EN-Bacillus and EN-Aliquat-0.5 to 3.0.

#### 2.4. Sample characterization

The surface area of the pure silica gel and encapsulated lipase samples was calculated using the Brunauer-Emmett-Teller (B.E.T.) method [22]. Pore volume and average pore diameter, based on BJH calculations [23], were evaluated by BET apparatus software (Model NOVA 1200-Quantachrome Analyzer), using N<sub>2</sub> adsorption at 77 K. Before analysis, samples were submitted to a thermal treatment at 60 °C, under vacuum, to eliminate any water existing within the pores of the solids. Scanning electron microscopy (SEM; model Hitachi SU-70), was used to characterize the surface of pure silica matrices and immobilized lipase samples.

#### 2.5. Enzymatic activity

Enzymatic activities of the free and immobilized lipase samples were assayed by the olive oil emulsion method according to a modification used by Soares *et al.* [24]. The substrate was prepared by mixing 50 mL of the olive oil with 50 mL of gum Arabic solution (7% w/v). The reaction mixture containing 5.0 mL of the oil emulsion, 4 mL of sodium phosphate buffer (0.1 M, pH 7.0) and either free (1.0 mL, 0.1 mg.mL<sup>-1</sup>) or immobilized ( $\approx$ 250 mg) lipase was incubated in a thermostated batch reactor for 5 min (free lipase) or 10 min (immobilized lipase) at 37°C. A blank titration was done with a sample where the enzyme was replaced with distilled water. The reaction was stopped by the addition of 2 mL of acetone-ethanol-water solution (1:1:1). The liberated fatty acids were titrated with potassium hydroxide solution (0.04M) in the presence of phenolphthalein as an indicator. All reactions were carried out in triplicate. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1  $\mu$ mol of free fatty acid per min ( $\mu$ mol.min<sup>-1</sup>) under the assay conditions (37°C, pH 7.0, 80 rpm), according to Eq.1.

$$A = \frac{(V_a - V_b) \times N \times 10^3}{t \times m} \quad (1)$$

Where: A is the lipase activity (U.g<sup>-1</sup>), V<sub>a</sub> is the volume of sample titrated (mL), V<sub>b</sub> is the volume of the blank titration (mL), N is the normality of the KOH solution (mol.L<sup>-1</sup>) and t is the reaction time in minutes. Analyses of hydrolytic activities carried out on the lipase loading solution and bioencapsulated preparations were used to determine the total activity recovery yield, Ya (%), according to Eq. (2),

$$Ya(\%) = \frac{U_s}{U_o} \times 100 \quad (2)$$

in which U<sub>s</sub> is the total enzyme activity recovered on the support and U<sub>o</sub> is the enzyme units offered for immobilization.

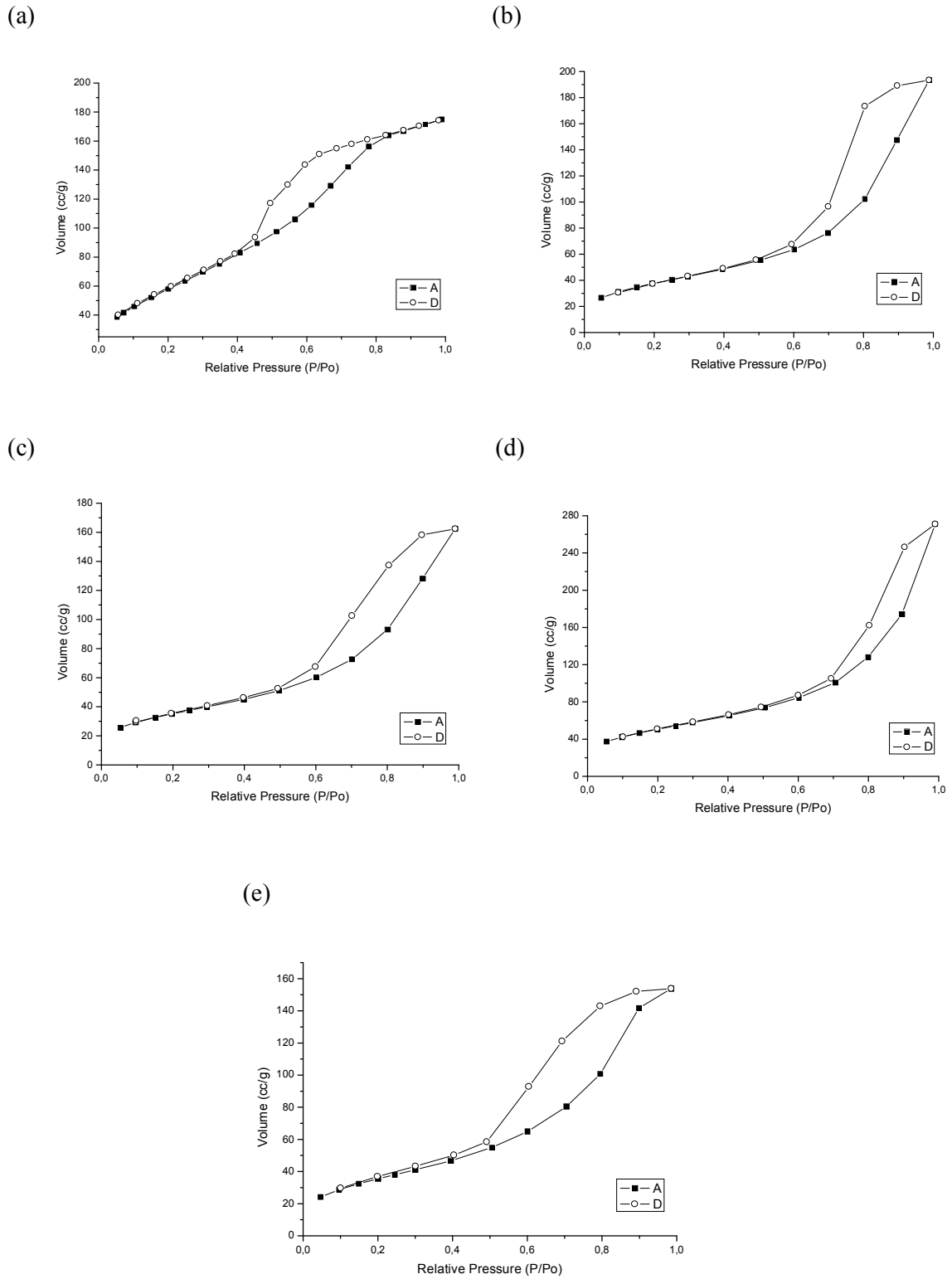
### 3. Results and Discussion

The incorporation of hydrophobic liquids in sol-gel matrices can be performed either by addition during the silica sol preparation [25, 26, 27] or together with the enzyme solution in gel formation [28]. In this work, the addition of Aliquat 336 at different concentrations was performed together with the enzyme solution containing lipase from *Bacillus* sp. ITP-001.

#### 3.1. Characterization of the porosity of hydrophobic matrices and immobilized biocatalyst

##### 3.1.1. Specific surface area and porous properties

The characterization of the porosity of hydrophobic matrices and immobilized biocatalysts is a complex issue when the total porosity, the pore size and the pore size distribution should be further analyzed. Methods based on gas adsorption are the most convenient for the study of the porous properties of solid materials, using volumetric measurements of the adsorbed gas quantities. The adsorption-desorption isotherms of N<sub>2</sub>, specific surface area, pore specific volume (V<sub>p</sub>) and mean diameter (d<sub>p</sub>) of the hydrophobic matrices and immobilized biocatalysts were determined from nitrogen adsorption-desorption measurements, which is a widely used method for the characterization of microporous and mesoporous materials. Shows the results with regard to the specific surface area (BET method), pore volume and mean pore diameter for sol-gel matrices and their derivatives. Surface area and mean pore diameter results indicated a significant influence of Aliquat 336 on the derivatives' properties. The nitrogen adsorption-desorption isotherms of the samples at -196°C are shown in Figure 8.



**Figure 8:** Nitrogen adsorption-desorption isotherms of the pure silica gel and immobilized samples .

The addition of Aliquat 336 resulted in an increase in the mean pore diameter for all immobilized derivatives; the largest pore sizes were obtained when Aliquat 336 was added at a concentration of 1.5% (w/v). The presence of the additive also led to larger pore sizes and pore volumes (Table 5). Hence, from the nitrogen adsorption-desorption measurements, it is clear that the additive acted as a pore forming agent. A similar profile was observed by Vila-Real *et al.* [29] who reported that the addition of ionic liquids in the sol-gel immobilization process plays an important role in enzyme performance by affecting the structural characteristics of the immobilized biocatalyst. This behavior was also reported by Zarcu *et al.* [28] during lipase encapsulation in a hydrophobic matrix with a high amount of hydrophobic groups, since the hydrophobic liquids induced significant changes on the porous structure of the biocatalyst.

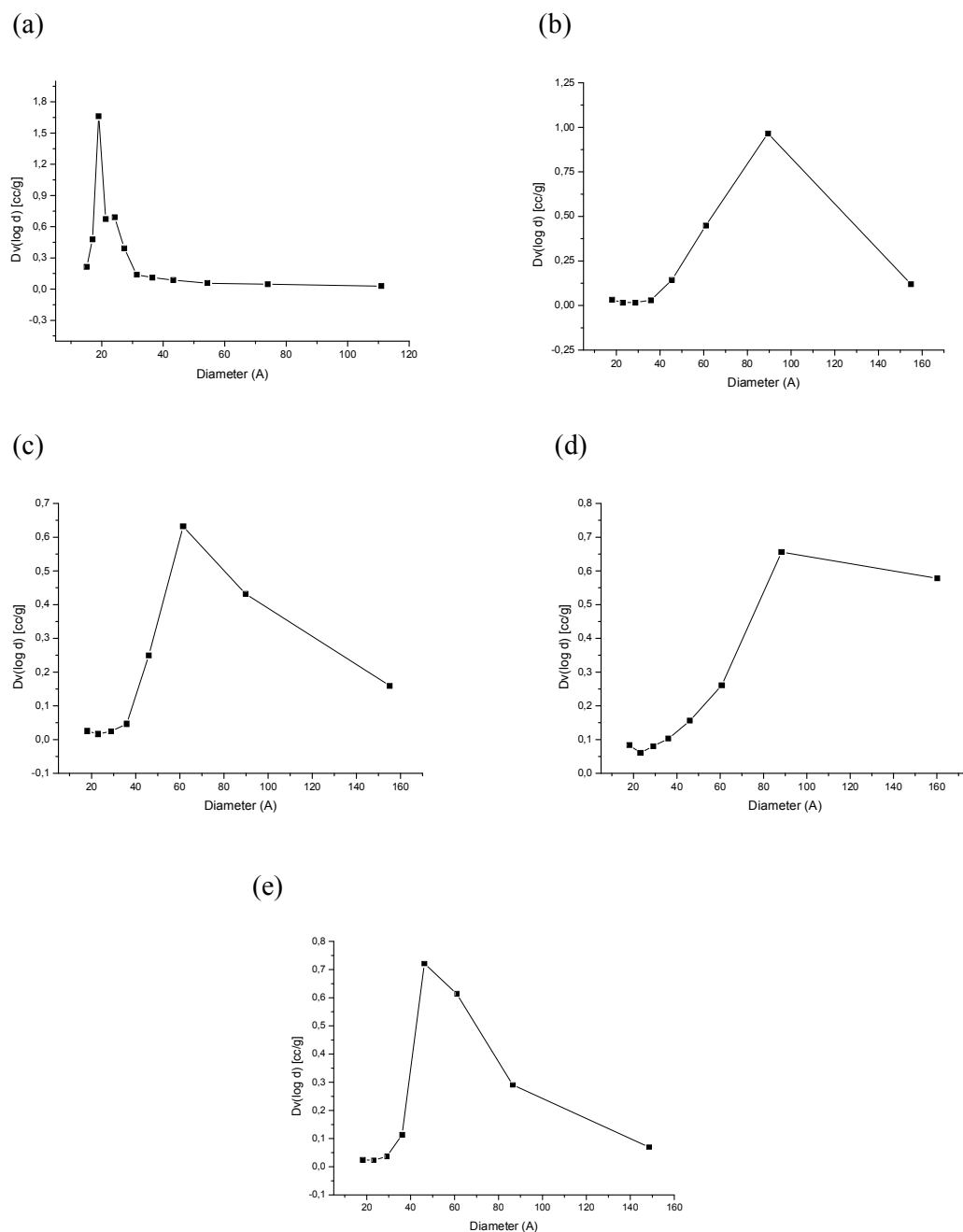
**Table 5:** Textural properties from nitrogen adsorption-desorption of the pure silica gel and immobilized samples.

<b>Samples</b>	<b>Surface area (m<sup>2</sup>.g<sup>-1</sup>)</b>	<b>Pore volume (cc.g<sup>-1</sup>)</b>	<b>Pore diameter (Å)</b>
Pure silica	224	0.21	35
EN-Aliquat-0.5	137	0.30	87
EN-Aliquat-1.0	128	0.24	79
EN-Aliquat-1.5	183	0.36	92
EN-Aliquat-3.0	131	0.25	73

On the other hand, the results show that the addition of Aliquat 336 decreased the specific surface area, in agreement with results reported by Soares *et al.* [24] using a commercial lipase from *Candida rugosa* and polyethylene glycol as an additive. Immobilized *Candida rugosa* in sol-gel matrices using PEG as the additive showed lower values of surface area in comparison with samples without the addition of additives (348 and 607 m<sup>2</sup>.g<sup>-1</sup>, respectively).

This behavior occurred due to partial adsorption of the enzyme on the external surface of the hydrophobic matrix (EN-Bacillus and EN-Aliquat), as can be seen in the scanning electron microscopy analysis. The pure silica showed a type IV isotherm with a hysteresis loop, which are typically exhibited by mesoporous solids [30]. The results observed for pure silica in the N<sub>2</sub> adsorption-desorption tests concur with those of the isotherms with H2 hysteresis loops, which is a characteristic of mesoporous materials and usually associated with pores with narrow necks and wide bodies. Similar results were obtained for the immobilized lipase samples. However, in the samples containing the enzyme and additive Aliquat (0.5 to 3.0%, w/v), the adsorbed volume was greater than that in pure silica. However, in EN-Aliquat-1.5, the magnitude of the adsorbed volume became larger, indicating that an ideal amount of the additive in the preparations of the immobilized samples has a maximum leading to materials with bigger pored, and thereafter decreases. For the immobilized derivatives, the isotherms were type IV isotherms with H4 hysteresis loops, usually associated with slit-like pores, or with H3 hysteresis loops, usually associated with plate-like particles [31].

This indicates that the addition of the Aliquat 336 not only increased the mean pore diameter and pore volume of the immobilized enzyme, but also led to the formation of pores with features and shapes different than those obtained with pure silica. The pure silica sample had a pore diameter of approximately 35 Å while the immobilized samples in presence of the additive had pore diameters in the 70-90 Å range (Table 5), which are mesoporous in nature. In agreement with these results, the use of hydrophobic additives caused an enlargement of pores from 18 to 57Å in the lipase fixed by the sol-gel technique. From the pore size distribution (Figure 9), it can be observed that the pure silica samples (Figure 9a) had a maximum peak about in 2 nm, indicating a microporous structure, while the EN-Aliquat-0.5, EN-Aliquat-1.0, EN-Aliquat-1.5, EN-Aliquat-3.0 (Figure 9b-e, respectively) samples show maximal peaks in the 5-9 nm range, typical of mesoporous materials. The results show that the addition of Aliquat to pure silica led to an increase in pore size. Thus, from the textural properties of the materials analyzed, it is clear that the Aliquat addition led to mesoporosity in silica-based materials by acting as a pore-forming agent.



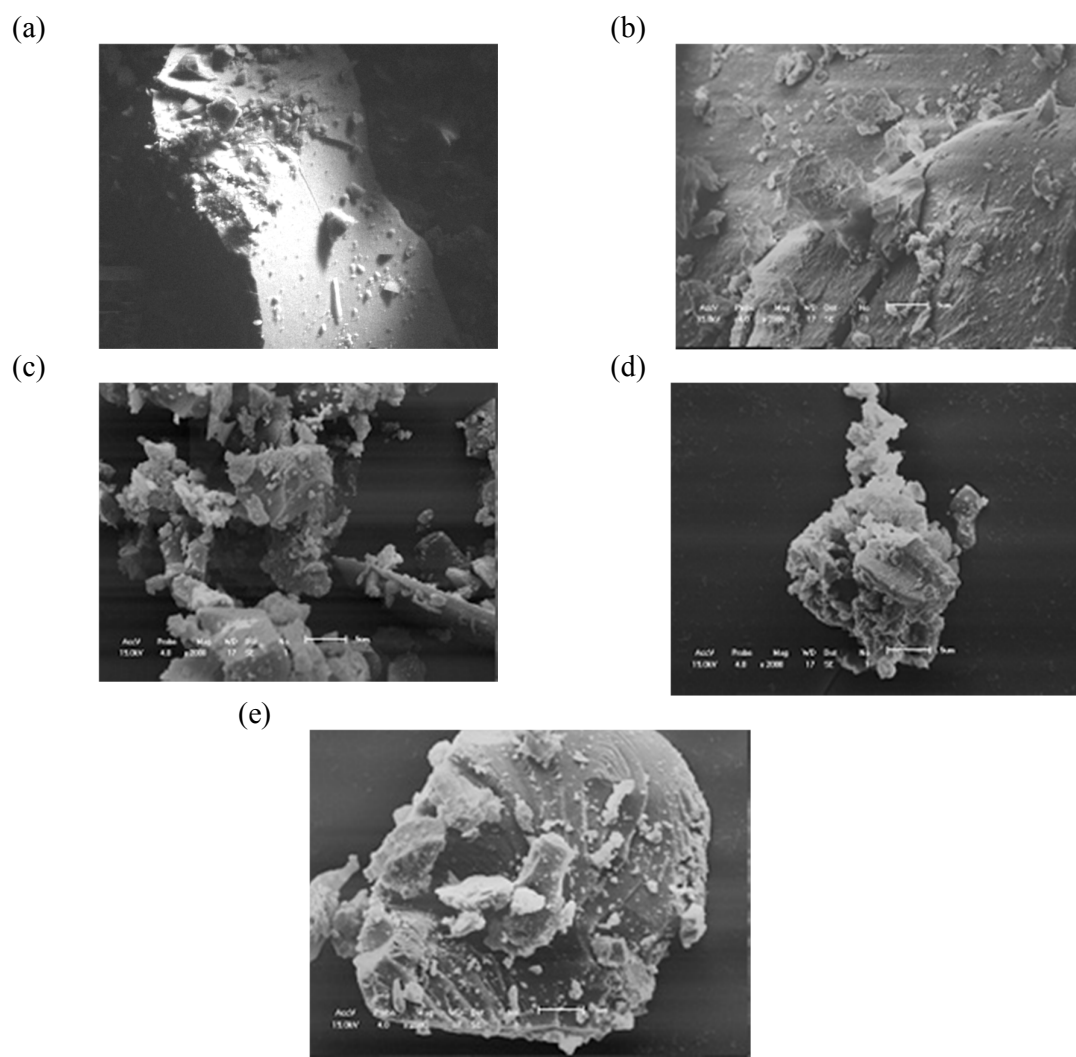
**Figure 9:** Pore size distribution for: (a) pure silica gel, (b) EN-Aliquat-0.5, (c) EN-Aliquat-1.0, (d) EN-Aliquat-1.5 and (e) EN-Aliquat-3.0.

### 3.1.2. Scanning electron microscopy (SEM)

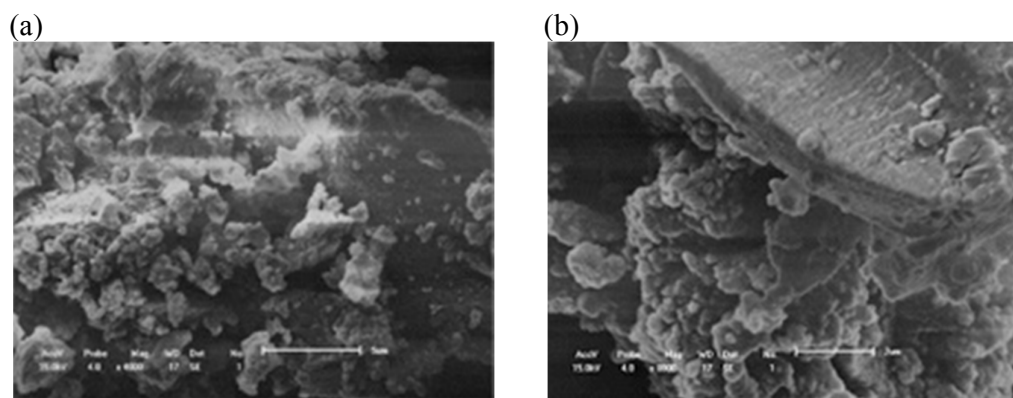
SEM micrographs of the pure silica gel and immobilized lipases, in the presence or absence of the Aliquat, are shown in Figure 10. From these images, it can be observed that the surface morphology had an irregular pattern for sol-gel matrices and their derivatives. Consequently, such images neither allowed us to identify the pore size nor to determine the size distribution. By analyzing the micrographs of pure silica gel (Figure 10a), one can



observe the presence of a rigid superficial structure, probably forming only one block. Addition of the enzyme and Aliquat 336 induced a modification of the morphology of the pure silica sol-gel. The biocatalyst morphology presented a superficial structure of greater porosity with more rounded shapes, although pores were still irregular. The crystals were composed of large accumulations of porous particles conferring higher porosity to the immobilized samples, as shown in Figure 11(a) and (b) for EN-Aliquat-1.5(x4000) and EN-Aliquat-1.5(x8000), respectively. These results are in agreement with those obtained by determining the specific surface area and porous properties. However, it could be observed by SEM that some particles were formed on the surface of EN-Aliquat biocatalysts with a structure similar to those obtained in pure silica. This indicates that during the preparations of biocatalysts, at some stage, homogeneity was not complete as would be expected with the sol-gel technique.



**Figure 10:** Scanning electron micrographs for: (a) pure silica gel (x2000), (b) EN-Aliquat-0.5 (x2000), (c) EN-Aliquat-1.0 (x2000), (d) EN-Aliquat-1.5 (x2000) and (e) EN-Aliquat-3.0 (x2000).

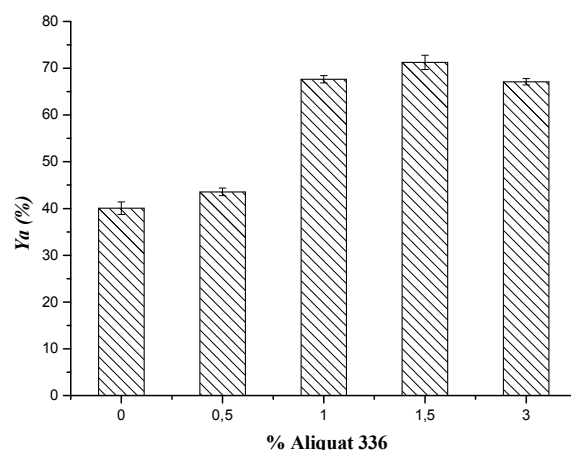


**Figure 11:** Scanning electron micrographs for: (a) EN-Aliquat-1.5 (x4000), (b) EN-Aliquat-1.5 (x8000).

### 3.1.3. Enzymatic activity of the immobilized sol-gel

The utilization of Aliquat 336 as an additive was found to have multiple effects on both the entrapment process and the catalytic activity of the immobilized enzyme. The total activity recovery yield in the presence of different Aliquat 336 concentrations is plotted in Figure 12. All the immobilized biocatalysts prepared in the presence of Aliquat 336 showed higher yields ( $Y_a= 43$  to  $71\%$ ) than the immobilized derivative in the absence of this additive ( $Y_a= 40\%$ ). The highest value was attained ( $Y_a= 71\%$ ) for samples containing  $1.5\%$  (w/v) Aliquat; however, the total activity yield results were higher than  $67\%$  at concentrations above  $1\%$  (w/v) Aliquat 336, which favorably compares with the results reported by Soares *et al.* [32] that found  $Y_a= 59.6\%$  in the immobilization procedure of lipase from *Candida rugosa* on silica using PEG-1.500 as an additive.

The consistency of these results can be verified by the well-known fact that a more hydrophobic microenvironment is beneficial for lipase activity. The high activity values may be also associated with the pore size, since the enzymatic reaction rate depends on substrate accessibility by the enzyme. The limiting factor is the substrate diffusion rate to the enzyme inside the caged silica structure. Since the enzyme is already within the pore channels after gelatinization, the pore channels needed to be just wide enough to allow the diffusion of substrate molecules to, and of the product molecules away from, the enzyme caged within the matrix. With the addition of Aliquat 336 in the immobilization process of the lipase from *Bacillus* sp. ITP-001, the enlargement of pores could be clearly observed compared to pure silica.



**Figure 12:** Total activity recovery yield of samples from *Bacillus* sp. ITP-001 immobilized in the absence and presence of different concentrations of Aliquat.

#### 4. Conclusions

Lipase from *Bacillus* sp. ITP-001 was successfully immobilized on silica materials via the sol-gel process using tetraethylorthosilicate (TEOS) as the silica precursor and Aliquat 336 to enhance the hydrophobic character of the matrix. In this method, encapsulation of lipase from *Bacillus* sp. ITP-001 was used as a free agent or model of pore formation during the sol-gel process to obtain the host mesoporous silica material. By varying the Aliquat 336 concentration, immobilized biocatalysts were obtained with different values for surface area, pore volume and pore diameter. These parameters defined the enzymatic activity of the immobilized biocatalysts as well as total activity yield, since the activity of the immobilized enzyme is limited by the diffusion of substrate rate of the enzyme within the solid matrix. Thus, the immobilized biocatalysts in the presence of Aliquat showed a general broadening of pores (always above 73 Å and attaining a maximum value for EN-Aliquat-1.5% of 91 Å) compared to pure silica (35 Å). The total activity and total activity yield for samples containing 1.5% Aliquat 336 ( $Y_a=71\%$ ) were significantly higher than those in the absence of this additive. Therefore, a positive effect on the morphological structure and enzymatic activity of the immobilized biocatalyst was observed in the presence of Aliquat 336, making it an important additive for immobilization.

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## Artigo II

### **Protic ionic liquid as additive on lipase immobilization using silica sol-gel**

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## Abstract

The immobilization of lipase from *Burkholderia cepacia*, on silica using ionic liquids as additives to protect the inactivation of lipase by releasing alcohol and shrinking of gel during sol-gel process, was investigated in this study. The influence of various factors, such as alkyl chain length of protic ionic liquids (methylmonoethanolamine-based) and the concentration between 0.5 to 3.0% (w/v) was evaluated. The resulting hydrophobic matrices and immobilized lipases were characterized with regard to specific surface area (the BET method), adsorption-desorption isotherms, pore volume ( $V_p$ ) and size ( $d_p$ ) by nitrogen adsorption (the BJH method) and scanning electron microscopy (SEM), physico-chemical (Thermogravimetric – TG, Differential scanning calorimetry – DSC and Fourier transform infrared spectroscopy – FTIR) and the potential for ethyl esters and emulsifier production. The results of total activity yield ( $Y_a$ ) for matrices of immobilized lipase employing protic ionic liquids - PIL as additives always showed greater than 1000%, and better value was 1526% to system using 1.0% (w/v) of more hydrophobic nature of PIL, the C<sub>5</sub>. Compared with arrays of immobilized biocatalysts without additive, in general, immobilized biocatalysts in the presence of PIL increased the values of surface area (143 to 245 m<sup>2</sup>.g<sup>-1</sup>) and pore size (19 to 38 Å). The immobilized with PIL favors lower mass loss by TG curves (always less than 42.9%) when compared to the immobilized matrix without PIL (45.1%), except for the sample containing 3.0% of PIL (46.5%) verified by thermogravimetric analysis. Ionic liquids containing a more hydrophobic alkyl group in the cationic moiety are beneficial for the recovery of the activity of immobilized lipase. The physico-chemical characterization confirmed the presence of enzymes immobilized derivatives obtained in this study by identifying the presence of amino groups, and profiles of enthalpy changes of mass loss.

Keywords: lipase, immobilization, sol-gel, protic ionic liquids.

## 1. Introduction

Lipase (EC 3.1.1.3) is an important enzyme with a wide variety of applications in the food, fine chemical and pharmaceutical industry due to the multiplicity of that catalyzes reactions such as esterification, transesterification and hydrolysis. [1-5]. Among them, the lipase from *Burkholderia cepacia* is distinguished by the ability to carry out organic synthesis, a fact that gives the industry great interest [6]. Applications of lipase can be achieved more economically and efficiently by immobilization to enhance its activity, selectivity, and operational stability. Therefore, numerous efforts have been focused on the preparation of lipases in immobilized forms, which involve a variety of both support materials and immobilization methods [6,7].

Compared to chemical methods for the immobilization of enzymes, physico-chemical methods, especially the sol-gel encapsulation, may have a greater commercial potential because they are simpler, less expensive and can maintain catalytic activity [8]. A very large number of biomolecules have been immobilized by the sol-gel technique and generally giving a better enzymatic activity and stability, however, there are some disadvantages in the process of immobilization sol-gel [9]. One is the shrinking of the gel during the process of condensation and drying, which can cause denaturation of enzymes. In addition, a slow diffusion of molecules of substrate for the enzyme within the sol-gel matrix hinders the catalytic activity of the immobilized enzyme in materials with a pore diameter smaller than 20 Å [10].

One way to overcome these drawbacks could be the use of additives to stabilize enzymes and assist in gel formation within arrays. Recently the use of aprotic ionic liquids have been reported as additives in the process of immobilization, which could increase the activity, the stability of immobilized enzymes by altering the hydration shell of the enzyme and reducing the shrinkage of the gel [11]. They can also affect the physical properties of the gel by participating in condensation reactions with free silanol groups [11].

The study of various additives such as ionic liquids (ILs) have recently been suggested as salts that can stabilize enzymes, protecting the hydration shell around the enzyme and/or conformational change leading to permanent activation of the enzyme [14]. Lee *et al.* [15] reported that in the process of immobilization of *Candida rugosa* lipase using the sol-gel technique showed high stability and increased enzyme activity about 10 times that of lipase in free form. The positive influence of the use of aprotic ionic liquids was also found in Zarcula *et al.* [16] using [C<sub>8</sub>Mim][BF<sub>4</sub>] in the process of immobilization of lipase from *Pseudomonas*



*fluorescens* in hybrid sol-gel matrices as an additive, the results obtained with the immobilized biocatalyst immobilization showed total income generally exceeding 100%.

Several studies were conducted using protic ionic liquids have been based on imidezolium cation and to a lesser extent using aprotic ionic liquids using alkyl pyridinium and trialkylamines[17]. The use of ionic liquids still has high costs of synthesis, hindering their industrial application [16]. However, Álvarez *et al.* [18] reports that the ionic liquids obtained from amines, organic and inorganic acids, the so-called protic ionic liquids (PIL), have low cost, simplicity of synthesis, favoring different applications and in industrial applications

Potential applications have been identified by the use of PIL with proteins, such as dissolving hydrophobic ligands (e.g., ferrocene) to incorporate them into a protein crystal, improved solubility of some proteins, improved monodispersity of proteins, as a precipitating agent, and as an additive [19]. In especial, the potential of ionic liquids leads to implementing them as additives in the process of immobilization sol-gel, addressed in to protect the inactivation of enzyme by releasing alcohol and shrinking of gel during the encapsulation process.

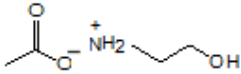
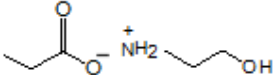
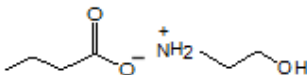
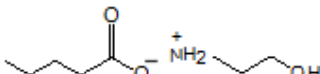
This study aimed to immobilize *Burkholderia cepacia* lipase in hydrophobic matrices obtained by sol-gel technique in the presence of various protic ionic liquids (C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub>), addition to varying the content of the added ionic liquid - C<sub>5</sub> (0.5 to 3.0%, w/v), hitherto not reported, with physico-chemical properties of ammonium-based, assessing the total yield of recovered activity, potential production of ethyl esters and emulsifiers, and physical-chemical derivatives immobilized.

## **2. Experimental procedures**

### *2.1. Materials and reagents*

Lipase from *Burkholderia cepacia* (Amano Lipase) was purchased from Aldrich. The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (New Jersey, United States) and used without further purification. Ethanol (minimum 99%), ammonia (minimum 28%), hydrochloric acid (minimum 36%) and gum Arabic were obtained from Synth (São Paulo, Brazil). Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. The protic ionic liquids view Table 6. Other chemicals were of analytical grade and used as received.

**Table 6:** Structure of protic ionic liquid used in this work.

Abbreviation	Organic acid	Structure (nomenclature)
C <sub>2</sub>	Acid acetic	 (N-methylmonoethanolamine acetate)
C <sub>3</sub>	Acid propionic	 (N-methylmonoethanolamine propionate)
C <sub>4</sub>	Acid butyric	 (N-methylmonoethanolamine butyrate)
C <sub>5</sub>	Acid pentanoic	 (N-methylmonoethanolamine pentanoate)

## 2.2. Encapsulation of lipase from *Burkholderia cepacia* in sol-gel matrices

The methodology previously established by Patent PI0306829-3 [20] was used and is briefly described as follows: 30 mL of TEOS were dissolved in 36 mL of absolute ethanol under an inert nitrogen atmosphere. To this, 0.22 mL of hydrochloric acid dissolved in 5 mL of ultra-pure water which was slowly added and the mixture was agitated (200 rpm) for 90 min at 35 °C. Added 870,71 U of lipase from *Burkholderia cepacia* solution containing 10 ml of water was simultaneously added 1% (w/v) of protic ionic liquids C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub>. As the PIL-C<sub>5</sub> has proven to be the best additive (see section 3.1), we evaluated the effect brought by varying the content of the additive added (0.5 to 3.0%, w/v). For obtained sol-gel were added to 1.0 mL of ammonium hydroxide dissolved in 6.0 mL of ethanol (hydrolysis solution) and the mixture was kept under static conditions for 24 hours to complete polycondensation. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 72 h. For comparison, the encapsulated (EN) lipase from *Burkholderia cepacia* was prepared similarly in the absence of IL (EN-AIL) and pure silica sol-gel (PS) was prepared in the absence of the enzyme and additive. The sol-gel matrix immobilized lipase was designated: EN-C<sub>2</sub>, EN-C<sub>3</sub>, EN-C<sub>4</sub>, EN-C<sub>5</sub> and EN-C<sub>5</sub>-0.5, EN-C<sub>5</sub>-1.0, EN-C<sub>5</sub>-2.0, EN-C<sub>5</sub>-3.0.

### 2.3. Enzymatic activity

Enzymatic activities of the free and immobilized lipase samples were assayed by the olive oil emulsion method according to a modification used by Soares *et al.* [21]. The substrate was prepared by mixing 50 mL of the olive oil with 50 mL of gum Arabic solution (7% w/v). The reaction mixture containing 5 mL of the oil emulsion, 4 mL of sodium phosphate buffer (0.1 M, pH 7.0) and either free (1.0 mL, 0.1 mg mL<sup>-1</sup>) or immobilized (≈250 mg) lipase was incubated in a thermostated batch reactor for 5 min (free lipase) or 10 min (immobilized lipase) at 37°C. A blank titration was done with a sample where the enzyme was replaced with distilled water. The reaction was stopped by the addition of 2 mL of acetone-ethanol-water solution (1:1:1). The liberated fatty acids were titrated with potassium hydroxide solution (0.04 M) in the presence of phenolphthalein as an indicator. All reactions were carried out in triplicate. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min (μmol.min<sup>-1</sup>) under the assay conditions (37°C, pH 7.0, 80 rpm).

Analyses of hydrolytic activities carried out on the lipase loading solution and bioencapsulated preparations were used to determine the total activity recovery yield,  $Y_a$  (%), in according to Eq. (1).

$$Y_a = \frac{U_s}{U_o} \times 100 \quad (1)$$

where: in which  $U_s$  is the total enzyme activity recovered on the support and  $U_o$  is the enzyme units offered for immobilization.

### 2.4. Ethyl esters and Emulsifier Production

Transesterification reactions catalyzed by lipase from *Burkholderia cepacia* were performed under the conditions proposed by Nouredini *et al.* [8]. The transesterification reaction was carried out in batch reactors, submerged in a thermostatic bath to keep the mixture at constant temperature and under agitation. The reaction was initiated by mixing soybean oil and ethyl alcohol (ratio 1:15.2), 0.075 g water, and lastly the free or immobilized *Burkholderia cepacia* (EN-AIL or EN-C<sub>5</sub>), as the PIL-C<sub>5</sub> has proven to be the best additive (see section 3.1). Added to each sample, equivalent to 38.3 U of enzymatic loading. Aliquots were taken out at different time intervals and were analyzed by GC gas chromatograph equipped with CARBOVAX (30 m x 0,25 mm x 0,25 μm) column. Column temperature was initially kept at 140 °C for 1 min, increased to 180 °C at 4 °C per minute, maintained for

2 min, increased to 230 °C at 10 °C per minute, and maintained for 10 min. Temperatures of injector and detector were set at 250 °C. Ethyl esters conversion (%) was defined as observed amount divided by theoretical amount when all soybean oil was converted.

The esterification activity of immobilized lipase was determined by the formation of isopropyl laurate in the reaction of isopropyl alcohol with lauric acid in the ratio (1:2,34) heptane to 52.5°C. The reaction was initiated by addition of 5.25% (w/v) of immobilized lipase and 10% (w/v) Molecular Sieves, (3x, 3.2 mm, Peppets - Sigma) to the reaction medium (5.0 g) at 150 rpm agitation for 10 minutes. The amount of isopropyl laurate formed was quantified by titration of NaOH (0.01 M). One unit of activity (esterification) was defined as the amount of enzyme that leads to the formation of 1 mol of isopropyl laurate per minute in the test conditions. The yield of esterification was quantified based on the sample blank.

### *2.5 Morphological and physicochemical properties*

The surface area of the pure silica gel and immobilized lipase deriveds was calculated using the Brunauer-Emmett-Teller method [24]. Pore volume and average pore diameter, based on BJH calculations for mesoporous samples, for microporous samples by t-method. Were evaluated by BET apparatus software (Model NOVA 1200e – Surface Area & Pore Size Analyzer, Quantachrome Instruments-version 11.0), using N<sub>2</sub> adsorption at 77 K. Before analysis, samples were submitted to a thermal treatment at 120 °C for 48 h, to eliminate any water existing within the pores of the solids.

The thermogravimetric (TG) curve was obtained in a Shimadzu DTG-60H Simultaneous DTA-TG apparatus, under a nitrogen atmosphere that started from room temperature and went up to 1000 °C at a heating rate of 20 °C.min<sup>-1</sup>. The DSC curve was obtained in a Shimadzu DSC-60, under a nitrogen atmosphere that started from room temperature and went up to 500 °C at a heating rate of 10 °C.min<sup>-1</sup>. Scanning electron microscopy (SEM; model Hitachi SU-70) was used to characterize the surface samples.

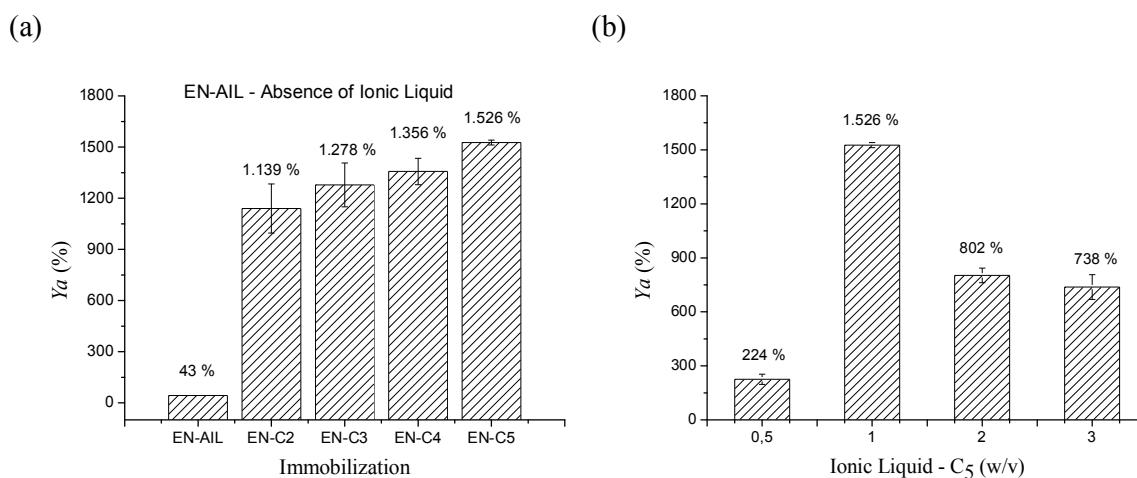
The samples of immobilized lipase in the presence and absence of additive (EN-AIL and EN- C<sub>5</sub>-1.0) were submitted to the FTIR analysis (spectrophotometer FTIR BOMEMMB-100). The spectra were obtained in the wavelength range from 400 to 4000 cm<sup>-1</sup>.

SEM was used to characterize the morphology of the immobilization procedures of the samples EN-AIL and EN-C<sub>5</sub>-1.0.

### 3. Results and Discussion

#### 3.1. Enzymatic activity of the immobilized sol-gel

The use of PIL as an additive was found to have various effects in both the process and retaining the catalytic activity of the immobilized enzyme. The results show that the increased alkyl chain of PIL, with the added content of 1.0% (w/v), enhances the yield of immobilization ( $Y_a$ ) with values always higher than 1000%. The absence of additives allow only find 43% yield of the enzymatic activity (Figure 13a). The increase in enzyme activity also occurs in immobilized lipase using additives, however, increase the number of carbon atoms (the additive) allows values near the enzymatic activity (Table 7). This is due to the use of an additive which acts as a template for the gelation and behaves as a stabilizer to protect the enzyme from inactivation by alcohol or heat released during the process [26].



**Figure 13:** Total activity recovery yield for enzyme *Burkholderia cepacia* encapsulated in sol-gel matrices: (a) Immobilized in the absence and presence of IL with different numbers of carbon atoms, (b) Immobilized in different concentrations of IL-C<sub>5</sub>.

The best recovery of activity was observed in the sample containing the ionic liquid of more hydrophobic character (IL-C<sub>5</sub>) employed during the immobilization process. The results demonstrate the positive effect, evidenced by the excellent values in the recovery of activity ( $Y_a = 1526\%$ ). The increased recovery of activity was positive effect up to the level of 1% (w/v) of the content of added IL-C<sub>5</sub> in the immobilization process.

Favorable results in the use of additives employed for the immobilization of lipases sol-gel, however, to values much lower than those found in our work was reported by Soares

*et al.* [2] found that  $Y_a = 60\%$  in the *Candida rugosa* lipase on silica using PEG-1500 as the additive. Zarcula *et al.* [16] observed recovery of lipase from *Pseudomonas fluorescens* always over 100% in hybrid sol-gel matrices using aprotic ionic liquid as an additive.

**Table 7:** Influence of alkyl chain lengths of the PIL, and concentration on recovery of total activity recovery yield of the immobilized samples.

Sample	Water Content (%)	A. Total (U)
EN-AIL	21.88	374.4
EN-C <sub>2</sub> *	11.10	9923.7
EN-C <sub>3</sub> *	15.77	12136.1
EN-C <sub>4</sub> *	14.20	11812.9
EN-C <sub>5</sub> *	10.70	13291.5
EN-C <sub>5</sub> -0.5**	7.71	1958.6
EN-C <sub>5</sub> -1.0**	10.70	13291.5
EN-C <sub>5</sub> -2.0**	13.69	6987.9
EN-C <sub>5</sub> -3.0**	18.21	6428.1

\*Immobilized with different alkyl chain lengths with PIL.

\*\* Immobilized in different concentrations of more hydrophobic character of PIL.

In this work were studied influence of the numbers of carbon atoms of the ionic liquids (methylmonoethanolamine-based) and verified that the best total total activity yield using more hydrophobic additive (IL-C<sub>5</sub>) to immobilize the enzyme from *Burkholderia cepacia*. In Table 7, derivatives immobilized with additive always presented values of water content below that the fixed assets without additive (21.88%), these results demonstrate the effects caused by the use of ionic liquid of hydrophobic character, however, the presence of water is really important because it formed a hydration shell around the enzyme inducing lipolytic activity, and possibly, the hydrophobicity of PIL provided this environment, inducing the presence of water the next most strongly bound enzyme. The results presented are consistent, because literature related that the hydrophilic nature of ionic liquids can inactivate lipases in a nonaqueous medium by taking the water that is essential for maintaining the active conformation of the enzyme [24]. This work has also been possible to verify the effect of additive concentration (IL-C<sub>5</sub>) in the process of immobilization observed that the content by addition of 1.0% (w/v), we observed an increase in the total activity recovery yield, and after the added portion in higher contents, this value decreases. This fact can be explained by the excess of PIL saturation providing the additive process (Figure 13b).

### 3.2. Ethyl esters and Emulsifier Production.

After the excellent results obtained in the sample recovery yield of enzyme activity (EN-C<sub>5</sub>-1.0), were observed, the potential reactions of esterification and transesterification for the synthesis of ethyl esters and emulsifiers (Table 8). For the production of ethyl esters, the lipase in its native form, showed 74.7% conversion in 48 h of reaction, whereas the sample immobilized in the presence of 1.0% of PIL has reached the maximum conversion of 46.5 % within 72 hours, however, the assets without the presence of PIL decreased drastically the conversion of triglycerides into ethyl esters to 1.3% in 48 hours.

In the same conditions reactions for ethyl esters production was used with *Burkholderia cepacia* immobilized in silica obtained by hydrolysis of the tetramethoxysilane (TMOS) and better ethyl esters formation of 65% [8]. The use of ionic liquids in transesterification reactions with lipases was reported by several research groups [25,26]. Ha *et al.*, [27] reports that the hydrophobic nature of ionic liquids favors the enzymatic transesterification reactions of soybean oil, increased the solubility of the reaction.

In this sense, we observed the positive effect caused by the addition of protic opening net in the conversion of triglycerides into ethyl esters for immobilization technique utilized in this work. However, the potential production of emulsifiers, verified by the esterification reaction catalyzed by derivatives and immobilized of lipase from *Burkholderia cepacia*, presents less than 5% conversion in the formation of isopropyl laurate.

**Table 8:** Transesterification reaction of soybean oil and ethanol, catalyzed by sol-gel immobilized lipase from *Burkholderia cepacia* absent of ionic liquids, the presence of 1.0% (w/v) ionic liquid and free enzyme.

Sample	Ethyl esters (%)	Reaction Time* (h)
Free enzyme	74.7	48
EN-AIL	1.3	48
EN-C <sub>5</sub> -1.0	46.5	72

\* Reaction time with higher conversion.

### 3.3. Morphological characterization of samples of lipase

#### 3.3.1. Specific surface area and porous properties

The addition of PILs changed the morphological structure of the immobilized biocatalysts (Table 9). Only eat IL addition of more hydrophobic (C<sub>4</sub> and C<sub>5</sub>) was observed

increase in surface area when compared with the pure silica matrix, however, compared with immobilized matrices without additive (EN-AIL), showed an increase in surface area, pore volume and average pore diameter for all immobilized derivatives. The largest pore size and surface area were obtained when the additive was increased C<sub>5</sub> in sol-gel process. The effect of concentration in the IL-C<sub>5</sub> biocatalysts can be seen in Table 9. At concentrations above 2.0% (w/v) had positive effect on the pore diameter (38 Å), however, immobilized matrices compared with 1.0% (w/v) showed lower surface area (245 m<sup>2</sup>.g<sup>-1</sup>). Therefore, in EN-C<sub>5</sub>-1.0, the magnitude of the volume adsorbed became larger, indicating that an optimal amount of the additive in the preparation of samples immobilized at a maximum, leading to materials with higher surface area, and then decreases, in fact, excess PIL may be blocking the pores causing the decrease in surface area (Table 9).

Thus, from the nitrogen adsorption-desorption measurements, it is clear that the additive acted as an agent of pore formation. A similar profile was observed by Vila Real *et al.* [24] who reported that the addition of IL in the process of immobilization sol-gel has an important role in the performance of enzymes, affecting the structural characteristics of the immobilized biocatalyst. Souza *et al.* [34] reported that the addition of Aliquat 336 (a quaternary salt) during the sol-gel process of immobilization of lipase from *Bacillus* sp. ITP - 001 modified the porous structure of the immobilized derivatives, such as enlargement of the pores (92 Å), surface area (183 m<sup>2</sup>.g<sup>-1</sup>) and pore volume (0.36 cc.g<sup>-1</sup>). This behavior was also reported by Zarcuła *et al.* [16] during the encapsulation of lipase in a hydrophobic matrix with a large amount of hydrophobic groups, since the hydrophobic liquids induce significant changes in the porous structure of the biocatalyst.

The consistency of these results can be verified by well-known fact that a more hydrophobic microenvironment is beneficial for lipase activity. The high values of total activity recovery yield may also be associated with the pore size and surface area, the rate of enzymatic reaction depends on the accessibility of the substrate by the enzyme. The limiting factor is the rate of diffusion of the substrate for the enzyme within the structure of silica cage. Once the enzyme is already within the channel pore after gelatinization, the pore channels needed to be just big enough to allow the diffusion of molecules of substrate and product molecules by far, the enzyme within the matrix cage [2]. With the addition of 1.0% (w/v) IL-C<sub>5</sub> in the process of immobilization of lipase from *Burkholderia cepacia*, enlargement of pores and increase in surface area can be clearly seen in relation to the biocatalyst without the ionic additive.



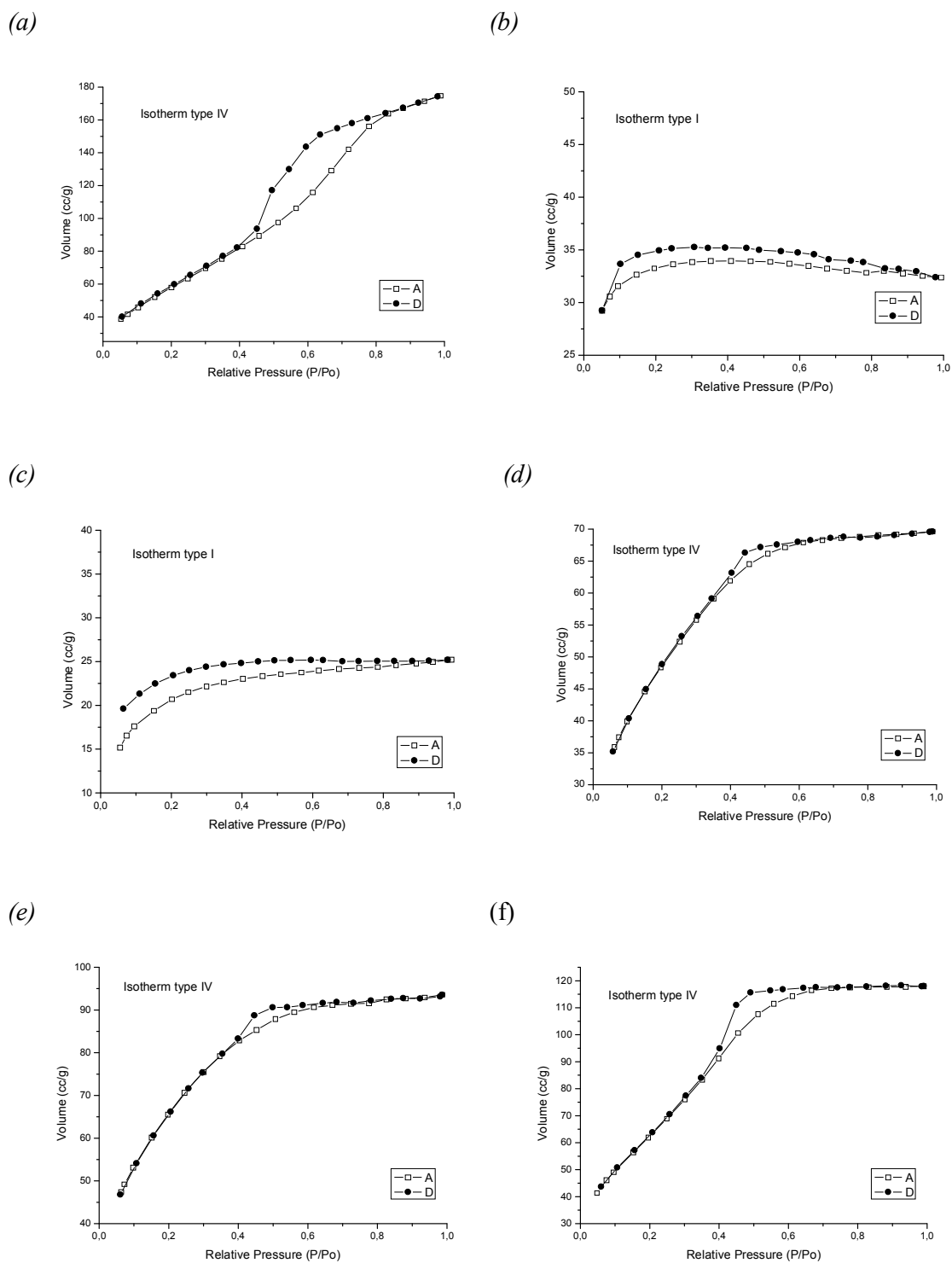
**Table 9:** Influence of concentration and the alkyl chain protic ionic liquid used as an additive in sol-gel immobilized lipase in the textural properties of adsorption-desorption of nitrogen.

Sample	Surface area (m <sup>2</sup> .g <sup>-1</sup> )	*Pore volume (cc.g <sup>-1</sup> )	Pore diameter (Å)
Pure silica	224	0.21	35
EN-AIL	143	0.05	19
EN-C <sub>2</sub>	72	0.005	19
EN-C <sub>3</sub>	176	0.02	24
EN-C <sub>4</sub>	241	0.04	24
EN-C <sub>5</sub>	245	0.08	30
EN-C <sub>5</sub> -0.5	164	0.02	26
EN-C <sub>5</sub> -1.0	245	0.08	30
EN-C <sub>5</sub> -2.0	181	0.15	38
EN-C <sub>5</sub> -3.0	121	0.08	38

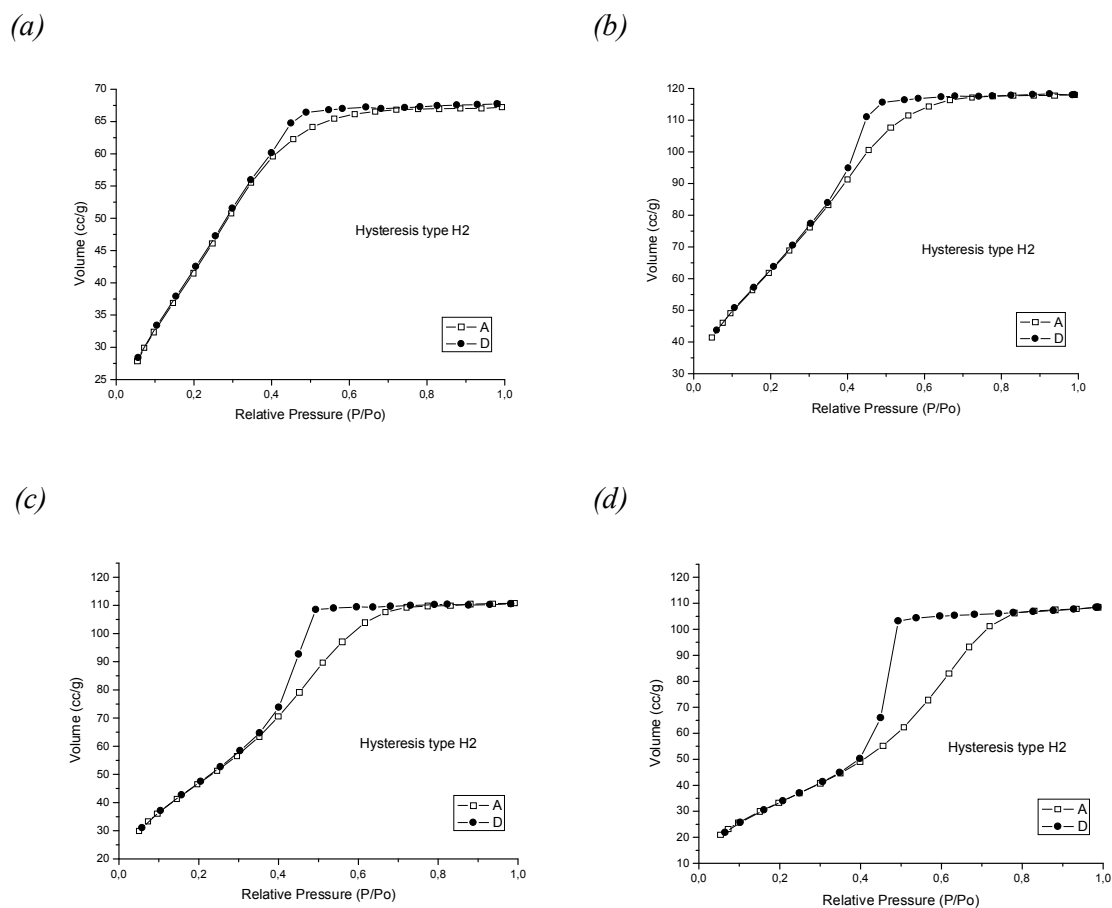
\*Pore volume calculated from nitrogen desorption.

The sample of pure silica (Figure 14a) showed a type IV isotherm with a hysteresis loop, which are usually exhibited by mesoporous solids [28]. The results observed for pure silica in the N<sub>2</sub> adsorption-desorption tests agree with those of the isotherms with H2 hysteresis loops, which is a characteristic of mesoporous materials and generally associated with pores with narrow necks and wide bodies [29]. For the sample EN-AIL and EN-C<sub>2</sub> (Figure 14b,c respectively), the isotherm is of type I, shows a rapid increase of gas adsorbed as it increases the pressure until a plateau. This isotherm is given to microporous solids having relatively small external surface and is also obtained when the adsorption form only a monolayer [30].

The matrix sol-gel immobilized of lipase from *Burkholderia cepacia* in the presence of PIL with different numbers of carbon atoms in its structure (Figure 14) and changing the concentration of the more hydrophobic ionic liquid (IL-C<sub>5</sub>), showed the type IV isotherms and loop hysteresis of type H2, which in most cases displays a prominent region in which the relative pressure and volume varies little adsorbed increases sharply [30]. However, as shown in Figure 15, increased concentration of IL present in fixed assets produced an enlargement of the hysteresis associated with the secondary process of capillary condensation, as a result brings more complete filling of the mesopore relative pressure less than 1 [30].



**Figure 14:** Nitrogen adsorption-desorption isotherms of the pure silica gel and immobilized samples: (a) PS, (b) EN-AIL, (c) EN-C<sub>2</sub>, (d) EN-C<sub>3</sub>, (e) EN-C<sub>4</sub>, (f) EN-C<sub>5</sub>.

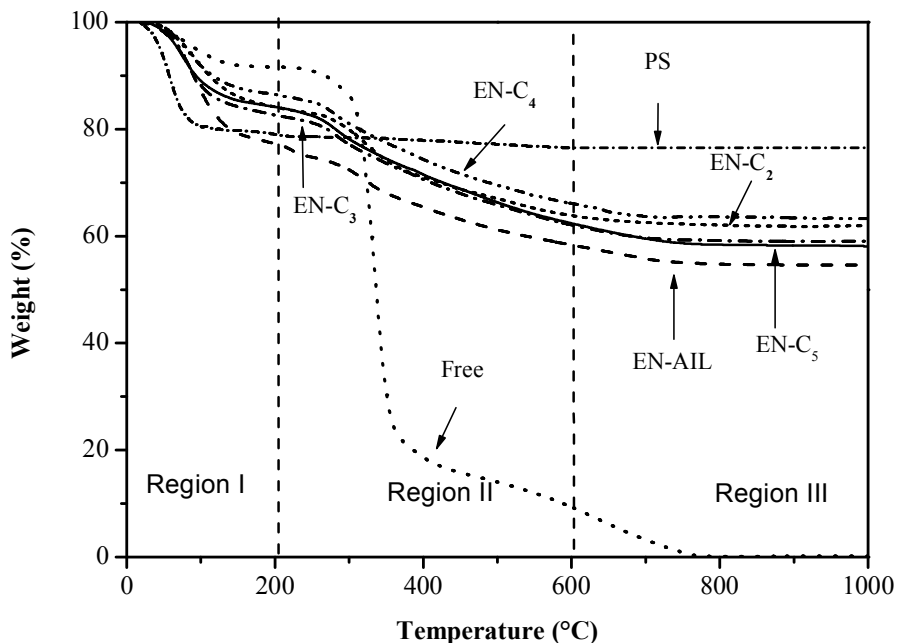


**Figure 15:** Nitrogen adsorption-desorption isotherms of the pure silica gel and immobilized samples: (a) EN-C<sub>5</sub>-0.5, (b) EN-C<sub>5</sub>-1.0, (c) EN-C<sub>5</sub>-2.0, (d) EN-C<sub>5</sub>-3.0.

### 3.3.2. Thermogravimetric analysis – TG

The mass loss of samples of pure silica (PS), biocatalyst free (free), immobilized with additive (EN-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub>, C<sub>5</sub> and EN-C<sub>5</sub>-0.5 to 3.0) and without additive (EN-AIL) were determined by thermogravimetric analysis (TGA). The weight loss obtained after heating the samples until 1000 °C are reported as TGA weight loss in Table 10. This weight loss observed is due mostly to the degradation of the enzyme present in the samples. The TGA curves for all the immobilized samples with different additives are shown in Figure 16. It is observed that the PS sample shows weight loss of only 22%. This weight loss can be attributed to the presence of un-reacted silanol groups from the TEOS, which are present in the silica, because of incomplete sol-gel reactions [31]. A part of this weight loss is also due to the removal of water molecules, which were tightly bound to the silica matrix [32]. Soares *et al.* [33] correlates a lower mass loss obtained by the additive is immobilized with a result of

increased thermal stability of the matrix, resulting from interactions between the silane precursors and the organic components (additives and lipase).



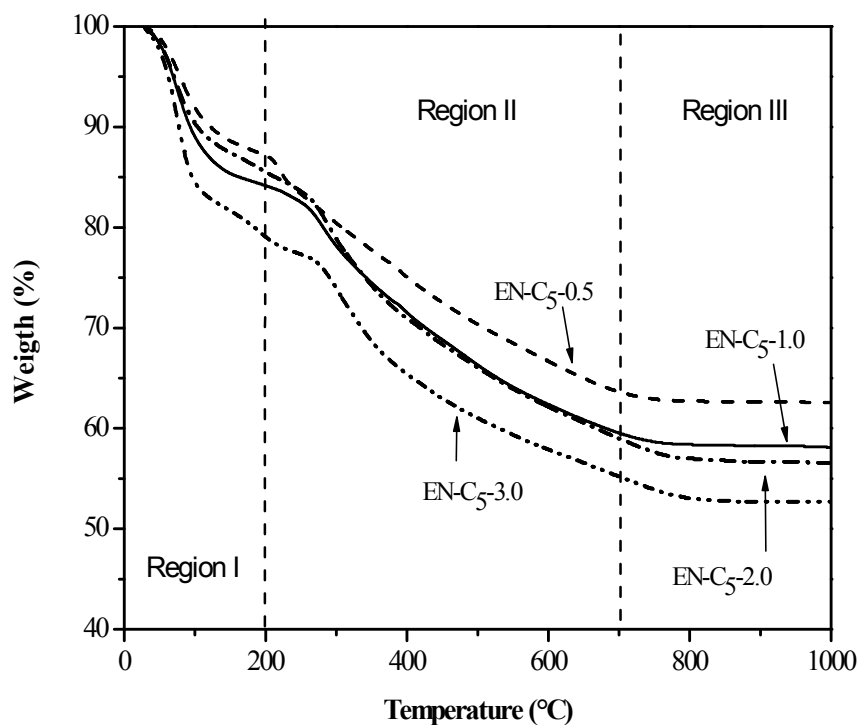
**Figure 16:** Thermogravimetric curve of samples the biocatalyst free (Free), without additive (EN-AIL) and immobilized with ionic liquid EN-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub> at 20 °C.min<sup>-1</sup> under nitrogen atmosphere.

The matrices of with immobilized IL-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub> showed values of mass loss close, however, the samples varying the concentration of IL-C<sub>5</sub> (Figure 17), we observed a trend of increasing mass loss, possibly influenced by the volume more protic ionic liquid. The thermographs were divided into three regions, region I in the weight loss is mainly associated with dehydration the water in the surface and decomposition of amino groups, usually of organic groups, the region is to understand the temperature of 200 °C. Weight loss associated with this region, may also be related to the presence of water ionic associated stoichiometrically to the cation or anion, this water may be fixed or random occupying positions in the lattice of sol-gel matrix. In region II, which comprises between 200 to 600 °C, is associated with condensation of silanols groups and some loss of organic constituents (C, H, O and N) in the form of volatiles either present or formed by the beginning of organics decomposition, including lipase, and in region III, the weight loss in this region is associated with final dehydroxylation reactions and definitive carbonisation of organic compounds,

including the lipase [33]. In the range above 750 °C is the thermal stability of the material or its complete breakdown as occurred with free sample of biocatalyst.

**Table 10:** Total loss of mass of the pure silica samples, free enzyme and immobilized of lipase from *Burkholderia cepacia*.

Samples	Total loss of mass (%)	Samples	Total loss of mass (%)	Samples	Total loss of mass (%)
Biocatalyst free	99.1	EN-C <sub>2</sub>	38.1	EN-C <sub>5</sub> -0.5	37.1
Pure silica	22.6	EN-C <sub>3</sub>	40.8	EN-C <sub>5</sub> -1.0	41.4
EN-AIL	45.1	EN-C <sub>4</sub>	36.6	EN-C <sub>5</sub> -2.0	42.9
		EN-C <sub>5</sub>	41.4	EN-C <sub>5</sub> -3.0	46.5

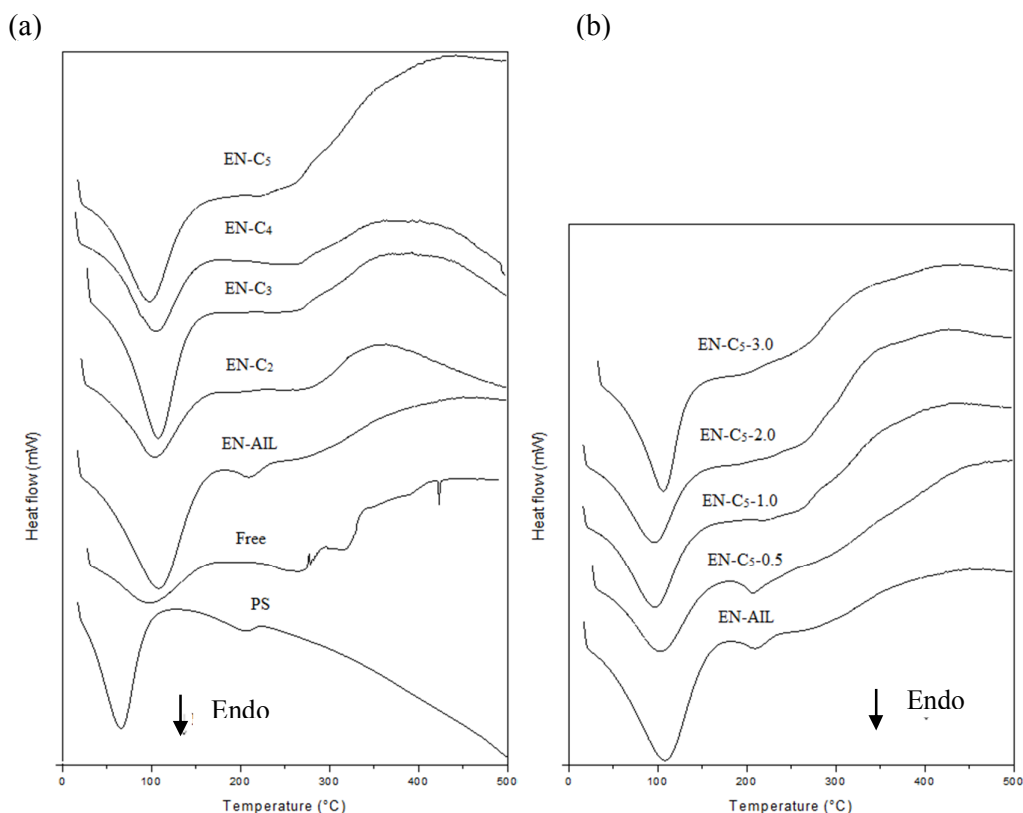


**Figure 17:** TG curve of samples immobilized with C<sub>5</sub> ionic liquid at 20°C.min<sup>-1</sup> under nitrogen atmosphere.

### 3.3.3. Differential scanning calorimetry -DSC

Differential scanning calorimetry or DSC is a technique in which a sample is subjected to controlled heating, and heat flow in and out of the sample is measured. Through this technique, phase transition in a different material is studied. These transitions give rise to endothermic or exothermic peak in the temperature range in a DSC scan.

The sample containing lipase from *Burkholderia cepacia* free enzyme (Figure 18a) showed a first endothermic peak temperature of 98°C and an enthalpy of 177.7 J.g<sup>-1</sup> the other peaks were less significant and are associated with decomposition of organic matter and loss of water. Figure 18 sample of pure silica (PS) showed only one endothermic transition with peak temperature of 65 °C and enthalpy of 364.3 J.g<sup>-1</sup>. Comparing the samples with the presence of additives immobilized C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub> with the sample without additive (EN-AIL) presented the results of changes in temperature of the exothermic peaks, since the increase in the number of carbon atoms of ionic liquids in the process of immobilization, increases the temperature of the exothermic peaks of samples (EN-C<sub>2</sub> = 364.9 °C, EN-C<sub>3</sub>= 392.5 °C EN-C<sub>4</sub>= 404.1 °C and EN-C<sub>5</sub>= 443.2 °C), DSC curves are shown in Figure 18(a).



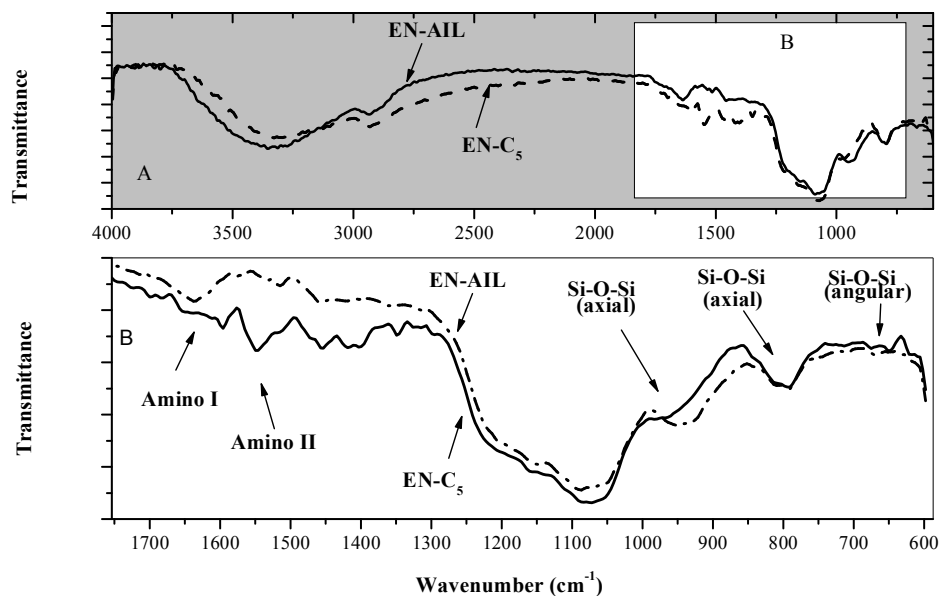
**Figure 18:** DSC curves at 10°C/min under nitrogen atmosphere of samples: (a) pure silica (PS), biocatalyst free (free), absence of IL (AIL) and EN-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub>; (b) immobilized in different concentrations of IL C<sub>5</sub>.

However, the main change caused by the increase of the concentration of IL-C<sub>5</sub> immobilized in the matrix is related to the exothermic peak, which will give it, increase energy enthalpy of these peaks (EN-C<sub>5</sub>-0.5=233.3 J.g<sup>-1</sup>, EN-C<sub>5</sub>-1.0=277.4 J.g<sup>-1</sup>, EN-C<sub>5</sub>-2.0=287.5 J.g<sup>-1</sup>, EN-C<sub>5</sub>-3.0=351.2 J.g<sup>-1</sup>). This behavior could be associated with the loss of water still present in the sample by means of so-called "water of hydration" and strongly

present in the lattice of fixed assets, since it is possible that water can be maintained on the surface of silica gel by forces different interaction, "dispersive forces" (eg, water physically connected), "polar forces" (eg, hydrogen from water on), or by "chemical forces" (eg. silanol groups that condense siloxane bonds with the release of water) [33]. This fact, which attributed to the use of additives such as ionic and hydrophobic case studied in this work, the influence of hydration layers and also the strengths of interactions between the enzyme and the support (Figure 18b).

### 3.3.4. Fourier transform infrared spectroscopy - FTIR analysis

Samples of lipase from *Burkholderia cepacia* lipase encapsulated in the absence of additive (EN-AIL) and presence (EN-C<sub>5</sub>-1.0) were characterized by FTIR, both samples showed the same characteristic FTIR spectrum (cm<sup>-1</sup>) 650 cm<sup>-1</sup> (Si-O-Si silica), 800 cm<sup>-1</sup> (Si-O-Si silica) and 950 cm<sup>-1</sup> (Si-O-Si silica) bands (Figure 19). As described in the literature [33], the lipase is two characteristic bands at 1600 and 1650 cm<sup>-1</sup> (primary and secondary amino groups) as exhibited in Figure 19. Those bands are also displayed in the spectra for the immobilized derivatives, revealing the presence of primary and secondary amino groups (lipase), particularly in samples EN-AIL and EN-C<sub>5</sub>-1.0 and more evident in the sample EN-C<sub>5</sub>-1.0 probably due to the positive effect of the ionic liquid.

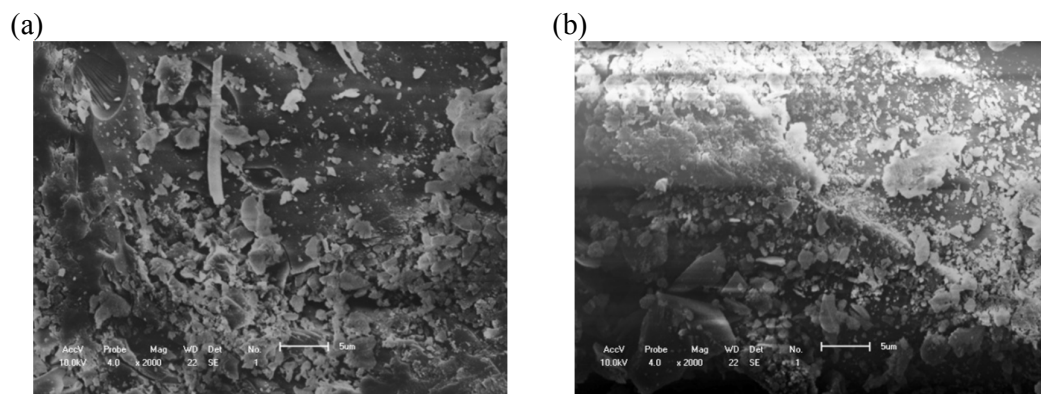


**Figure 19:** FTIR spectra for the immobilized of lipase from *Burkholderia cepacia* on silica gel in the absence and presence of protic ionic liquid (EN-AIL and EN-C<sub>5</sub>-1.0, respectively).

Consistency of results, which show higher peak intensity amino (corresponding to the presence of enzymes) in the sample immobilized lipase sol gel employing PIL as an additive, compared to the samples in the absence of additive (EN-AIL), can be verified by observing the values of conversion of ethyl esters seen in Table 8, where the results show higher values for samples immobilized using the ionic liquid. This same effect can also be verified concerning the analysis of enzymatic activity, previously seen in the Table 7.

### 3.3.5. Scanning electron microscopy (SEM)

SEM micrographs of the pure silica gel and immobilized lipases, in the presence or absence of the ionic liquid, are shown (Figure 20a, b). Conditions of porosity, surface area and pore volume gels containing lipase activity results in a favorable environment for the catalytic reactions. The 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 could be confirmed by the analysis of nitrogen adsorption. It can be observed that matrix immobilized in the absence of additive showed low surface porosity (Figure 20a). On the other hand the encapsulated lipase in the presence of protic ionic liquid (Figure 20b) showed a porous surface that appears to be more porous.



**Figure 20:** Scanning electron micrographs for: (a) EN-AIL (x2000) and (b) EN-C<sub>5</sub>-1.0 (x2000).

## 4. Conclusions

The lipases from *Burkholderia cepacia* immobilized by sol-gel technique in the presence of protic ionic liquids were successfully encapsulated. Assets in the presence of PIL, in general, showed total activity yield when more than 1000% compared with the immobilized missing additive. However, the number of carbon atoms characteristic of each IL was the



determining factor for these results, since the immobilized lipase with more hydrophobic nature of PIL (C<sub>5</sub>) showed the best total activity recovery yield, increase in surface area (245 m<sup>2</sup>.g<sup>-1</sup>) to a concentration of 1.0% (w/v) and also presented to 46.2% conversion of triglycerides into ethyl esters. Therefore, a positive effect on the morphological structure and interactions in the biocatalytic immobilized catalyst in the presence of a protic ionic liquid was observed more hydrophobic character, making it an important additive for the immobilization, since ease of synthesis, low cost and different applications of this IL new family, favors the industrial application.

### **Acknowledgements**

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## Artigo III

### **Protic ionic liquid applied to enhance the immobilization of lipase from *Bacillus* sp. ITP-001 in sol-gel matrices**

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## Abstract

The positive influence of using ionic liquids as an additive has been described in the literature for the process of lipase immobilization in hybrid sol-gel matrices. This work evaluated the capacity of protic ionic liquid (PIL) used as an additive to enhance the immobilization of lipase from *Bacillus* sp. ITP-001 in a sol-gel matrix. The immobilized derivatives were characterized with respect to the specific surface area (BET method), adsorption-desorption isothermic values, pore volume ( $V_p$ ) and size ( $d_p$ ) by nitrogen adsorption (BJH method), thermal analysis (thermogravimetric – TG, Differential scanning calorimetry – DSC) and full recovery of activity. The immobilized lipase derivatives were N-methylmonoethanolamine acetate (ITP-C<sub>2</sub>), N-methylmonoethanolamine propionate (ITP-C<sub>3</sub>), N-methylmonoethanolamine butyrate (ITP-C<sub>4</sub>) and N-methylmonoethanolamine pentanoate (ITP-C<sub>5</sub>). The results of the total recovery of activity for the samples encapsulated in PIL were always higher than those without the encapsulated additive ( $Y_a = 71\%$ ). The use of an ionic liquid of a more hydrophobic nature (IL-C<sub>5</sub>) generated the best results ( $Y_a = 305\%$ ) at concentrations of 0.5% (w/v) for ITP-C<sub>5</sub>. The positive effect of using PIL was also observed in the formation of the porous structure of the biocatalysts, as well as the increases in surface area (78 to 278 m<sup>2</sup>.g<sup>-1</sup>) and pore volume (0.018 to 0.414 cc.g<sup>-1</sup>). These results were also obtained using IL-C<sub>5</sub>, confirming the important role played by the mesoporous structure. Thermal analysis using TG/DSC verified the influence of water content on the hydration shell of the enzyme caused by the change in the alkyl chain of ionic liquids.

Keywords: lipase, immobilization, sol-gel, protic ionic liquids

## 1. Introduction

Immobilization of enzymes, in particular by the sol-gel technique, is well reported in the literature. A very large number of lipases have been successfully immobilized by sol-gel technique, highlighted by those from *Pseudomonas fluorescens*, *Penicillium roqueforti*, *Thermomyces lanuginosus* and *Burkholderia cepacia* [1,2] among others. Although lipases from *Bacillus* have been immobilized by the sol-gel technique, they present an excellent ability to catalyse the hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono and diglycerides as well as glycerol [3,4,5].

However, there are some disadvantages to the process of immobilization in sol-gel [1], such as the shrinking of the gel during the process of condensation and drying, which can cause enzyme denaturation. Moreover, a slow diffusion of substrate molecules within the sol-gel matrix hinders the catalytic activity of the immobilized enzyme in materials with a pore diameter smaller than 20 Å [6]. To this end, the use of aprotic ionic liquids (ILs) in the process of sol-gel immobilisation has recently been employed to minimise these effects, protecting the enzyme against inactivation caused by the release of alcohol during the formation and drying of the gel, thus increasing enzyme activity and stability (directly related to the hydration shell of the enzyme) and modifying the structure of the gel [7, 8, 9]. Hara *et al.* [10] also reported that the presence of ILs may cause a conformational change in the enzyme leading to permanent activation. The positive influence of using aprotic ILs was also reported by Zarcula *et al.* [11] using the IL [C<sub>8</sub>mim][BF<sub>4</sub>], more hydrophobic character of the process of sol-gel immobilized lipase from *Pseudomonas fluorescens*, results achieved of the total recovery of activity usually higher than 100%.

Recent reports have used aprotic ILs based on an imidezolium cation and, to a lesser extent, alkyl pyridinium and trialkylamines [12]. The use of ionic liquids still has high costs of synthesis, which hinders their industrial application [11]. Alternatively, there is interest in so-called protic ionic liquids (PIL), which are formed from amines, organic and inorganic acids, and have a low-cost and simple production method that favours their different applications and acceptance in industry [13]. Potential applications have been identified for PILs, such as in dissolving hydrophobic ligands (e.g., ferrocene) to incorporate them into a protein crystal, improving the solubility of some proteins, improving the monodispersity of proteins as a precipitating agent, as well as acting as an additive [14].

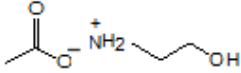
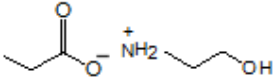
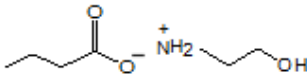
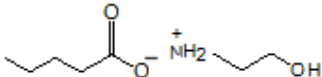
This work evaluated the capacity of PIL to modify lipase from *Bacillus* sp. ITP-001 immobilized in hydrophobic matrices obtained by the sol-gel technique. Characterization methods such as BET, TGA, DSC and the total activity recovery yield were employed.

## 2. Experimental procedures

### 2.1. Materials and reagents

The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (New Jersey, United States) and used without further purification. Ethanol (minimum 99%), ammonia (minimum 28%), hydrochloric acid (minimum 36%) and gum Arabic were obtained from Synth (São Paulo, Brazil). Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. The PILs (Table 11) used in this study were synthesised and supplied by the Department of Chemical Engineering-Salvador, Bahia. Other chemicals were of analytical grade and used as received.

**Table 11:** Structure of protic ionic liquid used in this work.

Abbreviation	Organic acid	Structure (nomenclature)
C <sub>2</sub>	Acid acetic	 (N-methylmonoethanolamine acetate)
C <sub>3</sub>	Acid propionic	 (N-methylmonoethanolamine propionate)
C <sub>4</sub>	Acid butyric	 (N-methylmonoethanolamine butyrate)
C <sub>5</sub>	Acid pentanoic	 (N-methylmonoethanolamine pentanoate)

### 2.2. Enzyme

Lipase was obtained by submerged fermentation of a *Bacillus* sp. ITP-001 strain recently isolated from soil with a history of contamination with oil (ITP, Aracaju, Sergipe, Brazil) [4]. Lipase obtained from the fermented medium was purified using an aqueous two-phase system (ATPS) containing polyethylene glycol (PEG, M<sub>w</sub> 8000, Merck) and potassium

phosphate buffer solution (30% w/v) as described by Barbosa *et al.* [4]. At the end of the purification steps, the lipase from *Bacillus* sp. ITP-001 obtained enzymatic activity of 997.5 U.g<sup>-1</sup>.

### 2.3. Encapsulation of lipase from *Bacillus* sp. ITP-001 in sol-gel matrices

The methodology previously established by Patent PI0306829-3 [15] was used and is briefly described as follows: 30 mL of TEOS were dissolved in 36 mL of absolute ethanol under an inert nitrogen atmosphere. To this, 0.22 mL of hydrochloric acid dissolved in 5 mL of ultra-pure water was slowly added and the mixture was shaken (200 rpm) for 90 min at 35 °C. The enzyme (2693.25 U) and additive (protic ionic liquid C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub>; C<sub>5</sub> at concentrations from 0.5 to 3.0%, w/v) were added to 10 mL of ultra-pure water; at the same time, 1.0 mL of ammonium hydroxide dissolved in 6.0 mL of ethanol was added (hydrolysis solution) and the mixture was kept under static conditions for 24 hours to complete polycondensation. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 72 h. For comparison, the encapsulated lipase from *Bacillus* sp. ITP-001 (ITP) was prepared similarly in the absence of IL (ITP-AIL) and pure silica sol-gel (PS) was prepared in the absence of the enzyme and additive. This yielded the following samples: PS, ITP-AIL, ITP-C<sub>2</sub>, ITP-C<sub>3</sub>, ITP-C<sub>4</sub>, ITP-C<sub>5</sub>, ITP-C<sub>5</sub>-0.5, ITP-C<sub>5</sub>-1.0, ITP-C<sub>5</sub>-2.0 and ITP-C<sub>5</sub>-3.0.

### 2.4. Enzymatic activity

Enzymatic activities of the free and immobilized lipase samples were assayed by the modified olive oil emulsion method used by Soares *et al.* [16]. The substrate was prepared by mixing 50 mL of the olive oil with 50 mL of gum Arabic solution (7% w/v). The reaction mixture containing 5 mL of the oil emulsion, 4 mL of sodium phosphate buffer (0.1 M, pH 7.0) and either free (1.0 mL, 0.1 mg.mL<sup>-1</sup>) or immobilized (≈250 mg) lipase was incubated in a thermostated batch reactor for 5 min (free lipase) or 10 min (immobilized lipase) at 37 °C. A blank titration was done with a sample where the enzyme was replaced with distilled water. The reaction was stopped by the addition of 2 mL of acetone-ethanol-water solution (1:1:1). The liberated fatty acids were titrated with potassium hydroxide solution (0.04 M) in the presence of phenolphthalein as an indicator. All reactions were carried out in triplicate. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min (μmol min<sup>-1</sup>) under the assay conditions (37 °C, pH 7.0, 80 rpm).

Analyses of hydrolytic activities carried out on the lipase loading solution and bioencapsulated preparations were used to determine the total activity recovery yield,  $Ya$  (%), according to Eq. (1).

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

in which  $U_s$  is the total enzyme activity recovered on the support and  $U_o$  is the enzyme units offered for immobilisation.

### 2.5. Morphological and physicochemical properties

The surface area of the pure silica gel and encapsulated lipase samples was calculated using the Brunauer-Emmett-Teller method [17]. Pore volume and average pore diameter were determined by BJH calculations for mesoporous samples and the t-method for microporous samples. The BET apparatus software (Model NOVA 1200e – Surface Area & Pore Size Analyzer, Quantschome Instruments-version 11.0) was employed, using  $N_2$  adsorption at 77 K. Before analysis, samples were treated at 120 °C for 48h to eliminate any water existing within the pores of the solids.

The thermogravimetric (TG) curve was obtained in a Shimadzu DTG-60H Simultaneous DTA-TG apparatus, under a nitrogen atmosphere that started from room temperature and went up to 1000 °C at a heating rate of 20 °C.min<sup>-1</sup>. The DSC curve was obtained in a Shimadzu DSC-60, under a nitrogen atmosphere that started from room temperature and went up to 500 °C at a heating rate of 10 °C.min<sup>-1</sup>. Scanning electron microscopy (SEM; model Hitachi SU-70) was used to characterize the surface samples.

The samples of pure silica matrices and immobilized lipase were submitted to the FTIR analysis (spectrophotometer FTIR BOMEMMB-100). The spectra were obtained in the wavelength range from 400 to 4000 cm<sup>-1</sup>.

## 3. Results and Discussion

### 3.1. Specific surface area and porous properties

Table 12 shows the porous modification caused by the addition of PIL to the process of sol-gel immobilization of the lipase from *Bacillus* sp. ITP-001. The results indicated that the ionic liquid with the most hydrophobic character, PIL-C<sub>5</sub>, achieved the best results for surface area and pore volume (278 m<sup>2</sup>.g<sup>-1</sup> and 0.414 cc.g<sup>-1</sup>, respectively). The increased alkyl chain lengths of the ILs in the process of immobilization increased the surface area and pore



volume, except for ITP-C<sub>4</sub>, although it gave values similar to those of the immobilized IL-C<sub>3</sub>. Moreover, increasing the alkyl chain lengths of the PIL enhanced the average pore diameter (30 Å to 48 Å) in the samples ITP-C<sub>2</sub> and ITP-C<sub>5</sub>, respectively. Although the derivatives without the immobilized additive showed lower values (ITP-AIL = 56 Å), increasing the concentration of the most hydrophobic ionic liquid (IL-C<sub>5</sub>) increased the average pore diameter values, exceeding even that of samples without the additives (Table 12).

**Table 12:** Influence of concentration and the alkyl chain length of PIL used as an additive in sol-gel immobilized lipase on the textural properties of adsorption-desorption of nitrogen.

Samples	Surface area (m <sup>2</sup> .g <sup>-1</sup> )	*Pore volume (cc.g <sup>-1</sup> )	Pore diameter (Å)
Pure silica	224	0.210	35
ITP-AIL	13	0.018	56
ITP-C <sub>2</sub>	78	0.027	30
ITP -C <sub>3</sub>	188	0.140	39
ITP -C <sub>4</sub>	144	0.016	34
ITP -C <sub>5</sub>	278	0.414	48
ITP -C <sub>5</sub> -0.5	278	0.414	48
ITP -C <sub>5</sub> -1.0	117	0.155	55
ITP -C <sub>5</sub> -2.0	78	0.068	45
ITP -C <sub>5</sub> -3.0	102	0.172	59

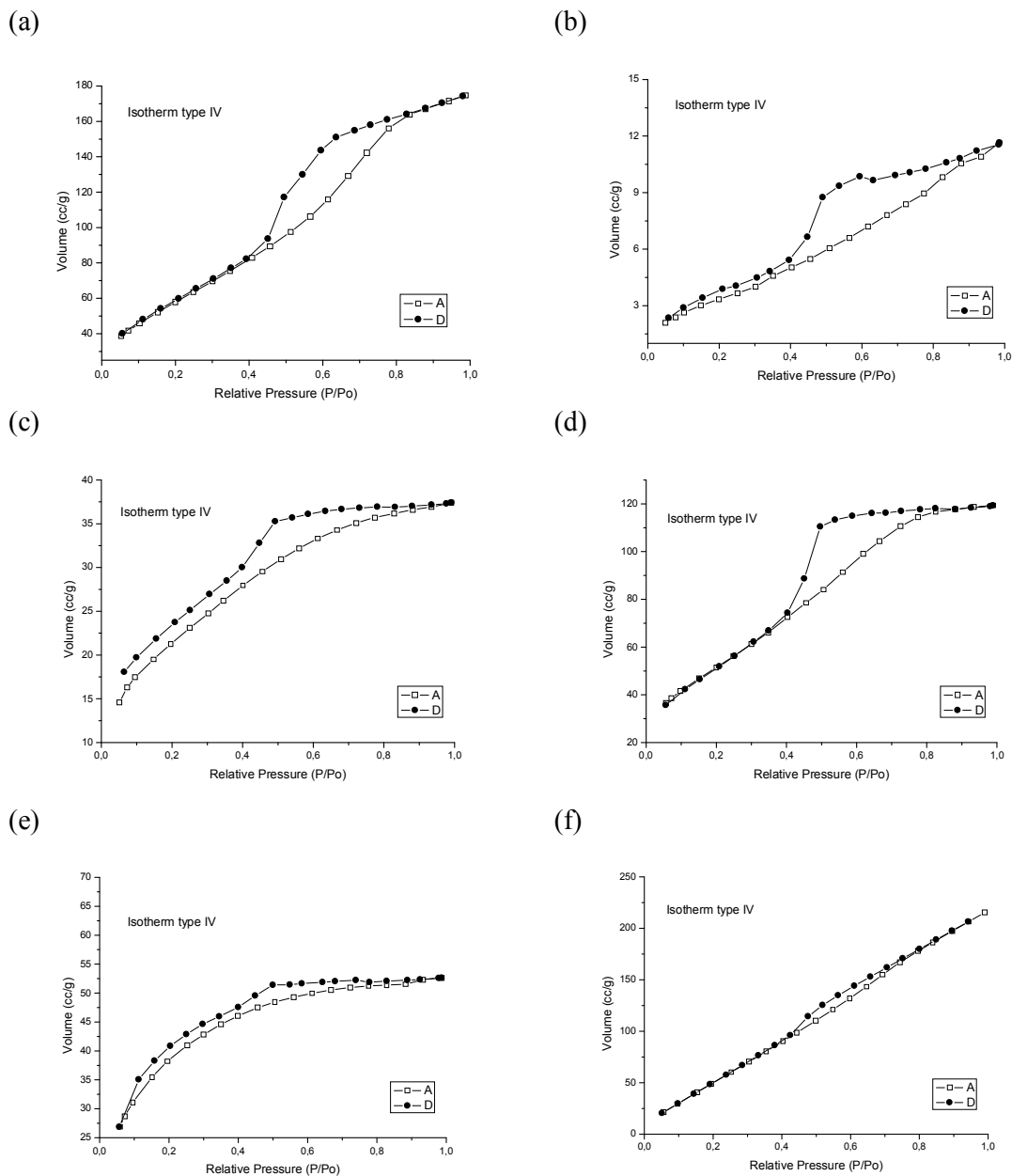
\*pore volume calculated from nitrogen desorption.

Raising the concentration of IL-C<sub>5</sub> in the immobilized sample also modified the porous structure. The values of surface area and pore volume decreased, with the exception of the sample containing 3.0% (w/v) of the ionic liquid, although this also showed reduced values of surface area and pore volume compared to the enzyme immobilized with IL-C<sub>5</sub>-0.5 (Table 12).

This structural change caused by the addition of IL is also reported by Zhou *et al.* [18], linking this to the formation of hydrogen bonds between the anion of ILs and the silanol groups of silica. Similarly, Vila Real *et al.* [17] reported that the addition of ILs to the sol-gel process played an important role in the formation of mesoporous matrices. Souza *et al.* [25] reported that the addition of Aliquat 336 (a quaternary salt) during the sol-gel process of immobilization of the lipase from *Bacillus* sp. ITP-001 modified the porous structure of the immobilized derivatives, such as an increase in the size of the pores (92 Å), surface area (183 m<sup>2</sup>.g<sup>-1</sup>) and pore volume (0.36 cc.g<sup>-1</sup>). Hydrophobic surfaces can have a positive influence on the reactivity of lipases by inducing interfacial activation, meaning that the lipase

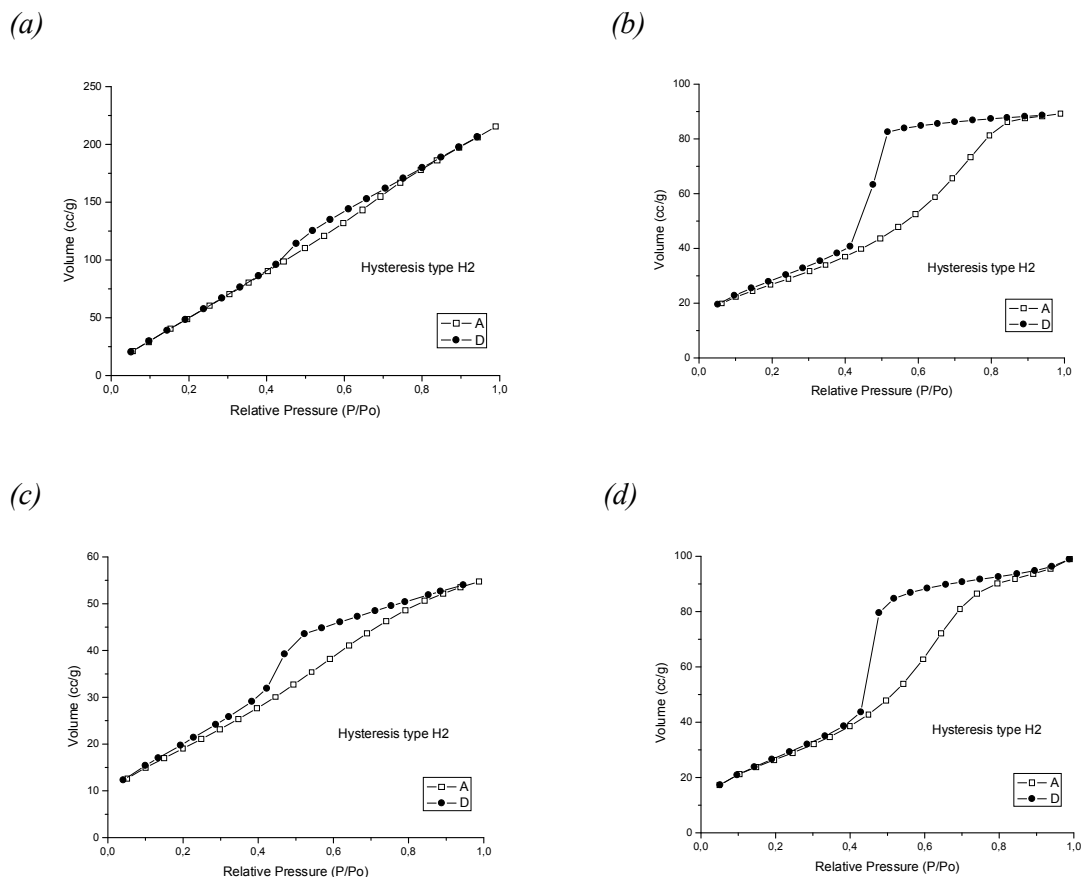
is in its active conformation. In the literature, it is also reported that IL can reduce the leaching of the enzyme used in different reactors [10].

The adsorption isotherms of pure silica derivatives (PS), as well as the immobilized lipase in the presence and absence of PIL are shown in Figure 21 and 22. All samples showed type IV isotherms with a hysteresis loop, which are usually exhibited by mesoporous solids [19]. These are of the type H2 hysteresis, which in most cases have a region in which the relative pressure and volume varies little adsorbed increases dramatically [20].



**Figure 21:** Nitrogen adsorption isotherms of the pure silica gel and immobilized samples: (a) PS, (b) ITP-AIL, (c) ITP-C<sub>2</sub>, (d) ITP-C<sub>3</sub>, (e) ITP-C<sub>4</sub> and (f) ITP-C<sub>5</sub>

As shown in Figure 22, an increased concentration of IL- $C_5$  present in the immobilized lipase sample produced an enlargement of the hysteresis associated with the secondary process of capillary condensation. This consequently elicits a more complete filling of the mesopores relative pressure less than 1 [21].



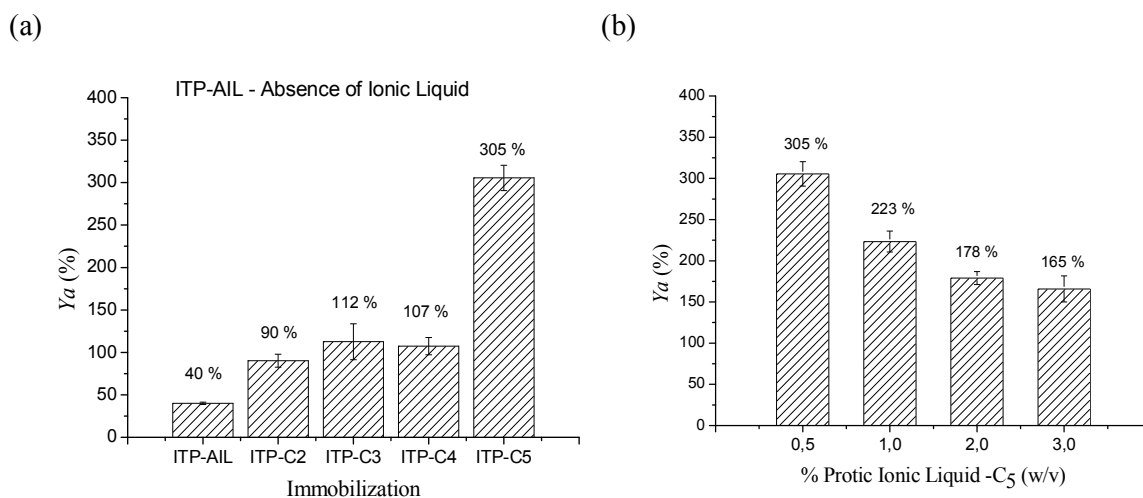
**Figure 22:** Nitrogen adsorption isotherms of the pure silica gel and immobilized samples: (a) ITP- $C_5$ -0.5, (b) ITP- $C_5$ -1.0, (c) ITP- $C_5$ -2.0 and (d) ITP- $C_5$ -3.0.

### 3.2. Enzymatic activity of the immobilized lipases

Lipase from *Bacillus* sp. ITP-001 was immobilized in sol-gel matrices obtained from a precursor of TEOS in the presence and absence of PILs at different concentrations. To test the enzymatic activity of the immobilized lipase, a modified olive oil emulsion method was used.

The enzyme immobilized with IL- $C_5$  demonstrated a full total activity yield of 305.5%, while the sample without PIL exhibited an efficiency of 40.1% (Figure 23a). Zarcula *et al.* [11] reported that the catalytic efficiency of lipase from *Pseudomonas fluorescens* could be

enhanced by the presence of hydrophobic alkyl groups in the sol-gel matrix. An ionic liquid with a hydrophobic characteristic, [C<sub>2</sub>mim] [NTf<sub>2</sub>], is used as an additive in sol-gel matrices and has been shown to act as an enzyme stabilizing agent, protecting the layer of hydration and/or conformational change, resulting in permanent activation of the enzyme [10]. This is supported by the well-known fact that the hydrophilic nature of ionic liquids can inactivate lipases in non-aqueous conditions [17], thus collaborating the findings of this study.



**Figure 23:** Total activity recovery yield of the lipase from *Bacillus* sp. ITP-001 encapsulated in sol-gel matrices: (a) immobilized in the absence and presence of IL with different numbers of carbon atoms, (b) immobilized with different concentrations of IL-C<sub>5</sub>.

Even if lower than the performance of the lipase immobilized with IL-C<sub>5</sub>, the value of the total activity yield (*Ya*) was 90.2% (sample ITP-C<sub>2</sub>), higher than the sample ITP-AIL (40.1%). In Figure 23b, it is noteworthy that 0.5% (w/v) of IL-C<sub>5</sub> was the ideal concentration in the process of immobilization; after that, the effect provided by the addition of the LIP did not favour the full recovery of activity. This same effect was observed for values of enzyme activity, which allowed an increase, the enzymatic activity, of 186.91 to 778.88 U.g<sup>-1</sup>, comparing the sample ITP-AIL and ITP-C<sub>5</sub>-0.5, respectively as shown in Table 13. The trends of these results may be related to the use of hydrophobic ILs that maintain strong links between the water molecules and the silica matrix or form a hydration shell around the enzyme. Moreover, enzymes can confuse the ILs with their natural substrate, which could explain the increase in activity and total activity recovery yield with the increasing hydrophobicity of the ILs in our study. This could also be related to the diffusion limitations in the porous substrate, which are directly related to the values of surface area, pore volume and pore diameter modified by the IL (Table 12).

Favourable results for the use of additives in sol-gel immobilization were also reported by Soares *et al.* [3], who found that  $Y_a = 60\%$  for the *Candida rugosa* lipase in silica using PEG-1500 as the additive. Zarcuła *et al.* [11] observed that the full recovery of activity of the lipase from *Pseudomonas fluorescens* was always over 100% in hybrid sol-gel matrices using aprotic ionic liquid as the additive. Lee *et al.* [21] reported that the process of immobilization of *Candida rugosa* lipase using the sol-gel technique showed high stability and increased enzyme activity about 10 times that of the lipase in free form.

**Table 13:** Influence of alkyl chain length and concentration of the PIL on recovery of total enzyme activity of the immobilized samples.

Sample	Activity (U.g <sup>-1</sup> )	Activity Total (U)
ITP-AIL	186.91	957.78
ITP-C <sub>2</sub> *	240.11	2429.42
ITP-C <sub>3</sub> *	259.27	3031.74
ITP-C <sub>4</sub> *	255.97	2889.83
ITP-C <sub>5</sub> *	778.88	8227.74
ITP-C <sub>5</sub> -0.5**	778.88	8227.74
ITP-C <sub>5</sub> -1.0**	605.01	6014.74
ITP-C <sub>5</sub> -2.0**	392.18	4820.79
ITP-C <sub>5</sub> -3.0**	481.66	4464.69

\*immobilized with different alkyl chain lengths with PIL.

\*\* immobilized in different concentrations of more hydrophobic character of PIL.

### 3.3. Morphological characterization of samples of lipase

#### 3.3.1. Thermogravimetric analysis – TGA

The loss in mass of sol-gel immobilized *Bacillus* sp. ITP-001 lipases in the presence and absence of PIL was determined by thermogravimetric analysis (TGA). TGA curves for all samples immobilized with different ILs are shown in Figure 25a. The results presented in Table 14 shows that the addition of PIL did not reduce the value of mass loss after heating to 1000 °C, which can be seen by comparing it with the immobilized lipase samples the absence of ionic liquid. However, it is possible to observe that the hydrophobic nature of the ILs

increased the entrapment of water molecules (Table 14). Furthermore, an increase in the PIL alkyl chain length hindered the release of these molecules, which may have been strongly linked to the matrix silica [22]. This was confirmed by the increase in final energy and enthalpy peak characteristic of dehydration. The values of the final temperature (*f*) of degradation increased to above 183 °C, possibly due to changes in water content and/or pore volume for lipase samples immobilized with more hydrophobic IL (Table 12 and 14). This could have been because of better functionalization over the internal mesoporous surface, confirmed by the results of BET and the full recovery of activity assays.

**Table 14:** Loss of total mass and partial derivatives of sol-gel immobilized lipase from *Bacillus* sp. ITP-001.

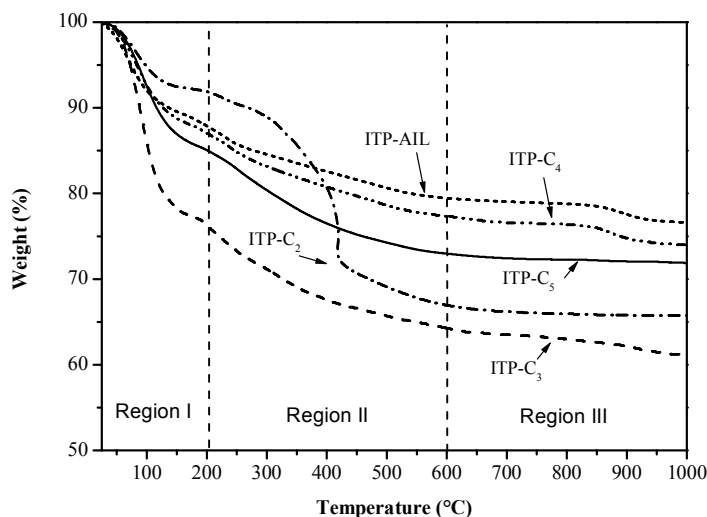
Samples	Water Content (%)	* Temp. (°C) ( <i>i</i> – <i>f</i> )	** Partial loss of mass (%)	Total loss of mass (%)
ITP-AIL	7.14	33.5 - 170.7	11.73	25.80
ITP-C <sub>2</sub>	11.86	24.0 – 183.7	14.99	29.89
ITP-C <sub>3</sub>	16.56	33.4 – 183.34	22.78	38.70
ITP-C <sub>4</sub>	8.91	30.6 – 205.7	13.32	23.70
ITP-C <sub>5</sub>	10.74	25.9 – 190.0	12.77	25.58
ITP-C <sub>5</sub> -0.5	10.74	25.9 – 190.0	14.60	28.07
ITP-C <sub>5</sub> -1.0	10.78	35.5 – 208.9	17.44	32.40
ITP-C <sub>5</sub> -2.0	10.96	33.6 – 242.8	18.42	31.50
ITP-C <sub>5</sub> -3.0	11.11	36.5 – 250.6	24.52	40.87

\* Initial temperature (*i*) and final (*f*) the main peak of degradation determined by analysis of the DTA curve.

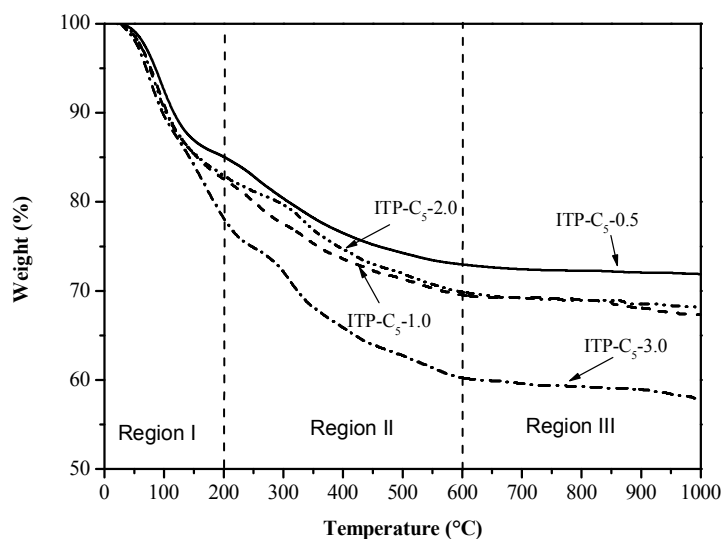
\*\* Mass loss occurred in derivative assets related to its higher peak degradation.

The thermograms were divided into three regions (Figure 24 and 25). In region I (up to temperatures close to 200 °C), weight loss was mainly associated with dehydration and decomposition of amino groups, usually of organic groups. Region II (temperature ranged from 200 to 600 °C) was associated with the condensation of silanol groups and some loss of organic components (C, H, O and N) in the form of volatile compounds present or formed until the onset of organic decomposition. This included the lipase and the presence of non-reacted silanol groups of the TEOS, which are present in the silica because of incomplete sol-gel reactions [23]. Region III was linked to weight loss associated with the final

dehydroxylation reactions [24] and the final carbonization of organic compounds, inducing thermal stability of the material or its complete degradation.



**Figure 24:** Thermogravimetric curve of samples the without additive (ITP-AIL) and immobilized with ionic liquid ITP-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub> at 20 °C.min<sup>-1</sup> under nitrogen atmosphere.

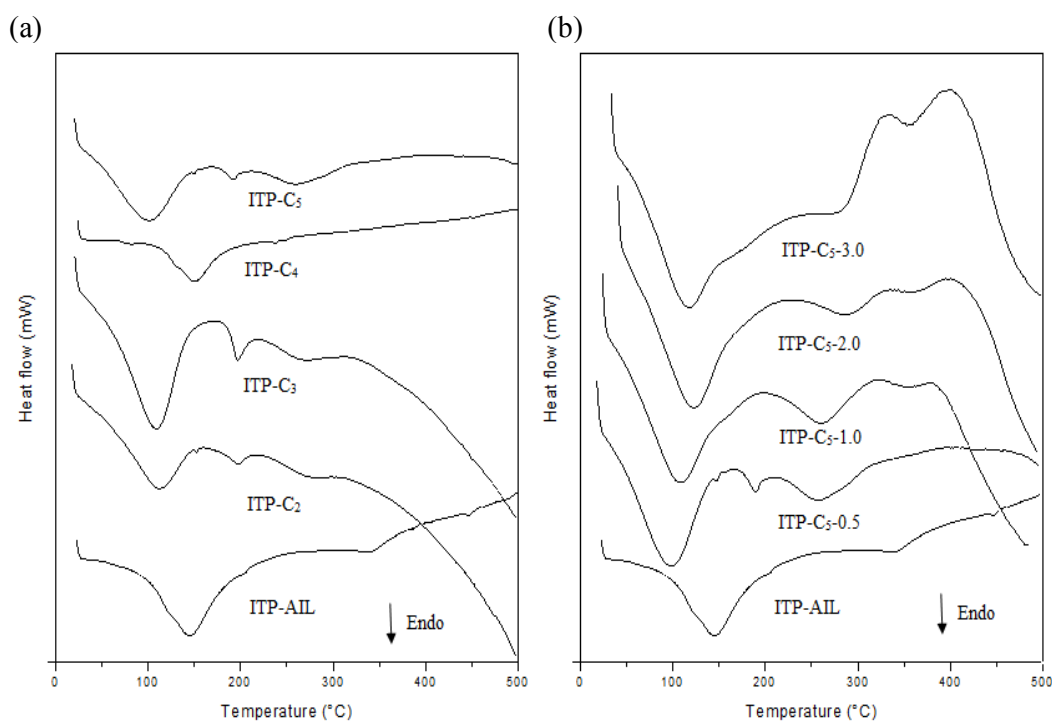


**Figure 25:** TG curve of samples the without additive (ITP-AIL) and immobilized with C<sub>5</sub> ionic liquid at 20 °C.min<sup>-1</sup> under nitrogen atmosphere.

### 3.3.2. Differential scanning calorimetry -DSC

Data from samples of sol-gel immobilized lipase in the absence and presence of IL were subjected to DSC calorimetric analysis to study the phase transitions. These transitions give rise to exothermic or endothermic peaks in the range of temperature in a DSC scan.

The DSC curves shown in Figure 26 complement the thermogravimetric curves discussed above and are consistent with the effect provided by the use of IL in sol-gel matrices. Comparing the samples immobilized in the presence and absence of additives, it is possible to observe an increase in energy enthalpy associated with endothermic peaks of  $105.2 \text{ J.g}^{-1}$  (in the sample ITP-AIL) until it reaches its maximum at  $462.9 \text{ J.g}^{-1}$  (in the sample ITP-C<sub>3</sub>). The other samples immobilized with PIL also increased their peaks, ITP-C<sub>2</sub>, C<sub>4</sub> and C<sub>5</sub> reaching 256.12, 121.3 and  $210.6 \text{ J.g}^{-1}$ , respectively. There was also an increase in the energy values of the endothermic peaks with increasing concentration of PIL: ITP-AIL, ITP-C<sub>5</sub>-0.5, ITP-C<sub>5</sub>-1.0, ITP-C<sub>5</sub>-2.0 and ITP-C<sub>5</sub>-3.0 gave values of 105.2 to 210.6, 403.4, 399.5 and 448, 15  $\text{J.g}^{-1}$ , respectively. This increase is directly associated with the loss of water still present in the sample, called "water of hydration", which is present on the surface of the silica gel with different strengths of interactions such as dispersion forces, polar forces and/or ionic strength [22].



**Figure 26:** DSC curves at  $10^\circ\text{C}/\text{min}$  under nitrogen atmosphere of samples: (a) ITP-AIL, ITP-C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub> and C<sub>5</sub>, (b) ITP-AIL and immobilized with C<sub>5</sub> ionic liquid.

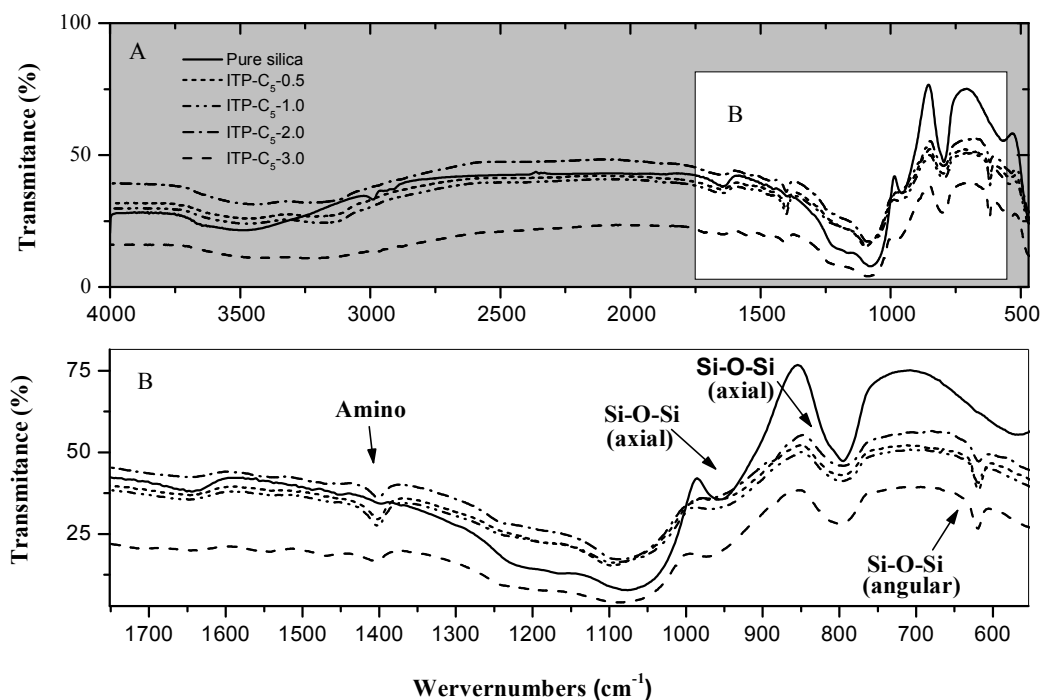
In fact, the use of ionic and hydrophobic additives, such as those studied here, influences the hydration shell of the enzyme and the forces of interactions between the enzyme and support.



We can conclude that these immobilized lipase samples with IL are stable at temperatures lower than those that can be tolerated by immobilized lipase samples without ionic liquid. Therefore, when the decomposition temperature is reached and the enzyme splits from the ionic liquid encapsulation, they leave the system almost at the same time, regardless of the difference in their melting point, as confirmed by the thermogravimetric analysis (Figure 24 and 26b).

### 3.3.3. Fourier transform infrared spectroscopy - FTIR analysis

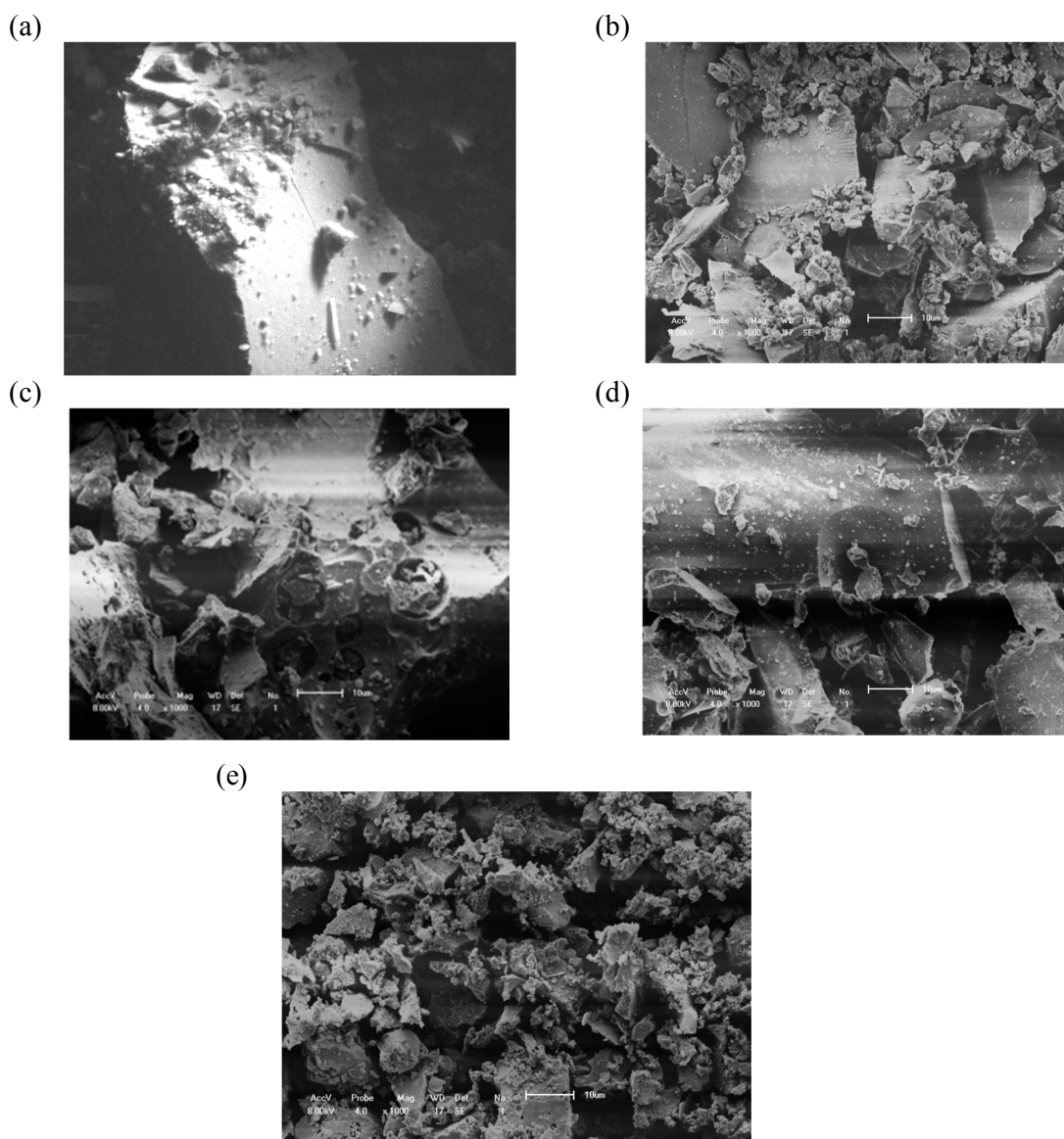
Samples of lipase from *Bacillus* sp. ITP-001 encapsulated in the absence of additive (ITP-AIL) and presence of different concentrations was characterized by FTIR, both samples showed the same characteristic FTIR spectrum ( $\text{cm}^{-1}$ )  $650 \text{ cm}^{-1}$  (Si-O-Si silica),  $800 \text{ cm}^{-1}$  (Si-O-Si silica) and  $950 \text{ cm}^{-1}$  (Si-O-Si silica) bands (Figure 27). The presence of *Bacillus* lipase in the presence of immobilized IL is confirmed by observing the amino group in the characteristic band at  $1400 \text{ cm}^{-1}$  as exhibited in Figure 27. This band, in a particular does not appear in the spectra of the sample PS (pure silica), showing the efficiency of the immobilization process using PIL.



**Figure 27:** FTIR spectra for the immobilized of lipase from *Bacillus* ITP-001 on silica gel in the presence of protic ionic liquid and pure silica.

### 3.3.4. Scanning electron microscopy (SEM)

SEM micrographs of pure silica gel and immobilized lipase in the presence of PIL are shown in Figure 28. From these images, one can observe the morphology of the surface had an irregular pattern for the sol-gel matrices and derivatives thereof. Consequently, these images are not able to identify the pore size, or to determine size distribution. By analyzing the micrographs of pure silica gel (Figure 28a), one can observe the presence of a rigid surface, probably forming a single block. The enzyme and the PIL induce a change in morphology of the sol-gel pure silica.



**Figure 28:** Scanning electron micrographs for: (a) pure silica gel (x2000), (b) ITP-C<sub>5</sub>-0.5 (x1000), (c) ITP-C<sub>5</sub>-1.0 (x1000), (d) ITP-C<sub>5</sub>-1.5 (x1000) and (e) ITP-C<sub>5</sub>-3.0 (x1000).

The morphology biocatalyst presented a higher porosity of the surface structure with more rounded shapes, although pores were still irregular. The crystals were composed of large accumulations of porous particles that increase the porosity to the immobilized samples, as shown in Figure 28 (b – e) samples and ITP-C<sub>5</sub>-0.5 (x1000), ITP-C<sub>5</sub>-1.0 (x1000), ITP-C<sub>5</sub>-2.0 (x1000), and ITP-C<sub>5</sub>-3.0 (x1000), respectively. These results are consistent with those obtained by determining the specific surface area and porosity properties. However, it was found that some particles by SEM were formed on the surface of material immobilized with PIL with a structure similar to those obtained in pure silica. This indicates that during the preparation of biocatalysts, at some stage, homogeneity was not complete, as expected with the sol-gel technique.

#### 4. Conclusions

Immobilization of lipase from *Bacillus* sp. ITP-001 using PIL as an additive in the sol-gel process was successful. The hydrophobic portion of the additive modified the surface area (from 13 to 278 m<sup>2</sup>.g<sup>-1</sup>) and pore volume (from 0.18 to 0.414 cc.g<sup>-1</sup>) of the lipase samples, while maintaining a mesoporous structure that facilitated access to the substrate. The change brought about by increasing the PIL alkyl chain length was also consistent with the results of activity (186.91 to 778.88 U.g<sup>-1</sup>) and total activity recovery yield (71.1 to 305.5%). These values reached the best values when using 0.5% (w/v) of IL-C<sub>5</sub>, compared to the immobilized sample without the additive (ITP-AIL). The thermal TGA/DSC analysis verified the influence of water content (essential for enzyme activation), caused by the change in the alkyl chain length of the ionic liquid. Therefore, the use of these additives could be considered in the process of immobilization.

#### Acknowledgements

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## Artigo IV

### **Employment of polyethylene glycol in the process of sol-gel encapsulation of *Burkholderia cepacia* lipase**

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## Abstract

Lipases from *Burkholderia cepacia* were encapsulated using polyethylene glycol (PEG,  $M_w$  1500) at various concentrations (0.5 to 3.0%, w/v) as an additive during the sol-gel immobilization process. The matrix immobilized in the presence and absence of additives were characterized by thermal analysis (thermogravimetric (TG) and differential scanning calorimetry (DSC)), scanning electron microscopy (SEM), enzymatic activity and total activity recovery yield ( $Y_a$ ). The addition of PEG increased the activity values, with  $Y_a$  just above 1.0% (w/v) in the presence of PEG. The additional of 1.0% (w/v) PEG increased enzymatic activity from 33.98 to 89.91  $U \cdot g^{-1}$  and the values of recovery yield were 43.0 to 91.4%, compared to values of the samples without PEG. PEG enhanced the thermal stability of the matrix structure in the temperature range 50 to 200 °C, as confirmed by TGA and DSC analyses. This was influenced by the presence of water bound to the matrix. The SEM micrographs clearly showed an increase in the number of deposition on the material surface, producing matrices with greater porosity.

Keywords: lipase, immobilization, sol-gel, polyethylene glycol.

## 1. Introduction

Applications of lipases in industrial processes are becoming increasingly important. The commercial values of these enzymes may be enhanced by using immobilisation techniques that maintain catalytic efficiency and operational stability. Therefore, numerous efforts have been focused on preparing immobilized lipase in ways that involve both a variety of support materials and immobilization methods [1,2]. Several different approaches have been used to immobilize the lipase from *Burkholderia cepacia* on various supports [3,4,5]. In the literature, use of additives in lipase immobilization on the surface of a solid support through hydrophobic interactions has been widely reported for applications of lipase in industrial processes [6-9]. Activation at the hydrophobic interface is a characteristic property of lipases, which was first reported by Sarda and Desnuelle in 1958 [10].

One alternative for the immobilization of enzymes is gel entrapment, which involves lesser structural strain for the enzyme as it is captured in its native form in a specific microenvironment that can enhance the stability and activity of the lipase. By contrast, in covalent attachment and cross-linking, the enzyme molecule can be bound to the carrier or another protein in a way that hinders the reaction by distorting the active conformation of the enzyme or blocking the access of the substrate to the active site [8].

Sol-gel coatings allow for coverage of complex structures and the target compound is deposited on the substrate surface of a colloidal suspension (sol) [11]. Hydrolysis and polycondensation of alkoxide precursors in solution at a low temperature is based on the sol-gel process, leading to the formation of a polymer network that allows the incorporation of a variety of compounds [11,12,13]. It is an alternative to produce materials exhibiting stable physical properties that cannot be obtained by other processes [14,15]. A very large number of biomolecules, including enzymes, antibodies, microorganisms, plant and animal cells, have been immobilized by the sol-gel technique [16-19]. This generally gives better enzyme activity and stability; however, there are some disadvantages in the process of sol-gel immobilization [20,21].

The use of additives in the process of sol-gel immobilization is one way of circumventing the negative effects caused during the shrinkage of the gel [12,22-24]. The use of macromolecules such as polyethylene glycol (PEG) in the process of immobilization has been reported to elicit a better distribution of the lipase on the mounting surface, allowing better contact between the water/oil interface and favouring necessary conditions for the hydrolytic activity of the immobilized lipase [12,25,27].



Through the careful selection of additives, these materials can be designed for specific applications and can produce useful, robust devices. Despite reports in the literature about the use of PEG for lipase immobilization, none have studied the effects of varying the concentration of the additive on immobilized lipases.

Therefore, the objective of this work was to use PEG as an additive at different concentrations during the sol-gel immobilization of lipase from *Burkholderia cepacia*. Moreover, morphological structure (scanning electron microscopy (SEM)), thermal properties (differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)) and full recovery of activity were characterized.

## **2. Experimental procedures**

### *2.1. Materials and reagents*

Lipase from *Burkholderia cepacia* (Amano Lipase) was purchased from Aldrich. The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (New Jersey, United States) and used without further purification. Ethanol (minimum 99%), ammonia (minimum 28%), hydrochloric acid (minimum 36%) and gum Arabic were obtained from Synth (São Paulo, Brazil). Polyethylene glycol (PEG) with a nominal molecular weight of 1500 g.mol<sup>-1</sup> was purchased from Sigma Chemical (Co., USA). Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. Other chemicals were of analytical grade and used as received.

### *2.2. Encapsulation of lipase from Burkholderia cepacia in sol-gel matrices.*

The methodology previously established by Patent PI0306829-3 [26] was used and is briefly described as follows: 30 mL of TEOS were dissolved in 36 mL of absolute ethanol under an inert nitrogen atmosphere. To this, 0.22 mL of hydrochloric acid dissolved in 5 mL of ultra-pure water was slowly added and the mixture was shaken (200 rpm) for 90 min at 35°C. The enzyme (2693.25 U) and additive (PEG, M<sub>w</sub> 1500, used at concentrations from 0.5 to 3.0% (w/v)) were added to 10 mL of ultra-pure water; at the same time, 1.0 mL of ammonium hydroxide dissolved in 6.0 mL of ethanol was added (hydrolysis solution) and the mixture was kept under static conditions for 24 hours to complete polycondensation. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 72h. For comparison, the encapsulated lipase from *Burkholderia cepacia* was prepared similarly in the absence of PEG (EN-AP) and pure silica sol-gel (PS) was prepared in the

absence of both the enzyme and additive. The samples with the presence of PEG were designated as EN-P0.5, EN-P1.0, EN-P2.0 and EN-P3.0.

### 2.3. Enzymatic activity

The determination of enzymatic activity of lipases with and without PEG was performed by the method described by Soares *et al.* [6], with some modifications. The substrate was prepared by mixing 50 mL of the olive oil with 50 mL of gum Arabic solution (7% w/v). The reaction mixture containing 5 mL of the oil emulsion, 4 mL of sodium phosphate buffer (0.1 M, pH 7.0) and either free (1.0 mL, 0.1 mg.mL<sup>-1</sup>) or immobilized ( $\approx$ 250 mg) lipase was incubated in a thermostated batch reactor for 5 min (free lipase) or 10 min (immobilized lipase) at 37 °C. A blank titration was done with a sample where the enzyme was replaced with distilled water. The reaction was stopped by the addition of 2 mL of acetone-ethanol-water solution (1:1:1). The liberated fatty acids were titrated with potassium hydroxide solution (0.04 M) in the presence of phenolphthalein as an indicator. All reactions were carried out in triplicate. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1  $\mu$ mol of free fatty acid per min ( $\mu$ mol.min<sup>-1</sup>) under the assay conditions (37 °C, pH 7.0, 80 rpm).

Analyses of hydrolytic activities carried out in the lipase loading solution and encapsulated preparations were used to determine the total activity recovery yield,  $Y_a$  (%), according to Eq. (1).

$$Y_a = \frac{U_s}{U_o} \times 100 \quad (1)$$

in which  $U_s$  is the total enzyme activity recovered on the support and  $U_o$  is the enzyme units offered for immobilization.

### 2.4. Sample characterization

Scanning electron microscopy (SEM; model Hitachi SU-70) was used to characterize the surface of pure silica matrices and immobilized lipase samples. The thermogravimetric (TG) curve was obtained in a Shimadzu DTG-60H Simultaneous DTA-TG apparatus, under a nitrogen atmosphere that started from room temperature and went up to 1000 °C at a heating rate of 20 °C.min<sup>-1</sup>. The DSC curve was obtained in a Shimadzu DSC-60, under a nitrogen atmosphere that started from room temperature and went up to 500 °C at a heating rate of 10 °C.min<sup>-1</sup>.

### 3. Results and Discussion

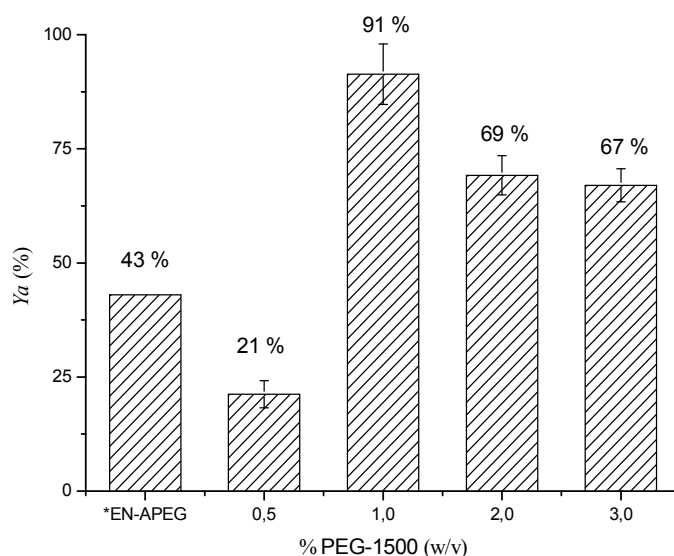
#### 3.1. Enzymatic activity of the immobilized lipase

The motivation for the use of additives in the process of sol-gel immobilization arose from the possibility of preserving the catalytic activity of the lipase from *Burkholderia cepacia* during the process of gel formation. Therefore, in this study, we evaluated the effect of PEG concentration on the process of encapsulation of lipase by measuring the catalytic activity of the enzyme Table 15.

**Table 15:** Influence of PEG concentration used for lipase encapsulation on enzyme activity.

Samples	Dry mass (g)	Enzymatic activity (U.g <sup>-1</sup> )	Total Activity (U)
EN-AP	14.11	33.98 ± 0,00	374.00
EN-P0.5	7.53	30.56 ± 5,25	184.69
EN-P1.0	9.65	89.91 ± 6,51	795.47
EN-P2.0	8.68	76.65 ± 4,75	602.42
EN-P3.0	8.21	76.84 ± 4,17	583.39

The use of low-molecular-weight additives such as 1.0% (w/v) PEG enhances catalytic activity, according to the literature, when the *Candida rugosa* lipase is covalently immobilized on silanized-controlled pore silica (CPS) [28,29]. We also observed the same trend; increasing PEG concentration during the sol-gel immobilization of the lipase increased enzyme activity from 33.98 to 89.91 U.g<sup>-1</sup>, when compared to the sample without PEG (EN-AP). The same effect was observed for the total activity yield recovered (43.0 to 91.4%), as seen in Figure 29. Raising the concentration of PEG induced a favourable condition for enzymatic activity. Decreases in catalytic activity and yield of enzymatic activity ( $Y_a$ ) were observed when the amount of PEG used was above its optimal level (1.0% w/v). This might have been due to reduced pore size and substrate accessibility. In accordance with our results, Soares *et al.* [12] also observed an increase in the yield of recovery of enzymatic activity of immobilized *Candida rugosa* lipase, reaching values of 60% in the presence of PEG; however, their values were lower than ours. Mohidem and Mat [25] found that the catalytic activity of laccase was enhanced using PEG of low-molecular weight (PEG,  $M_w$  600) during sol-gel immobilization.



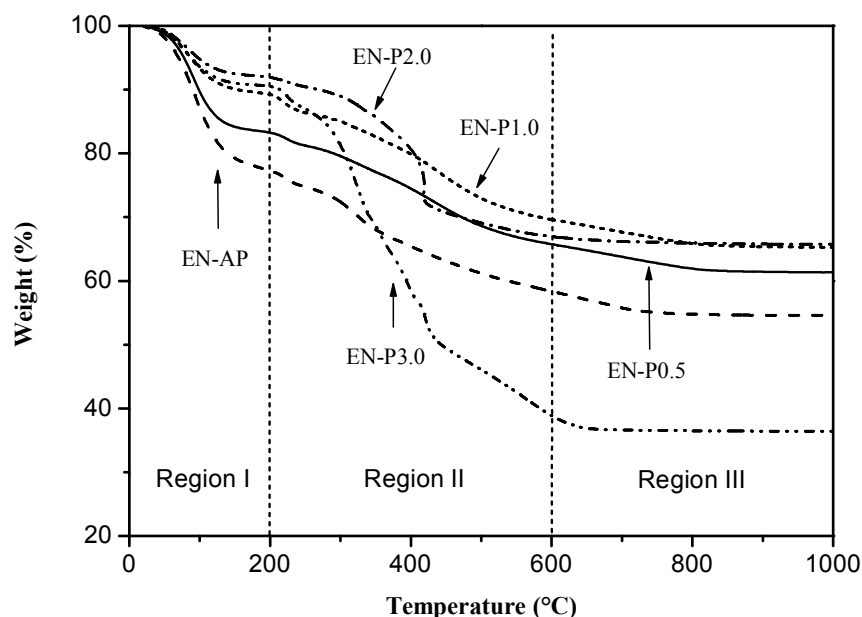
**Figure 29:** Total activity recovery yield of lipase from *Burkholderia cepacia* encapsulated in the absence and presence of different concentrations of PEG.

Our results showed the positive effect of PEG on the encapsulation process of lipase, confirming the literature data that indicated low-molecular-weight additives such as PVA, PEG and APTS to increase the catalytic activity of enzymes [25,29]. The presence of PEG during the immobilization process probably affects the moisture level of the lipase by modifying the hydrophobicity of the microenvironment, similar to that proposed Mohidem *et al.* [25].

The additive can also act as an agent model or modify the morphology of the pores of the gel, thereby facilitating the internal mass transfer and providing better accessibility of the substrate, similar to that reported by Yi *et al.* [30].

### 3.2. Thermogravimetric analysis – TGA

Figure 30 shows the TG curves for samples of immobilized enzyme in the presence and absence of PEG. The weight loss obtained after heating the samples to 1000 °C are reported as TG weight loss in Table 16.



**Figure 30:** TG curves of samples without PEG (EN-AP) and with PEG at  $20\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$  under a nitrogen atmosphere.

The thermograms were divided into three regions to illustrate which groups were lost in specific temperature ranges. Region I was mainly associated with dehydration, the decomposition of amino groups and the solvent adsorbed onto the surface of the material, usually organic groups. This region comprised temperatures close to  $200\text{ }^{\circ}\text{C}$ . We could observe that increasing the amount of PEG, which has hydrophobic characteristics, induced a lower loss in mass in this temperature range compared to the sample without the additive (EN-AP), as shown in Table 16. Rao and Kalesh [31], in agreement with the observed results, reported that only materials prepared with TEOS presented hydrophilic groups such as Si-OH on their surfaces. These groups can absorb moisture from the environment and therefore, undergo a significant loss of mass at temperatures between  $50$  and  $200\text{ }^{\circ}\text{C}$ . This was observed in the sample EN-AP, which exhibited the highest loss in mass ( $22.22\%$ ) and water content ( $21.88\%$ ) in this temperature range. This could have been due to the evaporation of water and/or alcohol groups that are produced from condensation reactions between the Si-OH groups. Thus, the presence of a higher amount of Si-OH groups in the matrix immobilized matrix does not promote enzymatic activity, as seen previously in Figure 29. This may be linked to the fact that a higher content of water is directed into the matrix, increasing the hydration shell of the enzyme and consequently, enhancing enzymatic activity.

In fact, the greatest loss in mass in this region (Region II in Figure 30) occurred when the lipase was immobilized with PEG.

Region II, which comprised temperatures between approximately 200 and 600°C, was associated with the loss of organic components (C, H, O and N), including lipases, in the form of volatile compounds present or formed at the beginning of organic decomposition, as well as the loss of unreacted silanol groups of TEOS present in the silica because of incomplete sol-gel reactions [32] and the removal of water molecules that were strongly linked to the silica matrix [33]. The results presented in this temperature range justified the use of PEG in lipase immobilization, as the lipase-PEG sample displayed greater weight loss in this region than in region I (Table 16), showing that the particle of interacted with the silica molecules of PEG.

The weight loss in region III was associated with dehydroxylation reactions [34], as well as the carbonization of organic compounds. At temperatures above 750 °C, the material was completely degraded.

### 3.3. Differential scanning calorimetry – DSC

Data of sol-gel encapsulated samples of *Burkholderia cepacia* lipase in the absence and presence of PEG were submitted to DSC calorimetric analysis to study the phase transitions. These transitions give rise to exothermic or endothermic peaks in a particular temperature range in a DSC scan.

The DSC curves shown in Figure 31 complemented the TG curves discussed earlier. The reduction of the enthalpy values was related to the increase in PEG concentration; values decreased from 530.34 to 345.79, 258.45, 153.27 and 196.79 J.g<sup>-1</sup> for EN-AP, EN-P0.5, EN-P1.0, EN-P2.0 and EN-P3.0, respectively. These results are consistent, since a greater amount of energy is required for decomposition and dehydration, especially at temperatures leading up to 200°C for lipases without PEG.

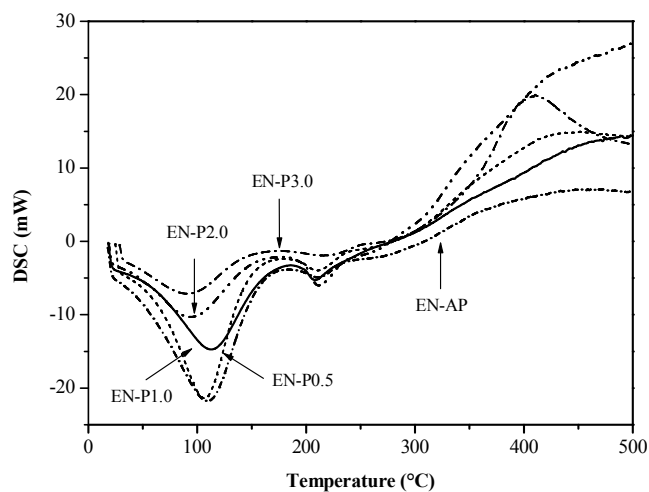
The endothermic and exothermic peaks (Figure 31) correlated with the decomposition of organic compounds, including lipase and silanol groups [32], as well as with the removal of water molecules, which were tightly bound to the silica matrix, the so-called "water of hydration". This is strongly linked to the surface of the silica gel [34], as discussed earlier.

**Table 16:** Loss of mass peaks associated with the degradation of lipase samples with or without PEG.

Samples	Water Content (%)	* Temp. (°C) (i) – (f)	** Partial loss of mass (%)	* Temp. (°C) (i) – (f)	** Partial loss of mass (%)	* Temp. (°C) (i) – (f)	** Partial loss of mass (%)	Total loss of mass (%)
EN-AP	21.88	26.94 – 194.71	22.22	204.85 – 604.27	18.93	657.49 – 812.59	2.10	45.12
EN-P0.5	19.72	31.57 – 191.53	16.23	203.16 – 647.59	18.31	679.85 – 840.22	2.79	38.37
EN-P1.0	8.29	38.36 – 187.84	10.20	194.38 – 617.56	20.06	651.37 – 873.32	3.77	34.74
EN-P2.0	9.46	33.38 – 186.04	8.18	200.75 – 617.67	25.68	633.90 – 910.38	0.84	35.18
EN-P3.0	7.48	33.48 – 183.17	9.26	199.93 – 531.89	46.59	545.59 – 701.93	6.25	63.48

\* Initial temperature (i) and final (f) the main peak of degradation determined by analysis of the DTA curve.

\*\* Samples of mass loss related to temperature range.

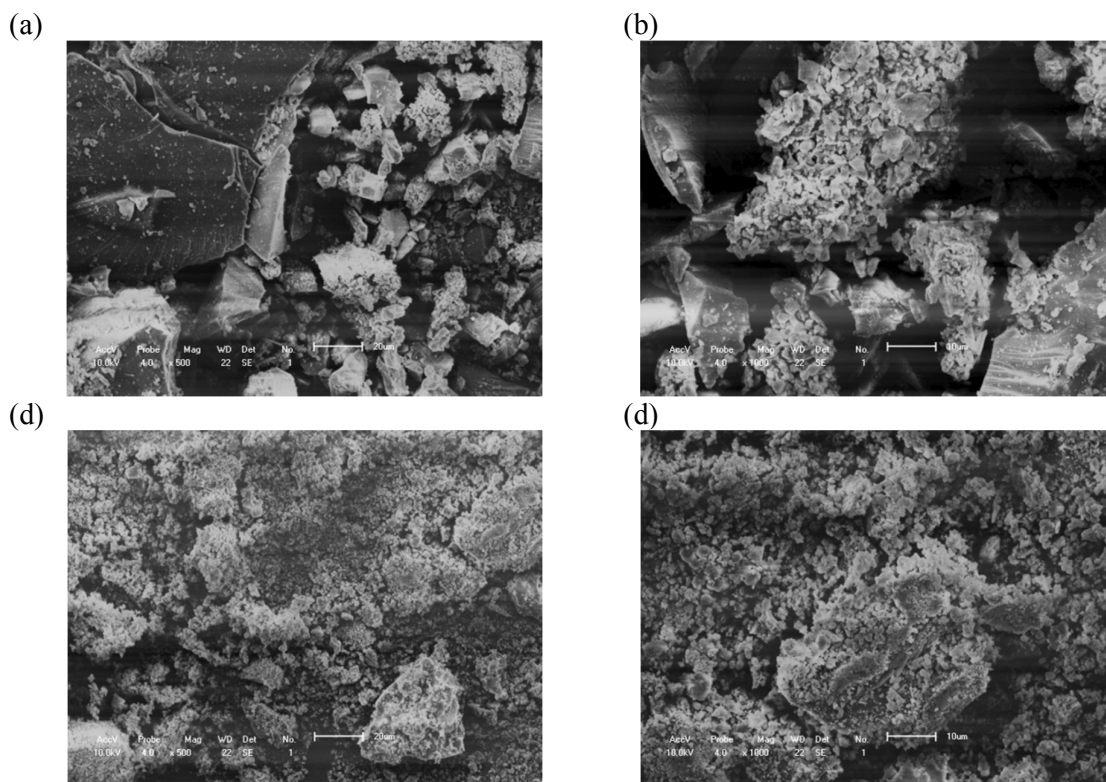


**Figure 31:** DSC curves at 10 °C/min under a nitrogen atmosphere of lipase samples in the presence of PEG at concentrations of 0.5 to 3.0% (w/v) (EN-P0.5, EN-P1.0, EN-P2.0 and EN-P3.0) or without PEG (EN-AP).

### 3.4. Scanning electron microscopy (SEM)

Micrographs of the immobilized lipases in the presence or absence of PEG are shown in Figure 32. SEM clearly showed that the coating on the support surface in the presence of PEG had an increased number of deposition on the surface of the material, giving higher porosity due to the large accumulation of particles as crystals (Figure 32c and d). This effect is possibly associated with the use of the additive during contraction, solidification and the aging steps of the gel, as observed when compared to the samples immobilized without PEG (Figure 32a and b).

The micrograph of the sample immobilized with PEG (Figure 32c and d) also showed that after coating, there was no continuous layer of the hybrid material. However, the surface appeared to be smoother with a relatively homogeneous appearance, which facilitates access to the substrate and consequently increases the recovery of enzymatic activity, as previously observed (Figure 29).



**Figure 32:** Scanning electron micrographs for derivatives immobilized in the absence of PEG (a) magnitude x500 and (b) magnitude x1000, and derivatives immobilized with PEG (c) magnitude x500 and (d) magnitude x1000.



#### 4. Conclusions

The immobilization of lipase from *Burkholderia cepacia* using polyethylene glycol as an additive in the sol-gel process was successful. The hydrophobic portion of the additive increased the porosity of the sample, which was verified by SEM micrographs. The addition of 1.0% (w/v) PEG increased enzymatic activity from 33.98 to 89.91 U.g<sup>-1</sup> and the recovery of activity from 43.0% to 91.4% when compared to samples without PEG. Increasing the concentration of PEG made the matrix structure more thermally stable in the temperature range of 50 to 200°C, and this was directly influenced by the presence of water bound to the matrix.

#### Acknowledgements

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## Capítulo IV

### CONCLUSÕES

As principais conclusões obtidas neste trabalho são apresentadas a seguir, separadas por assunto desenvolvido:

ARTIGO I – O emprego de Aliquat 336 no processo de imobilização da lipase de *Bacillus* sp. ITP-001 aumentou o rendimento de recuperação da atividade enzimática dos imobilizados de 40 para 71%. A concentração de 1,5% (m/v) de Aliquat 336 nos imobilizados favoreceu o aumento dos valores de área superficial, volume e diâmetro médio de poro, alcançando valores de 183 m<sup>2</sup>/g, 0,36 cm<sup>3</sup>/g e 92 Å, respectivamente. Portanto, um efeito positivo sobre a estrutura morfológica e a recuperação da atividade enzimática dos biocatalisadores imobilizados, foi observado na presença de Aliquat 336, tornando-se um aditivo importante para a imobilização.

ARTIGO II - O uso de líquidos iônicos próticos como aditivos no processo de imobilização sol-gel da lipase de *Burkholderia cepacia* apresentou aumento no rendimento de recuperação total da atividade enzimática e na estrutura porosa dos imobilizados, além de apresentar potencial para produção de ésteres etílicos via reação de transesterificação. Os imobilizados empregando aditivo apresentaram rendimentos de recuperação total da atividade enzimática, sempre superiores a 1000%. O aumento da cadeia alquílica do líquido iônico esteve diretamente relacionado com o aumento dos valores do rendimento de recuperação da enzima imobilizada, bem como, nos valores de área superficial, volume e diâmetro médio de poros. A concentração do líquido iônico prótico até o patamar de 1,0% (m/v) apresentou os melhores valores de recuperação total da atividade ( $Y_a = 1526\%$ ), estes derivados imobilizados apresentaram valores de área superficial igual a 245 m<sup>2</sup>/g, volume de poro igual a 0,08 cm<sup>3</sup>/g e uma estrutura mesoporosa com 30 Å.

ARTIGO III – A imobilização da lipase de *Bacillus* sp. ITP-001 empregando os líquidos iônicos prótico como aditivo no processo sol-gel foi bem sucedida. Os melhores resultados, comparados aos imobilizados sem aditivos, foram obtidos utilizando o LIP de caráter mais hidrofóbico (LI-C<sub>5</sub>) na concentração de 0,5% (m/v). Apresentando aumento na área superficial (de 13 para 278 m<sup>2</sup>/g), volume de poro (de 0,18 para 0,414 m<sup>3</sup>/g), além de manter uma estrutura de mesoporos nos imobilizados, também esteve diretamente associado

ao aumento da atividade enzimática (de 186,91 para 778,88 U/g) e no rendimento de recuperação total da atividade enzimática (de 71,1 para 305,5%).

ARTIGO IV – A imobilização da lipase de *Burkholderia cepacia* usando Polietilenoglicol como aditivos no processo sol-gel foi bem sucedida. A porção hidrofóbica do aditivo permitiu a modificação estrutural de ativos derivados, conferindo maior porosidade o que foi verificado pelas micrografias (MEV). O conteúdo de 1.0% (m/v) adicionado de PEG no processo de encapsulamento provocou o aumento, de 33.98 para 89.91 U.g<sup>-1</sup> na atividade enzimática, e 43.0% para 91.4% no rendimento total da recuperação da atividade, comparado as amostras na ausência de PEG. O aumento da concentração de PEG manteve a estrutura da matriz mais estável termicamente na faixa de temperatura entre 50-200 °C, influenciado diretamente pela presença de água ligada a matriz.

## Capítulo V

### SUGESTÕES PARA TRABALHOS FUTUROS

Embora nossos resultados tenham apresentado aumento na atividade catalítica e obtido elevados valores nos rendimentos de recuperação da atividade enzimática para os derivados imobilizados, principalmente pelo emprego dos líquidos iônicos próticos durante o processo de encapsulação sol-gel das lipases, é fundamental uma investigação mais profunda, buscando aperfeiçoar os parâmetros relativos a:

1. Caracterização bioquímica e estabilidade operacional;
2. Realizar experimentos adicionais variando a concentração da lipase encapsulada em matrizes hidrofóbicas utilizando a técnica sol-gel, para estabelecer condições favoráveis à modificação de óleos vegetais;
3. Realizar novos experimentos aplicando a lipase encapsulada em matrizes hidrofóbica utilizando a técnica sol-gel, em reações de esterificação;
4. Após o estabelecimento dessas condições complementar o estudo integral das etapas de um processo enzimático, com a aplicação em biorreatores.
5. Comparar os resultados obtidos na melhor condição experimental com o biocatalisador e posterior comparação com os valores obtidos neste trabalho.
6. Estudar a viabilidade econômica da metodologia desenvolvida em questão.

Desta forma, a imobilização de enzimas é realizada não só com o propósito de atender aplicações puramente científicas, como também visando ao uso comercial em processos contínuos.

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