Eliane Pereira Cipolatti

IMOBILIZAÇÃO DE LIPASES EM SUPORTES POLIMÉRICOS

Tese submetida ao Programa de Pós Graduação em Engenharia de Alimentos da Universidade Federal de Santa Catarina para a obtenção do Grau de Doutora em Engenharia de Alimentos
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Eliane Pereira Cipolatti

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“Somos do tamanho de nossos sonhos.”

(Fernando Pessoa)

RESUMO

O presente trabalho apresenta como objetivo principal a síntese de partículas poliméricas via miniemulsão para a imobilização das lipases de *Candida antarctica* B (CalB) e *Thermomyces lanuginosus* (TLL) e aplicação dos derivados enzimáticos na produção de ésteres etílicos de ácidos graxos. Na primeira etapa do trabalho, CalB foi imobilizada em nanopartículas de poliuretano via miniemulsão utilizando diisocianato de isoforona (IPDI) e poli(ε-caprolactona) (PCL530) e crodamol. A fase aquosa foi composta por água deionizada, SDS, PEG400 e enzima. Diferentes intensidades da sonda de ultrassom foram testadas (70 e 90%) por 1, 2 ou 3 min. A atividade enzimática foi determinada por esterificação do ácido láurico e n-propanol, e o maior valor alcançado foi de 21 U/mg (70% por 2 min), com Dp=158 nm. CalB também foi imobilizada em nanopartículas de polimetilmetacrilato (PMMA) via miniemulsão. AIBN e KPS foram testados como iniciadores. A atividade enzimática foi avaliada por hidrólise do pNPP. CalB imobilizada em PMMA manteve a atividade por 20 ciclos de hidrólise, com atividade relativa acima de 40%. Na segunda etapa do trabalho, o suporte de poliuretano (PU) foi sintetizado como citado anteriormente, com exceção do crodamol, e antes de utilizado, as partículas foram liofilizadas. TLL foi imobilizada em PU sintetizado com diferentes tamanhos de cadeia de PEG. A atividade enzimática foi determinada por hidrólise do pNPB. TLL-PU-PEG6000 apresentou os melhores resultados de Km (0,183 mM) e Vmax (45,79 μmol/min/mg). O derivado se destacou também da produção de ésteres etílicos (EE) de ácidos graxos (260 $mM.U^{-1}$). Os derivados foram recobertos com polietilenoimina (PEI) a 10 e 20% e trealose (10%). Os derivados recobertos com PEI 20% apresentaram os melhores resultados em termos de estabilidade à temperatura. O uso de agentes de ligação possibilitou um aumento de no mínimo 4 vezes na produção de EE de ácidos graxos. CalB também foi imobilizada em PU PEGuilado com diferentes cadeias de PEG. CalB-PU-PEG6000 apresentou os melhores valores de Km (0,815 mM) e Vmax (41,15 μmol/min/mg). O recobrimento com trealose possibilitou maior estabilidade térmica. CalB-PU-PEG400 apresentou os melhores valores de produção de EE, 43,72 e 16,83 $mM.U^{-1}$ EE-EPA e DHA, respectivamente. C imobilizada em PU-PEG400, 4000 e 6000 também foi aplicada na hidrólise do etil éster de (R,S)ácido mandélico, apresentando razões enantioméricas satisfatórias (E>20). Foi possível imobilizar as lipases CalB e TLL nas partículas poliméricas via miniemulsão com elevadas porcentagens de imobilização e atividade recuperada. Elevadas concentrações de EE de EPA e DHA foram obtidas com os derivados propostos.

Palavras-chave: Lipases. Polimerização em miniemulsão. Ésteres etílicos de ácidos graxos.

ABSTRACT

This current work shows as the main objective, the synthesis of polymeric particles via miniemulsion for the immobilization of *Candida antarctica* lipase B (CalB) and *Thermomyces lanuginosus* lipase (TLL) and application of the enzymatic derivatives for production of ethyl esters of fatty acids. In the first phase of the work, CalB was immobilized on polyurethane nanoparticles via miniemulsion using isophorone diisocyanate (IPDI) and poly(ε-caprolactone) (PCL530) and crodamol. The aqueous phase was comprised of deionized water, SDS, PEG400 and enzyme. Differents ultrasound power intensities were tested (70 and 90%) by 1, 2 or 3 min. The enzyme activity was determined by esterification of lauric acid and n-propanol, and the highest value achieved was 21 U/mg (70% for 2 min), with Dp=158 nm. CalB was also immobilized on polymethylmethacrylate (PMMA) nanoparticles via miniemulsion. AIBN and KPS were tested as initiators. The enzymatic activity was evaluated by hydrolysis of pNPP. CalB immobilized on PMMA retained its activity over 20 cycles of hydrolysis, with relative activity above 40%. In the second phase of the work, the polyurethane support (PU) was synthesized as previously mentioned, with the exception of crodamol, and before using it, the particles were lyophilized. TLL was immobilized on synthesized PU with different sizes of PEG chain. The enzymatic activity was determined by hydrolysis of pNPB. TLL-PU-PEG6000 showed the best results of Km (0.183 mM) and Vmax (45.79 mmol/min/mg). The derivatives also highlighted the production of ethyl esters (EE) of fatty acids (260 $mM.U^{-1}$). The derivatives were coated with polyethyleneimine (PEI) at 10 and 20%, and trehalose (10%). The derivatives coated with PEI 20% showed the best results in terms of temperature stability. The use of linking agents has enabled an increase of at least 4 times the production of EE of fatty acids. CalB was also immobilized on PU PEGylated with different PEG chains. CalB-PU-PEG6000 showed the best values of Km (0.815 mM) and Vmax (41.15 mmol/min/mg). The coating with trehalose allowed greater thermal stability. CalB-PU-PEG400 showed the best values of EE production, 43.72 and 16.83 $mM.U^{-1}$ of EE-EPA and DHA, respectively. Calb immobilized on PU-PEG400, 4000 and 6000 was also applied for the hydrolysis of the (R,S) mandelic acid ethyl ester, presenting satisfactory enantiomeric ratios (E> 20). It was possible to immobilize the CalB and TLL lipases in polymeric particles via miniemulsion with high percentages of immobilization and recovered activity. High concentrations of EE of EPA and DHA were obtained with by proposed enzymatic derivatives.

Keywords: Lipase. Miniemulsion polymerization. Ethyl esters of fatty acids.

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LISTA DE ABREVIATURAS E SIGLAS

PU – Poliuretano
IPDI - Diisocianato de isoforona
PEG - Poli(etilenoglicol)
PCL - Poli(ε-caprolactona)
PMMA- Polimetilmetacrilato
MMA - Metilmetacrilato
SDS - Dodecil sulfato de sódio
Dp - Diâmetro médio das partículas
FTIR – do inglês *Fourier transform infrared spectra* (Espectroscopia de infravermelho com transformada de Fourier)
MEV - Microscopia Eletrônica de Varredura
PdI - Índice de polidispersão
PEI - Polietilenoimina
p-NPB - p-nitrofenil butirato
p-NPP - p-nitrofenil palmitato
EPA - Ácido eicosapentaenóico
DHA - Ácido docosahexaenóico
AIBN - azobisisobutironitrila
KPS- persulfato de potássio
EE – éster etílico

SUMÁRIO

DIAGRAMA CONCEITUAL DO TRABALHO

IMOBILIZAÇÃO DE LIPASES EM SUPORTES POLIMÉRICOS

Por quê?

- Importância da imobilização de enzimas para aplicação industrial;
- Investigar o uso de polímeros sintetizados por miniemulsão na imobilização de lipases.

Quem já fez?

- Polímeros sintetizados via miniemulsão são utilizados na liberação controlada de fármacos, engenharia de tecidos, odontologia e inúmeras aplicações biotecnológicas.

Hipótese?

- A imobilização de lipases em suportes poliméricos obtidos via miniemulsão pode ser realizada de maneira eficiente?
- É possível aplicar esses derivados em reações de catálise interessantes industrialmente?

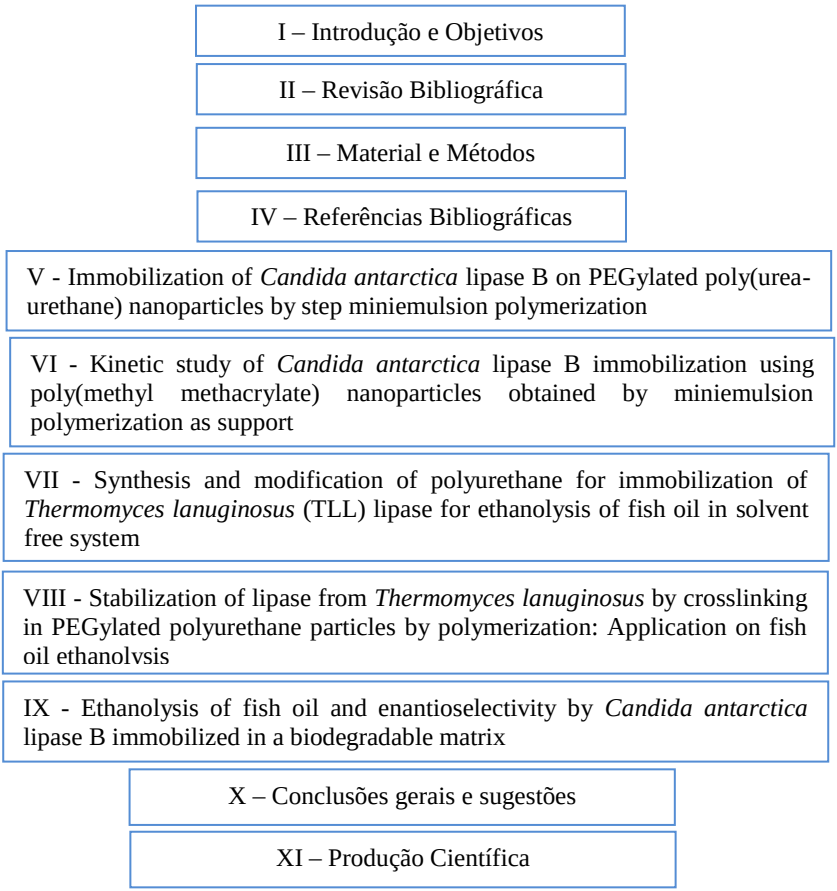
Como fazer?

- Estudar o processo de interação polímero-enzima;
- Avaliar em qual etapa do processo a enzima deve ser adicionada;
- Analisar modificações na atividade enzimática;
- Aplicar o derivado obtido em reações de produção de ésteres etílicos de EPA e DHA;
- Aplicar o derivado obtido em outras reações de interesse industrial.

ORGANOGRAMA DO DOCUMENTO DE TESE

O presente documento foi organizado conforme Figura 1.1. No Capítulo I foi descrito a introdução e objetivos do trabalho. O Capítulo II traz uma breve revisão sobre os temas abordados. O Capítulo III traz a descrição dos materiais e métodos utilizados. As referências bibliográficas dos capítulos I, II e III estão no Capítulo IV. Os resultados foram divididos nos Capítulos V, VI, VII, VIII e IX. O Capítulo X traz as conclusões gerais e sugestões para trabalhos futuros. Por fim, o Capítulo XI apresenta as produções científicas obtidas ao longo do desenvolvimento do trabalho.

Figura 1.1 – Organograma do documento de tese



1. INTRODUÇÃO

Processos de imobilização permitem que a enzima se torne mais estável e resista a condições mais bruscas, como uma ampla faixa de pH e temperatura, podendo, também, preservar a atividade da enzima por vários ciclos. A imobilização enzimática surge como uma alternativa para a aplicação desses biocatalisadores na indústria, tanto alimentícia como química e farmacêutica. Para que o emprego de enzimas imobilizadas torne-se um processo economicamente viável, deve-se considerar o tipo de suporte empregado, assim como o método de imobilização, que influenciará diretamente na posterior atividade e reuso do biocatalisador. A imobilização de enzimas tem sido largamente estudada, porém, há ainda a necessidade de desenvolvimento de técnicas simples e de baixo custo que possam ser utilizadas de maneira eficiente. (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004; GUISÁN, 1988; JOSE et al., 2015; MATEO et al., 2006).

A utilização de polímeros tais como o poliuretano (PU) como suporte para a imobilização de enzimas é relatada na literatura (CUI et al., 2013; NICOLETTI et al., 2015), porém o uso de PU sintetizado a partir de miniemulsão ainda é pouco estudado para este fim, embora tenha uma ampla aplicação no campo da biomedicina (VALÉRIO; ARAÚJO; SAYER, 2013; WANG; GRAYSON, 2012). A síntese de PU por miniemulsão permite a obtenção de partículas pequenas do polímero (elevada área superficial) em uma única etapa e fácil controle dos monômeros utilizados. Além disso, esta técnica permite a obtenção de um polímero resistente, podendo ser biodegradável, biocompatível, e, adicionalmente, pode ser considerada uma técnica de baixo custo (DE LIMA et al., 2013; GAUDIN; SINTES-ZYDOWICZ, 2008b; VALÉRIO et al., 2014; ZANETTI-RAMOS; LEMOS-SENNA; CRAMAIL, 2008; ZANETTI-RAMOS et al., 2009).

Outros polímeros sintetizados via miniemulsão podem ser utilizados na imobilização de enzimas, como o polimetilmetacrilato (PMMA). Este polímero sintético apresenta aplicações biotecnológicas e biomédicas, devido ao seu caráter biocompatível e resistência mecânica (CERQUEIRA et al., 2015; LI; HU; LIU, 2004). Biocompatibilidade, resistência mecânica e baixo custo são características comuns ao PU e PMMA que tornam esses polímeros interessantes para aplicação na imobilização de lipases.

Lipases são as enzimas mais utilizadas na síntese orgânica, sendo aplicadas em inúmeras preparações comerciais, devido a sua ampla especificidade e maior estabilidade (em comparação com outras

enzimas) (DE CASTRO et al., 2004; FERNANDEZ-LAFUENTE, 2010; KAPOOR; GUPTA, 2012; RODRIGUES; FERNANDEZ-LAFUENTE, 2010). As lipases vêm conquistando uma faixa crescente do mercado global de enzimas. Embora muitas das aplicações industriais destas estejam concentradas nas indústrias de detergentes, novas aplicações estão ganhando destaque, com possibilidades de aplicação na indústria farmacêutica, química fina, cosméticos, oleoquímica, couros, polpa de celulose e papel, e no tratamento de resíduos industriais (DE CASTRO et al., 2004; KAPOOR; GUPTA, 2012). A lipase B de *Candida antarctica* (CalB) é particularmente interessante devido a sua capacidade de catalisar diversas reações, resistência a solventes orgânicos, estabilidade térmica, estereoespecificidade e enantiosseletividade (FORDE et al., 2010; MCCABE; TAYLOR, 2004). Outra lipase que merece destaque é a proveniente de *Thermomyces lanuginosus* (TLL) por ser aplicada em inúmeras reações de biotransformação, também por apresentar estabilidade térmica e ser enantiosseletiva (FERNANDEZ-LAFUENTE, 2010; RODRIGUES et al., 2009).

A utilização destas lipases imobilizadas na produção de ésteres etílicos de ácidos graxos é uma aplicação interessante. O uso desses ésteres é uma alternativa ao consumo de ácidos graxos, que pode também ser feito através do óleo de peixe ou do ácido graxo livre. Porém, a quantidade máxima de triglicerídeos de PUFAs (sigla em inglês para ω-3 *polyunsaturated fatty acids*/ ácidos graxos polinsaturados) em tais produtos é de 18-25%, o que é considerado baixo. Logo, para enriquecer os níveis de PUFAs em alimentos, EPA (ácido eicosapentaenóico) e DHA (ácido docosahexaenóico) podem ser estabilizados como ésteres etílicos. Embora os ácidos graxos livres e triglicerídeos possam ser metabolizados mais rapidamente e completamente do que os ésteres etílicos, os ácidos graxos são mais facilmente oxidados. Atualmente, a maioria dos concentrados comerciais são derivados de ésteres etílicos de omega-3 (DE LEONARDIS; PIZZELLA; MACCIOLA, 2008; MORENO-PÉREZ; GUISAN; FERNANDEZ-LORENTE, 2014; MORENO-PEREZ et al., 2015). Logo, o presente trabalho contribui tanto no estudo do processo de imobilização, com a proposta de uso de um polímero sintetizado por uma via ainda pouco explorada na imobilização de biocatalisadores, como na aplicação na reação de catálise com triglicerídeo rico em ácidos graxos poli-insaturados.

1.1 OBJETIVOS

1.1.1 Objetivo Geral

O presente trabalho apresenta como objetivo principal a síntese de partículas poliméricas via miniemulsão para a imobilização das lipases de *Candida antarctica* B e *Thermomyces lanuginosus* e aplicação na produção de ésteres etílicos de ácidos graxos.

1.1.2 Objetivos Específicos

- Estudar a imobilização de *Candida antarctica* lipase B (CalB) em nanopartículas de poliuretano PEGuiladas sintetizadas via miniemulsão, verificando o efeito das condições operacionais, como intensidade da sonda de ultrassom e tempo de sonicação, assim como a presença de crodamol na síntese das partículas.

- Estudar a imobilização de CalB em nanopartículas de polimetilmetacrilato (PMMA), avaliando a concentração de crodamol e tipo de iniciador (azobisisobutironitrila - AIBN e persulfato de potássio – KPS) na cinética de polimerização via miniemulsão do metil metacrilato), distribuição do tamanho de partícula, potencial zeta e em termos de atividade enzimática.

- Estudar a imobilização de *Thermomyces lanuginosus* (TLL) em partículas de poliuretano PEGuiladas obtidas por miniemulsão, rerificando o efeito do tamanho de cadeia do PEG (400, 4000 ou 6000) na imobilização e análise da estabilidade à temperatura e pH, determinação dos parâmetros de imobilização e parâmetros cinéticos e aplicação dos derivados obtidos na etanólise do óleo de peixe.

- Avaliar o uso de agentes de estabilização (trealose e polietilenoimina) na imobilização de *Thermomyces lanuginosus* pela análise da estabilidade dos derivados obtidos frente à temperatura e pH, parâmetros de imobilização e parâmetros cinéticos e aplicação dos derivados recobertos na etanólise de óleo de peixe.

- Imobilizar CalB em poliuretano PEGuilado com diferentes tamanhos de PEG (400, 4000 e 6000) sintetizado via miniemulsão pela análise do uso de agentes de ligação (trealose e polietilenoimina) no processo de imobilização e aplicação dos derivados na produção de ésteres etílicos de ácidos graxos e a possível aplicação dos derivados na separação dos enantiômeros do ácido mandélico.

2 REVISÃO BIBLIOGRÁFICA

O objetivo deste capítulo é apresentar uma breve revisão dos principais conceitos abordados no trabalho, como características gerais das lipases, métodos de imobilização de enzimas, algumas aplicações das enzimas imobilizadas, considerações sobre polímeros e a importância dos ésteres etílicos de ácidos graxos.

2.1 LIPASES

Lipases (EC 3.1.1.3, triacilglicerol acilhidrolases) são enzimas que catalisam as reações de hidrólise de gorduras e óleos com subsequente liberação de ácidos graxos livres, diglicerídios, monoglicerídios e glicerol. As lipases podem alcançar massa molecular entre 20 e 75 kDa com cerca de 300 resíduos de aminoácidos, atividade na faixa de pH que varia de 4 a 9 e temperatura entre 25 e 70 °C, sendo as lipases microbianas as que possuem maior estabilidade térmica (DE CASTRO et al., 2004; KAPOOR; GUPTA, 2012).

Estas enzimas são as mais utilizadas na síntese orgânica, por serem aplicadas em inúmeras preparações comerciais, devido a sua ampla especificidade e maior estabilidade (em comparação com outras enzimas). Embora muitas das aplicações industriais das lipases estejam concentradas nas indústrias de detergentes, novas aplicações estão ganhando destaque, como na indústria farmacêutica, química fina, cosméticos, oleoquímica, couros, polpa de celulose e papel, e no tratamento de resíduos industriais (DE CASTRO et al., 2004; KAPOOR; GUPTA, 2012; STERGIOU et al., 2013). Também é aplicada na maturação acelerada de queijos, panificação, produção de óleos e gorduras estruturados (uso nutricional e tecnológico), produção de surfactantes não iônicos, síntese de aromas, produção de compostos oticamente ativos e resolução de racematos (KOBLITZ, 2008). Além de serem utilizadas eficientemente na produção de biodiesel (AGUIEIRAS; CAVALCANTI-OLIVEIRA; FREIRE, 2015)

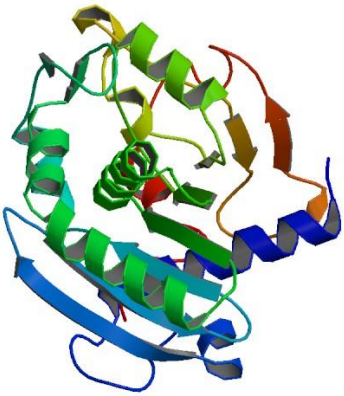
As lipases são amplamente encontradas na natureza, podendo ser obtidas de fontes animais, vegetais e microbianas. Numerosas espécies de leveduras (ex: *Candida rugosa* (DALMAU et al., 2000), bactérias (ex: *Burkholderia* sp. (LIU et al., 2012) e fungos filamentosos (ex: *Geotrichum candidum* (MALDONADO et al., 2012); *Rhizopus arrhizus* (RAJENDRAN; THANGAVELU, 2009) são produtores de lipases.

2.1.1. Lipase de *Thermomyces lanuginosus* (TLL)

Lipase de *Thermomyces lanuginosus* (TLL), anteriormente *Humicola lanuginosa,* encontra-se disponível comercialmente na forma livre (Lipolase 100 L-Novozym) ou imobilizada (Lipozyme TL IM). Seu sítio ativo é composto pela tríade catalítica Ser-His-Asp. TLL é uma das lipases com maior tendência a formar aglomerados biomoleculares (FERNANDEZ-LAFUENTE, 2010; PALOMO et al., 2003a).

A atividade e estabilidade elevadas da TLL, permitem sua utilização em vários meios de reação, de sistemas bifásicos água-solvente orgânico à sistemas livres de solventes. A TLL é muito utilizada na hidrólise e transesterificação de óleos e gorduras. Embora seja uma enzima bastante estável, qualquer melhoria adicional em sua estabilidade, como processos de imobilização, podem ser interessantes para ampliar ainda mais a sua gama de aplicações (FERNANDEZ-LAFUENTE, 2010; RODRIGUES et al., 2009). Um esquema da estrutura desta lipase desenvolvido por cristalografia por Brzozowski et al (2000) está disponível no banco de dados *ProteinDataBase* (*http://www.rcsb.org/pdb)* e está apresentado na Figura 2.1. Nela está demonstrada sua forma ativada interfacialmente.

Figura 2.1 – Esquema estrutural da lipase de *Thermomyces lanuginosus* (BRZOZOWSKI et al., 2000)

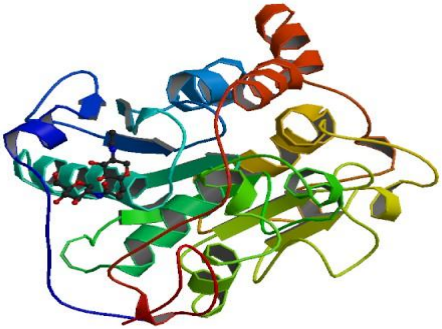


2.1.2. Lipase de *Candida antarctica* fração B (CalB)

Lipase B de *Candida antarctica* (CalB) é um dos biocatalisadores mais utilizados em síntese orgânica, devido à sua capacidade de atuação sobre vários substratos, tolerância em meios não aquosos e resistência à desativação térmica (LUTZ, 2004; McCABE; TAYLOR, 2004). CalB é uma enzima extracelular e encontra-se disponível no mercado na forma livre ou imobilizada (Novozym 435). Esta não é tão eficiente quanto outras enzimas na hidrólise de triglicerídeos, no entanto, é altamente estereoespecífica no sentido da síntese e hidrólise de ésteres, isso ocorre possivelmente devido ao espaço limitado disponível em sua parte hidrofóbica (IDRIS; BUKHARI, 2012), que aparece como uma particularidade desta enzima, que ao contrário da maioria das lipases, não apresenta ativação interfacial, sendo o acesso ao sítio ativo feito por um canal estreito com uma pequena hélice de elevada mobilidade perto deste sítio (JAEGER; REETZ, 1998).

O sítio ativo da CalB é composto pela tríade catalítica serina, histidina e ácido aspártico (Ser-His-Asp) (UPPENBERG et al., 1994). O resíduo nucleofílico serina esta localizado ao lado do C-terminal da fita β5 em um pentapeptídeo GXSXG altamente conservado, formando uma característica principal - β em torno de α, designada como a cavidade nucleofílica (DE CASTRO et al., 2004). O nucleófilo catalítico (serina) é responsável pela catálise e está unido por ligações de hidrogênio a um resíduo de histidina; o resíduo carboxilado ligado ao mesmo resíduo de histidina poderá ser um aspartato ou glutamato (JAEGER; REETZ, 1998). A estrutura cristalográfica da CalB mostra uma grande variação de conformação no ambiente de seu sítio ativo, o que pode explicar a grande variação na especificidade de substratos destas enzimas (UPPENBERG et al., 1995). Um esquema da estrutura da CalB foi desenvolvido por UPPENBERG et al (2004) e está apresentado na Figura 2.2. Assim como a estrutura da TLL, essa figura está disponível em *ProteinDataBase* (http://www.rcsb.org/pdb) bem como outras modificações estruturais das lipases citadas.

Figura 2.2 - Esquema estrutural da lipase B de *Candida antarctica* (UPPENBERG et al., 1994)



O uso da TLL e CalB em reações de catálise pode ser melhorado com processos de imobilização. Na literatura há inúmeros trabalhos com a imobilização das enzimas citadas (NICOLETTI et al., 2015; ONDUL; DIZGE; ALBAYRAK, 2012; VALÉRIO et al., 2015a). Porém, como não existe um processo padrão de imobilização de enzimas, há a recorrente necessidade do desenvolvimento de técnicas de imobilização e diferentes suportes para este fim.

2.2. IMOBILIZAÇÃO DE ENZIMAS

De acordo com a 1ª Conferência em Engenharia de Enzimas (Henniker, Estados Unidos, 1971): "Enzimas imobilizadas são catalisadores fisicamente confinados ou localizados em uma região definida do espaço, com retenção de suas atividades catalíticas, e que podem ser utilizados repetida e continuamente" (KATCHALSKI-KATZIR; KRAEMER, 2000). O principal interesse em imobilizar uma enzima é obter um biocatalisador com atividade e estabilidade que não sejam afetadas durante o processo, em comparação à sua forma livre. Idealmente, a enzima imobilizada deverá exibir uma atividade catalítica superior (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004).

Processos de imobilização podem permitir que a enzima se torne mais estável e resista à condições mais bruscas, como uma ampla faixa de pH e temperatura, podendo, também, preservar a atividade da enzima por vários ciclos. A imobilização enzimática surge como uma alternativa para a aplicação desses biocatalisadores na indústria, tanto

alimentícia como química e farmacêutica (GUISAN; BLANCO, 1987; PESSELA et al., 2007).

Existem inúmeras técnicas de imobilização e diferentes suportes, a escolha vai depender das peculiaridades e aplicações específicas do biocatalisador em questão. Segundo IDRIS e BUKHARI (2012), as enzimas imobilizadas devem apresentar algumas características gerais para que sejam utilizadas em nível industrial:

- alta atividade;
- alta seletividade (reduzindo reações paralelas);
- alta estabilidade (permitindo sua reutilização);
- custo-benefício;
- segurança durante o uso;
- ser considerado novo (para o reconhecimento como propriedade intelectual).

A escolha do método vai depender da enzima a ser imobilizada e sua finalidade, ou seja, um importante requisito para imobilização de enzimas é de que a matriz deve fornecer um ambiente inerte e biocompatível, isto é, não deve interferir com a estrutura nativa da proteína, que, assim, possa comprometer sua atividade biológica (MITCHELL et al., 2002).

As lipases estão entre as enzimas mais utilizadas em processos de imobilização, devido a sua tendência em se adsorver em suportes hidrofóbicos, característica essa que pode ser utilizada para desenvolver protocolos de imobilização específicos para estas enzimas (FERNANDEZ-LORENTE et al., 2008). Adicionalmente, muitos tipos de suportes e materiais podem ser encontrados na literatura e descritos como eficientes na imobilização de lipases, como suportes magnéticos, poliméricos, fibras de carbono, quitosana, grafeno, etc (CIPOLATTI et al., 2014a; PURI; BARROW; VERMA, 2013; TAN; FENG; JI, 2012; TANG; QIAN; SHI, 2007; YILMAZ; SEZGIN; YILMAZ, 2011)

A ligação entre esses suportes e a enzima pode ser feito por adsorção, ligação covalente, iônica, encapsulamento, entre outras técnicas mais sofisticadas de imobilização (BARBOSA et al., 2011; FERNANDEZ-LORENTE et al., 2008; GUISÁN, 1988; KIM; GRATE; WANG, 2006). A ligação por adsorção é a técnica mais simples e permite imobilizar enzimas em suportes sólidos através de ligações de baixa energia, como van der Waals ou hidrofóbicas, ligações de hidrogênio e iônicas, entre outras (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004). Porém, se o interesse do pesquisador estiver também relacionado ao suporte utilizado, esta é uma técnica muito interessante, por permitir a dessorção da enzima com aplicação de

gradientes de detergentes (FERNANDEZ-LORENTE et al., 2008; KHARRAT et al., 2011).

Pode ser formada também uma ligação covalente entre a enzima e um suporte insolúvel em água, ou ainda pela formação de ligações cruzadas com a matriz (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004). A formação de ligação covalente enzima-suporte é forte e irreversível, apresentando uma maior estabilidade operacional, porém ao ser constatada a desnaturação da enzima, o suporte é juntamente descartado (CADENA et al., 2010).

A imobilização de lipases por adsorção, ligação covalente e/ou multifuncional em matrizes poliméricas são apontadas como técnicas promissoras para aplicação em meio orgânico, e muitas vezes, apresentam aumento da atividade e estabilidade da enzima (DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004).

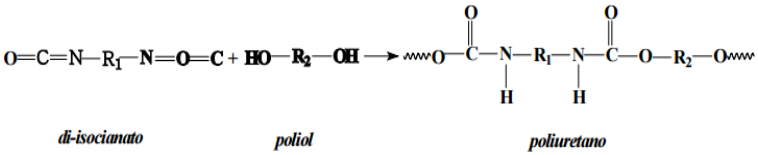
Muitas são as técnicas e os suportes reportados na literatura, resultando numa infinidade de formas de se imobilizar uma enzima. O uso de partículas nanométricas para este fim tem sido extensamente estudado. Nanopartículas utilizadas como suportes para enzimas foram alvo de um recente estudo publicado por nosso grupo de pesquisa: *Current status and trends in enzymatic nanoimmobilization*, publicado na *Journal of Molecular Catalysis B: Enzymatic* em 2014. Nele estão apresentadas algumas vantagens em relação ao uso de nanopartículas, como menor resistência à transferência de massa, carga eficaz de enzima, elevada área superficial e minimização dos problemas difusionais. Algumas desvantagens também são apontadas, como custo do processo de fabricação, aplicação em larga escala e dificuldade na separação do meio reacional.

2.3 POLIURETANO (PU)

Poliuretano é muito utilizado em pesquisas na área de imobilização de enzimas, principalmente na forma de espuma (NICOLETTI et al., 2015; SILVA et al., 2013). A estrutura geral dos poliuretanos é caracterizada por suas unidades monoméricas principais, diisocianatos (NCO) e polióis (OH), formando ligações uretano (Figura 2.3). Este polímero, que não contêm as unidades uretano repetidas de modo regular, pode conter hidrocarbonetos alifáticos e aromáticos, grupos éster, éter, ureia e amida (WEGENER et al., 2001). PU apresenta características interessantes como durabilidade, flexibilidade, bioestabilidade, além de não ser tóxico e ser biocompatível, podendo ser

sintetizado de forma a torná-lo biodegradável (ZANETTI-RAMOS; LEMOS-SENNA; CRAMAIL, 2008; ZANETTI-RAMOS et al., 2006), características importantes e cada vez mais exploradas pelos pesquisadores preocupados com saúde e meio ambiente. Considerando o exposto, poliuretano se torna interessante na aplicação como carreador de fármacos, engenharia de tecidos, desenvolvimento de dispositivos médicos e imobilização de enzimas (CIPOLATTI et al., 2014b; VALÉRIO; ARAÚJO; SAYER, 2013; VALÉRIO et al., 2014).

Figura 2.3 – Esquema da formação de poliuretano (SOARES, 2012).



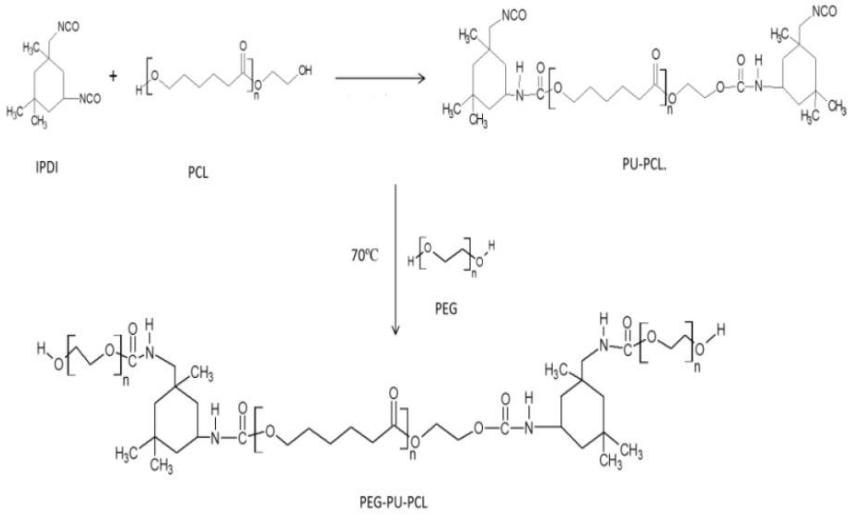
Poliuretano também pode ser obtido via miniemulsão (como descrito no item 2.6.), sendo possível a escolha dos monômeros utilizados. Os monômeros utilizados neste trabalho foram o diisocianato de isoforona – IPDI - (5-isocianato-1-(metilisocianato)-1,3,3'- trimetil ciclohexano) e PCL (policaprolactona), ainda foi utilizado polietilenoglicol (PEG) para garantir uma maior resistência mecânica ao PU formado.

O IPDI é uma molécula assimétrica, cicloalifática, comercializado na forma de mistura de isômeros (25/75 cis/trans). Possui quatro grupos NCO diferentes, dois são grupos NCO alifáticos e secundários, e outros dois são primários, mas estericamente impedidos, o que torna o IPDI o isocianato menos reativo dentre os comerciais (VILAR, 2008). O poliol utilizado no trabalho foi o PCL, um dos monômeros mais utilizados na síntese de poliuretanos biodegradáveis, uma vez que é aprovado pela *Food and Drug Administration* (FDA). Ao ser degradado, as ligações éster do PCL podem ser hidrolisadas, e há a produção de 6-hidroxi-hexanóico, um metabólico não tóxico (DA SILVA et al., 2010; ZHANG et al., 2012).

Como já mencionado, a adição de PEG na síntese de poliuretano confere resistência as partículas, principalmente quando produzidas por miniemulsão. Este polímero se liga à superfície das

partículas por reação de seus grupos hidroxilas terminais com o grupamento isocianato (Figura 2.4). Encontra-se disponível comercialmente em diversas massas moleculares (de poucas centenas a milhares de Dáltons). PEG é biodegradável, aprovado para consumo interno pelo FDA, possuindo grande importância para as áreas biomédicas e de biomateriais (OTSUKA; NAGASAKI; KATAOKA, 2003). Poliuretano PEGuilados são utilizados como carreadores de fármacos (CHERNG et al., 2013; VALÉRIO et al., 2014).

Figura 2.4 – Esquema da formação das partículas de PU-PEGuiladas (Valério, 2013).



2.4 POLIMETILMETACRILATO (PMMA)

Os metacrilatos são ésteres insaturados ($H_2C=C(CH_3)COOR$, no qual a natureza do grupo R, normalmente, é o que determina as propriedades físicas dos monômeros e seus respectivos polímeros. Estes monômeros apresentam alta versatilidade por serem líquidos e com ponto de ebulição elevado, se polimerizam facilmente entre si ou com uma série de acrilatos. Conforme o método de polimerização escolhido é possível obter cadeias poliméricas com diferentes estruturas, o que resulta em propriedades físicas bem específicas e controladas. Há

possibilidade de inserção de outros grupos ao polímero, o que torna esse polímero particularmente interessante (ARAUJO, 1996).

Polimetilmetacrilato apresenta grande importância comercial devido a sua boa transparência ótica e grande resistência ao impacto. A presença do grupo metila no carbono α do PMMA é o que lhe concede maior estabilidade térmica, dureza e rigidez em relação aos outros poliacrilatos (PÉREZ; LÓPEZ-CABARCOS; LÓPEZ-RUIZ, 2006; ARAUJO, 1996).

O polimetilmetacrilato pode ser sintetizado via miniemulsão e é apontado como suporte para enzimas (JENJOB et al., 2012; LI; HU; LIU, 2004). É um polímero biocompatível, utilizado na liberação controlada de fármacos e em muitas aplicações biomédicas e biotecnológicas (CUNHA et al., 2014; LI; HU; LIU, 2004). Este polímero pode ser sintetizado via radicalar. A polimerização via radical livre ocorre através da adição sucessiva de moléculas (insaturadas ou cíclicas) de monômeros a um radical propagador. Este mecanismo permite a obtenção de polímeros com elevada massa molecular (ARAUJO, 1996). A obtenção dos radicais ocorre pela decomposição térmica de um iniciador. Este deve ter uma ligação química suficientemente frágil para decompor homoliticamente, mas deve, por outro lado, ser suficientemente estável à temperatura ambiente para poder ser armazenado em segurança (SZWARC, 1956). O AIBN (azobisisobutironitrila) é um dos iniciadores mais utilizados, este se decompõe com a perda de N_2 (ROMIO et al., 2009) Outra iniciador muito utilizado é o persulfato de potássio (KPS).

2.5. UTILIZAÇÃO DE SUPORTES SINTETIZADOS VIA MINIEMULSÃO PARA IMOBILIZAÇÃO DE ENZIMAS

Considerando a fácil manipulação das condições e dos monômeros utilizados durante o processo de miniemulsão, além do baixo custo dos polímeros, este pode ser um método promissor na síntese de suportes para imobilização de enzimas.

Miniemulsão é classicamente definida como uma dispersão aquosa de gotículas de óleo relativamente estável dentro de uma faixa de tamanhos de 50-500 nm, preparadas por um sistema contendo óleo, água, surfactante e um agente "cosurfactante" (LANDFESTER et al., 1999). A polimerização em miniemulsão tem por objetivo iniciar a polimerização quando as gotas já estão estáveis, evitando nucleações secundárias e minimizando o transporte de massa (ANTONIETTI;

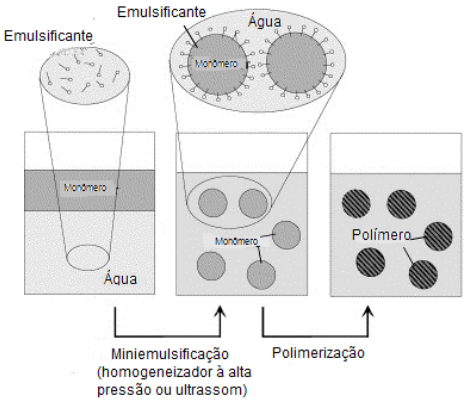
LANDFESTER, 2002). Esta técnica permite que fármacos, óleos ou outras substâncias possam ser incorporados às gotas, mantendo suas características desde a dispersão até a obtenção das nanopartículas (LANDFESTER, 2009; VALÉRIO; ARAÚJO; SAYER, 2013; VALÉRIO et al., 2014). Normalmente, o preparo das nanopartículas em sistemas de miniemulsão inclui três etapas: pré-emulsão de duas fases heterogêneas para preparar (macro)emulsões, homogeneização das emulsões brutas para obter as miniemulsões e a reação para originar as nanopartículas (QI; CAO; ZIENER, 2014). A Figura 2.5 mostra as etapas envolvidas na polimerização via miniemulsão. As nanopartículas podem ser formadas com a utilização de homogeneizador à alta pressão ou ultrassom. Este método pode ser utilizado para o encapsulamento de materiais em nanopartículas poliméricas. (LANDFESTER, 2009).

Além disso, a miniemulsão traz a vantagem de que o produto final pode ser obtido em uma etapa de reação (VALÉRIO; ARAÚJO; SAYER, 2013)(VALERIO et al., 2013). A síntese das nanopartículas de PU em uma etapa consiste em adicionar os monômeros (diisocianato e polióis), extensor de cadeia e demais componentes da reação ao reator simultaneamente para formação do produto final (BOCK, 2001).

As miniemulsões podem ainda ser classificadas como diretas ou inversas, dependendo da polaridade das fases dispersa e contínua. Na miniemulsão direta, a polaridade da fase contínua é maior que na fase dispersa, enquanto que na miniemulsão inversa a polaridade da fase contínua é menor do que na fase dispersa. Em miniemulsão direta, uma solução aquosa de surfactante é comumente utilizada como fase contínua. Já em miniemulsões inversas, uma solução hidrofóbica de surfactante é utilizada como fase contínua. Solventes hidrofóbicos mais utilizados são o ciclohexano, tolueno, hexadecano e Isopar M (uma mistura de hidrocarbonos de C12-C14). Sistemas com miniemulsões diretas são utilizadas para preparo de nanopartículas hidrofóbicas, enquanto que a inversa produz partículas hidrofílicas (QI; CAO; ZIENER, 2014)

A polimerização em miniemulsão se destaca, ainda, por apresentar vantagens como o uso não excessivo de tensoativo, estabilidade coloidal suficiente e incorporação de compostos hidrofóbicos, sendo ainda considerada uma tecnologia de baixo custo (LANDFESTER et al., 1999; ROMIO et al., 2009).

Figura 2.5 - Princípio da polimerização em miniemulsão (ANTONIETTI e LANDFESTER, 2002).



A Tabela 2.1 apresenta algumas matrizes utilizadas em miniemulsões e algumas formas de síntese desses polímeros. Embora sucinta, nos dá a ideia do quanto é possível modificar as formas de se produzir polímeros por miniemulsão, seja pelos reagentes empregados ou pelas condições do processo, como temperatura, tempo de reação, intensidade da sonda de ultrassom, entre outros.

Tabela 2.1 – Aplicações e condições reacionais de polímeros obtidos por miniemulsão.

Matriz	Condições reacionais	Aplicação	Referência
Magnetita em poliuretano	Fase dispersa:IPDI e crodamol. Fase aquosa:1,6-hexanodiol e SDS. Mistura das fases por 20 min e sonicação por 180s a 70% de amplitude → adição de nanopartículas magnéticas recobertas com ácido oleico.	-	(CHIARADIA et al., 2015)
Magnéticas em poliestireno	Fluido magnético e SDS→ agitação 15 min e ultrassom a 600 W. Adição de ácido acrílico e divinilbenzeno → agitação por 2h. Adição de AIBN → 70ºC/3h.	Adsorção de proteína de soro bovino	(LIU et al., 2014)
Poliuretano	-Fase orgânica: IPDI e óleo castor, óleo de açaí e PEG (400, 600 ou 1000). Fase aquosa: Tween 80→ agitação por 5 min. Após: sonicação por 120s à 70% de amplitude. Polimerização: 70°C/3h. -Fase aquosa:SDS e PEG(400, 600 ou 1000). Fase orgânica: IPDI, PCL530 e Crodamol em ciclohexano →30 min/40ºC. Polimerização 70°C/3h.	Incorporação de óleos vegetais	(VALÉRIO et al., 2014)
Poliuretano PEGuiladas	IPDI, PEG400 e óleo de oliva adicionados à solução aquosa com Tween 80 (temperatura ambiente →18000 rpm/15 min. Após: 800 rpm/60ºC por 4h	Imobilização de peroxidase para detecção de dopamina	(FRITZEN-GARCIA et al., 2013)
PMMA e quitosana	Quitosana em ácido acético a 65°C → MMA and APS (Persulfato de amônio) a 65°C/4h.	Imobilização de α-chimotripsina	(ABD EL-GHAFFAR; HASHEM, 2013)
Magnéticas em poliestireno	Fluido magnético disperso em solução aquosa de KPS → sonicação por 10 min → temperatura ambiente por 15h. Adição de Span80, dodecil benzeno sulfonato de sódio, etanol, acrilamida e ácido acrílico → 70ºC/10h a 300 rpm sob nitrogênio	-	(SUN et al., 2007)

A síntese de polímeros via miniemulsão pode ser uma alternativa interessante para uso em imobilização de enzimas. Porém, poucos trabalham foram encontrados com a utilização dessa técnica para este fim. Fritzen-Garcia et al. (2013) estudaram a adsorção de peroxidase em nanopartículas de poliuretano PEGuiladas para aplicação na detecção de dopamina. Os autores utilizaram uma mistura contendo diisocianato de isoforona, polietilenoglicol (PEG400) e óleo de oliva. A quantidade de enzima adsorvida foi de 22,5 mg/g de nanopartículas. Trabalhos como o de Liu et al. (2014) estudaram a síntese, via miniemulsão, de microesferas de estireno-ácido acrílico/Fe_3O_4 com grupos carboxílicos na superfície. As microesferas apresentaram boa capacidade de adsorção da albumina de soro bovino (105 mg/g).

Processos que utilizam emulsões para o preparo de polímeros casca-núcleo são mais facilmente encontrados (CUNHA et al., 2014), como o realizado por JENJOB et al. (2012). Os autores prepararam uma emulsão a partir de água e MMA em reator a 80 °C com nitrogênio, posteriormente KPS foi adicionado e o processo de polimerização foi conduzido na mesma temperatura por 3 h. Quitosana foi utilizada para recobrir o PMMA, formando uma estrutura casca-núcleo, que foi utilizada na imobilização de lipase de *Candida rugosa.*

O presente trabalho não descarta a importância dos polímeros casca-núcleo e sua eficiência já comprovada na imobilização de enzimas, mas aponta a síntese de partículas poliméricas por miniemulsão como promissora na imobilização de enzimas, embora pouco tenha se encontrado na literatura sobre o método proposto na imobilização de enzimas.

Dentro deste contexto, a síntese de nanopartículas via miniemulsão de um polímero com qualidade ambiental e custo-benefício favorável, como o poliuretano e o polimetilmetacrilato, aliaria as vantagens atribuídas às nanopartículas, com as do processo de síntese. Essas nanopartículas de PU e PMMA produzidas por polimerização em miniemulsão podem ser utilizadas na imobilização de enzimas, surgindo como um método inovador e eficiente para a manutenção da atividade e estabilidade destes biocatalisadores.

2.6 SÍNTESE DE ÉSTERES ETÍLICOS DE ÁCIDOS GRAXOS

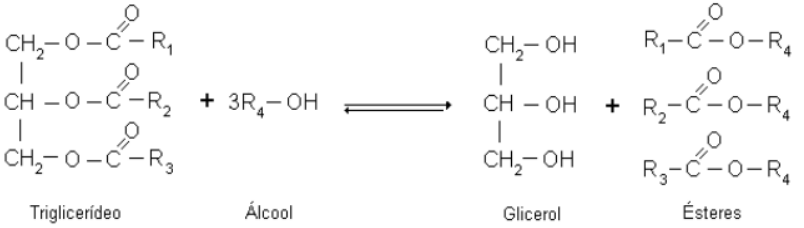
Os óleos de peixes contêm grandes quantidades de ácidos graxos poli-insaturados de cadeia longa ω-3, estes são oriundos do ácido linolênico proveniente principalmente das plantas as quais esses animais de alimentam. Os ácidos eicosapentaenóico (EPA) e docosaexaenóico (DHA) são os principais bioativos desta classe. Esses importantes ácidos graxos estão presentes em óleos de pescado em baixas quantidades (18-25%) (DE LEONARDIS; PIZZELLA; MACCIOLA, 2008; MARIA et al., 2006; MORENO-PEREZ et al., 2015). Adicionalmente, EPA e DHA são essenciais para o desenvolvimento do cérebro e sistema nervoso, apresentam um papel importante na prevenção e tratamento de doença arterial coronariana, hipertensão, artrite e outras doenças inflamatórias (SHIN et al., 2012; SIMOPOULOS, 1999).

Óleos e gorduras podem ser modificados por hidrogenação, interesterificação e fracionamento para alterar suas caraterísticas físico-químicas e garantir sua aplicação em nível industrial. Algumas das reações que podem ser aplicadas são: acidólise, glicerólise e transesterificação. A alcoólise permite a hidrólise da molécula do triglicerídio, gerando uma mistura de ésteres metílicos ou etílicos dos ácidos graxos (KOBLITZ, 2008; MARIA et al., 2006; De CASTRO et al., 2004)

Os ésteres etílicos (EE) produzidos por via enzimática surgem como alternativa para obtenção de lipídios estruturados específicos, possibilitando baixa temperatura no processo, o que protege os ácidos graxos poli-insaturados da oxidação, e possibilita também o uso de reagentes não agressivos (MARIA et al., 2006; SHIN et al., 2012; VALVERDE et al., 2014). A etanólise enzimática seletiva permite a separação entre EPA e DHA, o que é muito interessante uma vez que esses compostos apresentam estrutura similar e há dificuldade na separação destes por protocolos físico-químicos (MORENO-PÉREZ; GUISAN; FERNANDEZ-LORENTE, 2014).

A etanólise de óleo é realizada eficazmente com solventes orgânicos considerando que estes podem modular e melhorar a seletividade e atividade das enzimas. Por outro lado, solventes orgânicos polares são prejudiciais à estabilidade da enzima, e isto deve ser considerado no decorrer da reação (MORENO-PÉREZ; GUISAN; FERNANDEZ-LORENTE, 2014). A Figura 2.6 mostra uma reação genérica de um triglicerídeo com um álcool.

Figura 2.6 - Reação de transesterificação de um triglicerídeo com álcool (ABBASZAADEH et al., 2012)



Considerando que quanto mais seletiva a reação mais interessante industrialmente, uma vez que normalmente o foco está em um dos dois ácidos graxos (EPA ou DHA), é feito o cálculo de seletividade, que nada mais é do que a relação entre etíl ésteres (EE): EE-EPA e EE-DHA (MORENO-PÉREZ; GUISAN; FERNANDEZ-LORENTE, 2014), onde, dentro desse conceito, há, obviamente, a busca por valores elevados deste parâmetro.

Um interessante estudo feito por Moreno-Pérez et al (2014) mostra as diferenças nos valores de seletividade ao se utilizar diferentes enzimas, e utilizando diferentes métodos de imobilização, em reações com ciclohexano ou álcool terc-amílico. Lipases de *Candida antarctica* fração B (CalB), *Thermomyces lanuginosus* (TLL) e *Rhizomucor miehei* (RML) imobilizadas em Sepabeads C18, com o uso do álcool na reação, apresentaram seletividades de 1, 8 e 13, respectivamente. Enquanto que a resposta ao se aplicar os mesmos derivados na reação, agora com ciclohexano, aumenta para 3, 29 e 13. Diferentes tipos de suportes também afetaram a seletividade das reações, por exemplo, TLL imobilizada em Duolite e Lewatit, com ciclohexano, apresentou valores de seletividade de 12 e 4. Os autores estudaram ainda diferentes recobrimentos dos suportes, com consequente modificação nos valores de seletividade. Por exemplo, TLL imobilizada em Sepabeads C18 e com 3 distintas modificações (PEI, aminação e dextrana) apresentaram valores de 20, 8 e 19 de seletividade.

2.7 RESOLUÇÃO DE ENANTIÔMEROS

Lipases são enzimas que agem eficientemente na resolução de moléculas quirais. A maioria das substâncias com atividade biológica, como fármacos, pesticidas e agro-químicos, são moléculas quirais. Estas

moléculas normalmente possuem um ou mais centros quirais, necessitando a resolução óptica para a obtenção de um produto enantiomericamente puro (KOBLITZ, 2008; MIŠL'ANOVÁ; HUTTA, 2003). O ácido mandélico tem sido objeto de muitos estudos estereoquímicos utilizando lipases (CAO et al., 2014; PALOMO et al., 2002; TULASHIE; LORENZ; SEIDEL-MORGENSTERN, 2010). Os isômeros puros de R e S mandélico são muito utilizados em síntese orgânica. Por exemplo, o ácido (R) mandélico é um intermediário versátil no preparo de cefalosporinas sintéticas (grupo de antiobióticos relacionados com a penicilina), agentes antitumorais e antiobesidade (HUMMEL; SCHUTTE; KULA, 1988; KIM et al., 2000).

A qualidade do produto da resolução racêmica é caracterizada pelo excesso enantiomérico, parâmetro que se refere a pureza optica. Porém um parâmetro muito importante que deve ser considerado é a razão enantiomérica (E). Um valor de E elevado para um determinado derivado é fundamental para o sucesso da resolução enantiomérica. Valores de E inferiores a 15 tornam a reação inviável em termos práticos. Valores entre 15-30 são considerados moderados a bons. Acima desses valores, E é considerado excelente. Valores de E superiores a 200 não podem ser determinados com precisão devido aos métodos comumente utilizados (RMN, CLAE ou CG) (FABER, 1997; SIH et al., 1992 citados por MANOEL, 2011). A técnica mais amplamente utilizada no controle de produtos farmacêuticos e medicamentos de qualidade é a cromatografia líquida de alta eficiência (CLAE) (MIŠL'ANOVÁ; HUTTA, 2003).

Torres et al. (2006) ao imobilizar CalB em CNBr obtiveram valor de razão enantiomérica de 8,5; 9,0 e 12 para os pHs de 5, 7 e 9, respectivamente, utilizando éster metílico de ácido mandélico e conduzindo a reação a 25 °C. Os autores observaram que uma menor temperatura possibilitou o aumento no valor de E: a 4°C o valor obtido foi de 13 e a 37 °C foi de apenas 6. Os autores verificaram, ainda, que mudanças no derivado afetam os valores de E obtidos.

2.8 CONSIDERAÇÕES A RESPEITO DO ESTADO DA ARTE

O presente trabalho visa agregar conhecimento na área de imobilização de enzimas, com o estudo das interações enzima-polímero e sua aplicação numa reação de interesse para indústria. Considerando que os polímeros sintetizados por miniemulsão possuem muitas aplicações tecnológicas e biomédicas (LANDFESTER, 2009;

VALERIO et al., 2013), porém ainda são pouco explorados como suportes para a imobilização de enzimas, o ineditismo e contribuição do trabalho são inquestionáveis.

A aplicação de suportes biodegradáveis em reações como a de produção de ésteres etílicos de ácidos graxos visa aliar as características ambientais inerentes da biodegradabilidade do polímero, e os benefícios do uso de reações sem o uso de solventes orgânicos. Porém, deve ser utilizado com cautela para garantir que nenhum vestígio de polímero fique na reação, e após a desnaturação da enzima, o polímero possa ser reutilizado ou degradado.

O estudo pretende também servir de base para futuros usos de derivados obtidos via miniemulsão, e a maneira com que estes possam ser utilizados, tanto no processo de imobilização quanto na reação de catálise.

3. MATERIAL E MÉTODOS

3.1 MATERIAL

O material utilizado no desenvolvimento do trabalho está listado e devidamente referenciado no início de cada capítulo referente aos resultados (Capítulos V, VI, VII, VIII e IX).

3.2 DIÁLISE DAS ENZIMAS *Candida antarctica* Fração B (CalB) E *Thermomyces lanuginosa* (TLL)

A etapa de diálise fez-se necessária para remoção de estabilizantes e possíveis impurezas do preparado comercial. A diálise foi realizada em solução tampão fosfato 50 mmol.L^{-1}, pH 7,0, por 24 h. Após, a enzima foi congelada (-80 ºC), liofilizada por 24 h e armazenada sob refrigeração (4 ºC) para posterior análise.

3.3 SÍNTESE DAS PARTÍCULAS DE POLIURETANO (PU) PEGUILADAS

Nanopartículas de poliuretano foram sintetizadas por polimerização em miniemulsão (VALÉRIO et al., 2014). A fase aquosa foi preparada com 10% em massa do surfactante (SDS) em relação à fase orgânica e PEG400 (10% em massa em relação à fase monomérica). A fase orgânica foi preparada com solução de IPDI e PCL530 com razão molar (NCO:OH) de 2,5. O IPDI foi dissolvido em 2 mL de ciclohexano e pré-reagido com PCL sob agitação magnética por 30 min a 40 ºC. A fase aquosa foi adicionada lentamente à fase orgânica sob agitação magnética por 5 min. A miniemulsão foi então preparada por sonicação da emulsão anterior em reator encamisado com sonda de ultrassom (Fisher Scientific, Dismembrator Ultrasonic 500, 400 W) em diferentes potências (70 e 90%) por um período de 1, 2 ou 3 min em banho de gelo. A reação de polimerização foi conduzida em temperatura constante de 70 °C durante 3 h em reator encamisado (50 mL) (Figura 3.1).

Figura 3.1 - Reator encamisado utilizado na etapa de polimerização das nanopartículas.

3.3.1 Síntese das partículas de poliuretano PEGuiladas com crodamol

Nesta etapa, o processo de síntese foi realizado como descrito no item 3.2, porém crodamol foi adicionado à fase orgânica. Crodamol GTCC, um triglicerídeo completamente saturado foi adicionado à fase orgânica (20% m/m) como um possível agente protetor da enzima. Este procedimento foi utilizado na primeira etapa do trabalho, onde a enzima CalB foi imobilizada durante o processo de síntese da miniemulsão.

3.3.2 Síntese das partículas de poliuretano PEGuiladas sem crodamol

O processo de síntese foi realizado como descrito no item 3.3. As condições da sonda de ultrassom foram 70 % por 2 min, em banho de gelo. Após a obtenção das partículas, estas foram congeladas (-80 ºC) e liofilizadas por 48 h para posterior uso.

3.4 IMOBILIZAÇÃO DAS ENZIMAS CalB E TLL NAS PARTÍCULAS DE POLIURETANO PEGUILADAS

3.4.1 Imobilização de CalB em nanopartículas de poliuretano PEGuiladas

A primeira etapa do trabalho consistiu na imobilização da enzima CalB nas nanopartículas de PU durante o processo de síntese das partículas poliméricas. A enzima foi adicionada à fase aquosa nas proporções de 5, 10 ou 20 % em massa (em relação à fase monomérica).

3.4.2 Imobilização das enzimas CalB e TLL nas partículas de poliuretano PEGuiladas

No desenvolvimento da segunda etapa do trabalho, as partículas foram produzidas e liofilizadas por 48 h. Após, foi realizada a imobilização das enzimas CalB e TLL nas partículas de PU. Partículas de PU-PEG (1 g) foram utilizadas no processo de imobilização. A mistura foi colocada em agitador rotatório para tubos à temperatura ambiente (25 ºC) até máxima imobilização (determinada quando a atividade enzimática permanece constante). Amostras foram retiradas periodicamente, e a atividade enzimática do sobrenadante e da suspensão foram medidas (URRUTIA et al., 2013).

3.5 CARACTERIZAÇÃO DAS NANOPARTÍCULAS DE POLIURETANO

Os produtos da etapa de polimerização foram verificados por espectroscopia de infravermelho com transformada de Fourier (FTIR), em modo de refletância total atenuada (ATR). Foi utilizada banda de absorção com a localização do pico a 2272 cm^{-1}, devido à vibração de alongamento dos grupos isocianato N=C=O, para identificação do IPDI. A banda de absorção com a localização de pico entre 1680-1650 cm^{-1} para identificação do grupo N-H de ureia e a banda de absorção entre 1740-1700 cm^{-1}, devido ao alongamento da vibração de C=O do grupo de uretano. Estas bandas foram utilizadas para identificar a PU ao final do tempo de reação (VALERIO et al., 2014).

3.6 MORFOLOGIA DA ENZIMA CalB IMOBILIZADA EM NANOPARTÍCULAS DE POLIURETANO

A morfologia da CalB imobilizada foi determinada utilizando microscópio ótico de fluorescência (Leica DM5500). As amostras foram preparadas com gotas da miniemulsão em uma suspensão aquosa diluída (1:10, v/v), adicionada de corante azul de metileno em uma lâmina de vidro, à temperatura ambiente.

3.7 SÍNTESE DAS PARTÍCULAS DE POLIMETILMETACRILATO (PMMA) E IMOBILIZAÇÃO DA ENZIMA CalB

Para a síntese das partículas de PMMA foi utilizada uma fase orgânica composta por metilmetacrilato (MMA), crodamol, e AIBN, de acordo com a Tabela 3.1, em seguida foi realizada agitação magnética por 20 min em banho de gelo. Após, a fase orgânica foi adicionada à fase aquosa (água, SDS como surfactante, enzima CalB e KPS conforme Tabela 3.1). A dispersão foi agitada por 10 min em agitador magnético antes de sonicação com sonda de ultrassom (Fisher Scientific, Sonic Dismembrator Model 500) por 2 min e amplitude de 70% para o preparo da miniemulsão. A miniemulsão foi colocada em banho de gelo durante a sonicação, evitando polimerização precoce. As reações de polimerização foram realizadas em ampolas submersas de 20 mL imersas em banho termostático à temperatura constante de 70 °C. Amostras foram removidas periodicamente e solução de hidroquinona 1% foi adicionada.

Tabela 3.1 – Formulação da polimerização em miniemulsão com MMA.

	Componente (g)			
Experimento	AIBN	KPS	Crodamol	CalB
1	-	0.01	2.7	-
2	0.015	-	2.7	-
3	-	0.01	2.7	0.15
4	-	0.01	2.7	0.3
5	-	0.01	2.7	0.6
6	0.015	-	2.7	0.15
7	0.015	-	2.7	0.3
8	0.015	-	2.7	0.6
9	0.015	-	1.35	0.3
10	-	0.01	1.35	0.3

*Todas as reações foram conduzidas com 90% de água destilada, 3 g de MMA e 0,06 g de SDS.

3.8 CARACTERIZAÇÃO DAS PARTÍCULAS DE PMMA

A conversão de MMA em PMMA foi calculada com base em análises gravimétricas. Para essas medidas, amostras foram retiradas em diferentes intervalos de tempo, colocadas em cápsulas de alumínio com massa conhecida contendo 0,2 g de solução aquosa de hidroquinona 1%. Essas cápsulas contendo o polímero foram secas a 60 °C até massa constante. A conversão foi determinada pela razão entre o teor de polímero experimental e teórico, descontando valores dos componentes não voláteis (emulsificante, enzima, crodamol e hidroquinona utilizada para parar a reação).

O potencial Zeta das nanopartículas do polímero foi medido por dispersão dinâmica de luz (DLS—Malvern Instruments, Zeta Sizer Nano S). Microscopia eletrônica de transmissão (TEM 100 kV—JEM-1011) foi utilizada para investigar a morfologia das partículas. Amostras foram preparadas na proporção 1:10 (polímero:água deionizada).

3.9 ESTUDO DOS SUCESSIVOS CICLOS DE USO DA ENZIMA CalB EM PMMA

Este estudo de reciclo foi realizado com a coleta do substrato (pNPP) residual (sobrenadante) e a suspensão da reação (PMMA-CalB) após de 30 min de hidrólise a 37 °C através de centrifugação a 13.000

rpm por 30 min. Após a centrifugação, o sobrenadante foi recolhido e um novo substrato foi adicionado à reação, realizado agitação e nova centrifugação. Os experimentos de reciclo foram conduzidos até a atividade enzimática de hidrólise apresentar queda de 50% em relação à inicial.

3.10 MEDIDA DOS DIÂMETROS MÉDIOS DAS NANOPARTÍCULAS DE PU E PMMA

Os diâmetros médios (médias de intensidades – Dpz) das nanopartículas foram medidos utilizando equipamento de dispersão dinâmica de luz (DLS, Zetasizer Nano S, from Malvern). Ambos os polímeros foram diluídos em água destilada para realização das medidas para evitar problemas de difusão.

3.11 DETERMINAÇÃO DA ATIVIDADE ENZIMÁTICA

Diferentes métodos de atividade enzimática foram utilizados ao longo do desenvolvimento da tese.

3.11.1 Atividade de esterificação

A atividade de esterificação da lipase foi determinada pelo consumo de ácido láurico na reação de esterificação entre este ácido e o álcool propílico (razão molar ácido láurico-álcool de 3:1) à temperatura de 60 °C utilizando reator encamisado, com a enzima a 5 % (m/m) mantida sob agitação por 40 min. A reação iniciou-se pela adição da enzima ao meio reacional. Alíquotas de 150 µL foram retiradas, em triplicata, no tempo zero e ao final da reação, e adicionadas em uma solução de 20 mL de acetona-etanol (1:1) v/v com a finalidade de cessar a reação e de extrair os ácidos restantes. A quantidade de ácido láurico consumida foi determinada pela titulação com NaOH 0,01 N. Uma unidade de atividade (U/g) foi definida como a quantidade de enzima que conduz ao consumo de 1 µmol de ácido láurico por minuto nas condições experimentais descritas. A atividade enzimática (AE) foi calculada de acordo com a equação 1 (OLIVEIRA et al., 2006).

$$AE = \frac{(Vb - Va).M.1000.Vf}{t..m.Va} \qquad (1)$$

Sendo:
AE: atividade de esterificação (U/g);
Va: volume de NaOH gasto na titulação da amostra retirada após 40 min (mL);
Vb: volume de NaOH gasto na titulação da amostra retirada no tempo 0 (mL);
M: molaridade da solução de NaOH;
Vf: volume final de meio reacional (mL);
t: tempo (min);
m: massa da amostra utilizada na reação (g);
Va: volume da alíquota do meio reacional retirada para titulação (mL).

3.11.2 Hidrólise do *p*-nitrofenil palmitato (pNPP)

A atividade de hidrólise do *p*-nitrofenil palmitato (*p*-NPP) foi medida em solução 0,5% de *p*-NPP (m/v) em etanol. O aumento da absorbância causada pela liberação do *p*-nitrofenol na hidrólise do p-NPP foi medida espectrofotometricamente. Na reação foi utilizado 1 mL de tampão fosfato de sódio 50 mM, pH 7,0 e incubação em banho termostatizado por 5 min à 30 ºC. Após, foi adicionado 2 mL de Na_2CO_3 0,5 M seguido por centrifugação à 10.000 rpm por 10 min. O sobrenadante foi diluído e realizada leitura no espectro a 410 nm. Uma unidade de atividade enzimática (U) foi definida pela quantidade de enzima que catalisa a produção de 1 mmol de *p*-nitrofenol por minuto sob as condições experimentais (CHIOU; WU, 2004).

3.11.3 Hidrólise do *p*-nitrofenil butirato (pNPB)

A atividade enzimática, através da hidrólise do *p*-nitrofenil butirato (*p*-NPB), foi determinada pelo aumento da absorbância a 348 nm produzida pela liberação do *p*-nitrofenol na hidrólise de 0,4 mM de p-NPB em tampão fosfato de sódio 25 mM a pH 7 e 25 °C. A análise foi realizada utilizando espectrofotômetro com célula termostática e agitação magnética contínua (500 rpm) por 2,5 min. A atividade enzimática foi calculada com ε=5.150 $M^{-1}.cm^{-1}$. Atividade enzimática (U) foi definida como μmol de *p*-NPB hidrolisado por minuto por mg de enzima sob as condições descritas (MORENO-PÉREZ et al., 2014).

3.12 CÁLCULOS DOS PARÂMETROS DE IMOBILIZAÇÃO

A porcentagem de imobilização (I) e atividade recuperada (AR) foram calculadas pelas equações (2) e (3), respectivamente.

$$I = \frac{A - B}{A} \qquad (2)$$

$$AR = \frac{C \times 100}{A \times I} \qquad (3)$$

Onde: I é a porcentagem de imobilização, A é a atividade oferecida ao suporte na imobilização, B é a atividade enzimática do sobrenadante no final do processo de imobilização e C é a atividade do derivado no final do processo de imobilização (BENASSI et al., 2013).

3.13 DETERMINAÇÃO DO TEOR DE PROTEÍNA

A concentração de proteína nos extratos enzimáticos foi quantificada pelo método de Bradford (BRADFORD, 1976) utilizando como padrão uma curva de albumina de soro bovino, y=0,9944x+0,0186 (mg/mL).

3.14 SÍNTESE DE ETIL ESTERES (EE) DE ÁCIDOS GRAXOS (OMEGA 3)

As enzimas CalB e TLL imobilizadas na segunda etapa do trabalho (onde a imobilização ocorreu nas partículas poliméricas liofilizadas) foram utilizadas na produção dos ésteres etílicos. Para isto 0,1 g de derivado com atividade conhecida foram adicionados foram adicionados em solução contendo 0,701 mmol de óleo de peixe (óleo de sardinha obtido da Biotec BTSA, Espanha) (Tabela 3.2), 5,77 mmol de etanol, sem o uso de solventes orgânicos e 0,2 g de peneira molecular (3 Å).

A reação foi conduzida à 28 e 37 °C por 72 h sob agitação magnética constante. A síntese dos ácidos eicosapentaenóico (EPA) e docosahexaenóico (DHA) foi verificada em sistema HPLC (MORENO-PÉREZ; GUISAN; FERNANDEZ-LORENTE, 2014) utilizando RP-HPLC (Spectra PhysicSP 100 acoplado com detector UV Spectra Physic SP 8450) utilizando coluna de fase reversa (Ultrabase-C8, 150 × 4.6

mm, 5 µm). A vazão utilizada foi de 1,5 mL/min com fase móvel composta por acetonitrila/água/ácido acético (80:20:0,1) e pH 3. A detecção em UV foi realizada em 215 nm. As porcentagens foram calculadas com base nas áreas dos picos correspondentes ao EE-EPA (tempo de retenção de 10 min) e EE-DHA (tempo de retenção de 13 min). A Figura 3.2 mostra um exemplo de um cromotograma obtido após 48 h de reação com TLL imobilizada em PU-PEG4000. Como forma de comparação uma reação sem o derivado também está apresentada (Figura 3.3).

Figura 3.2 – Cromatograma da síntese de ésteres etílicos pelo derivado TLL-PU-PEG4000 após 48 h de reação.

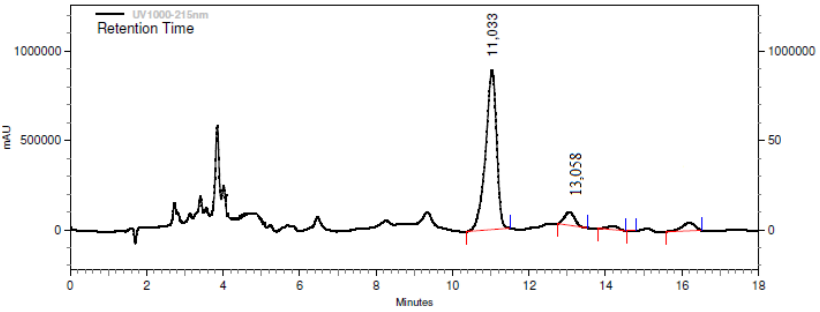


Figura 3.3 – Cromatograma da síntese de ésteres etílicos após 48h de reação sem derivado.

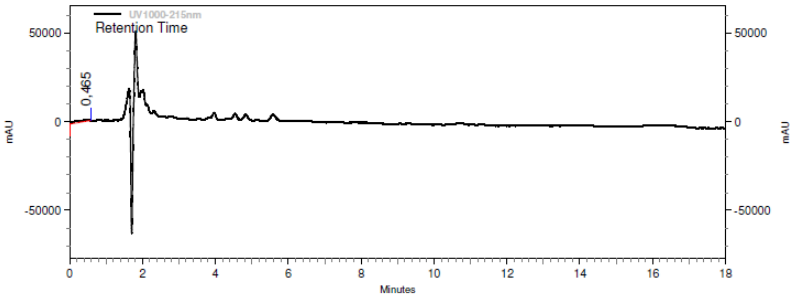


Tabela 3.2 – Composição do óleo de peixe utilizado no presente trabalho.

Componente (%)	
Ácido oleico	20
EPA	18
DHA	12
Ácido palmítico	17,5
Ácido palmitoleico	14
Ácido mirístico	7,3
Ácido esteárico	7
Ácido linolênico	2
Ácido linoleico	1
Ácido docosapentaenóico	0,95
Ácido araquidônico	0,25
Características	
Massa molecular	356,55 g/mol
Densidade	900 kg/m^3

3.15 HIDRÓLISE ENZIMÁTICA DO ÉSTER ETÍLICO DE (R,S) ÁCIDO MANDÉLICO

Ao final do trabalho foi realizado um breve estudo sobre a separação enantiomérica. Este estudo foi realizado com a enzima CalB imobilizada em PU-PEG400, 4000 e 6000. Para a reação de hidrólise, 0,1 g do derivado foi adicionado à 5 mL de uma solução de etil éster de (R,S) ácido mandélico 10 mM dissolvida em tampão fosfato pH 7,0 (25 mM) e a reação foi conduzida sob agitação leve à 37 °C. Foram coletadas amostras em diferentes tempos e o grau de hidrólise foi confirmado por HPLC através de coluna de fase reversa Kromasil C18 (25 cm×0.4 cm) (Spectra Physic SP 100 acoplada com detector UV Spectra Physic SP 8450). A fase móvel utilizada foi composta por acetonitrila (30 %) e tampão fosfato de amônio 10 mM. A vazão utilizada foi de 1,5 mL/min absorbância de 254 nm (TORRES et al., 2006a). Os tempos de retenção foram de 2,5 min para o ácido e 6,5 min para o éster.

A conversão do substrato em produto foi realizada por comparação das respectivas áreas.

3.16 DETERMINAÇÃO DO EXCESSO ENANTIOMÉRICO (EE)

O excesso enantiomérico foi determinado em coluna Chiracel OD-R (Spectra Physic SP 100 acoplada com detector UV Spectra Physic SP 8450). A fase móvel foi composta por acetonitrila (30%) e água MilliQ (70%) a pH corrigido para 2,3. O fluxo utilizado foi de 0,5 mL/min a 225 nm (PALOMO et al., 2003b). O excesso foi calculado pelas diferenças entre as respectivas áreas dos isômeros R e S.

4. REFERÊNCIAS BIBLIOGRÁFICAS

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5. RESULTADOS E DISCUSSÃO

Os resultados deste trabalho estão apresentados na forma de artigos científicos. Os referidos artigos estão apresentados nos itens Resultados I, II, III, IV e V. Os resultados apresentados nos itens I e II foram desenvolvidos no Laboratório de Engenharia Bioquímica (Engebio) na Universidade Federal de Santa Catarina, e contaram com o apoio do Laboratório de Controle de Processos (LCP). Os demais resultados foram obtidos no Departamento de Biotecnología y Microbiología de los Alimentos no Instituto de Investigación en Ciencias de la Alimentación CIAL-CSIC em Madrid (Espanha).

Em resultados I está apresentado o artigo "*Immobilization of Candida antarctica lipase B on PEGylatedpoly(urea-urethane) nanoparticles by step miniemulsion polymerization"* publicado na *Journal of Molecular Catalysis B: Enzymatic*. Resultados II referem-se aos estudos realizados com PMMA, "*Kinetic Study of Candida antarctica Lipase B Immobilization Using Poly(Methyl Methacrylate) Nanoparticles Obtained by Miniemulsion Polymerization as Support"* publicado na *Applied Biochemistry and Biotechnology*. Ambos os trabalhos estão relacionados com a imobilização da enzima durante a formação da miniemulsão.

Na segunda etapa do trabalho, o suporte foi obtido por miniemulsão e liofilizado para posterior uso (Resultados III, IV e V). Resultados III apresenta o estudo "*Synthesis and modification of polyurethane for immobilization of Thermomyces lanuginosus (TLL) lipase for ethanolysis of fish oil insolvent free system*" publicado na *Journal of Molecular Catalysis B: Enzymatic.* Um trabalho relacionado ao uso de agentes de ligação também foi realizado "*Stabilization of lipase from Thermomyces lanuginosus by crosslinking in PEGylated polyurethane particles by polymerization:Application on fish oil ethanolysis"*e submetido à *Biochemical Engineering Journal* (Resultados IV). Por último, foi desenvolvido o trabalho "Synthesis of ethyl esters of Omega-3 fatty acids and enzymatic hydrolysis of (R,S)-mandelic acid ethyl ester by *Candida antarctica* lipase B immobilized in a biodegradable matrix" e submetido à *Enzyme And Microbial Technology.*

RESULTADOS I

Immobilization of *Candida antarctica* lipase B on PEGylated poly(urea-urethane) nanoparticles by step miniemulsion polymerization

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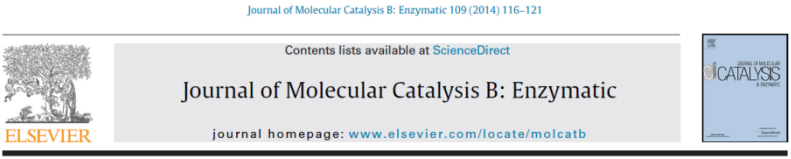

Immobilization of *Candida antarctica* lipase B on PEGylated poly(urea-urethane) nanoparticles by step miniemulsion polymerization

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Abstract

In this work, *Candida antarctica* lipase B (CalB) was immobilized in PEGylated poly(urea-urethane) nanoparticles by step miniemulsion polymerization. The nanoparticles were synthesized using isophorone diisocyanate (IPDI) and polycaprolactone diol (PCL530) as monomers and crodamol as hydrophobe. The aqueous phase was composed by DI water, surfactant (SDS), PEG400 and free enzyme. The miniemulsion was prepared by sonication at different power intensity (70 and 90%) for 1, 2, and 3 min. The enzymatic activity was determined by esterification of lauric acid and n-propyl alcohol. The thermal stability was evaluated at different temperatures and times. The highest enzyme activity (21 U/mg) was obtained at 70% ultrasound power intensity for 2 min, resulting in nano-size particle with an average diameter of 158 nm. After 3 h of incubation, the relative activities of the immobilized and free enzymes were 71.5 and 64.5%, respectively, at 40 °C. FTIR spectrum and optical fluorescence microscopy images confirmed the immobilization of enzyme in PEGylated poly(urea-urethane) nanoparticles.

Keywords: Poly(urea-urethane), miniemulsion, nanostructures, lipase CalB

5.1. INTRODUCTION

Nanostructures are very attractive materials for enzymatic immobilization processes since they have ideal characteristics to determine biocatalysts efficiency, including specific surface area, mass transfer resistance and effective enzyme loading [1]. The development of polyurethanes (PU) has drawn much attention in the last decades due to their excellent physical properties, biocompatibility and biodegradability. Their potential utilizations have been discussed for a variety of pharmaceutical, medical, and cosmetic applications such as drug encapsulation and controlled release [2-4]. The use of PU nanoparticles for enzyme immobilization deserves growing attention due to the high potential of application of such nanostructured material, as well as to improve the knowledge about the mechanisms involved in the immobilization and the effect on enzyme activity. PU nanoparticles have been synthesized using several techniques such as miniemulsion, solvent evaporation and interfacial polymerization [2,3,5-7]. Miniemulsion technique has the advantage of obtaining nanoparticles in a single step without the use of organic solvents. Miniemulsions (also frequently known as nanoemulsions) can be defined as dispersions of relatively stable oil droplets in water with a size range between 50 to 500 nm [8-10]. The oil nanodroplets are obtained by the application of high shear in a system containing oil, water, surfactant and co-stabilizer, also known as ultra-hydrophobe [9-10]. In principle, polymerization occurs in oil, or hydrophobic monomers, nanodroplets allowing the encapsulation of different compounds via step or chain polymerizations. As reported by Tiarks and Landfester [11], when PU nanoparticles are prepared by direct oil in water miniemulsion polymerization two reaction paths may occur: 1) poly-addition of isocyanate with the polyol to form urethane and 2) hydrolysis of isocyanate to form urea with CO2 release.

This second reaction path results in a loss of stoichiometry, as well as the formation of polymers with a lower molar mass [3,12,13]. The urea formation also gives rigid segments to the polymer chain and may reduce the hydrolytic degradation reaction in poly(urea-urethane), PUU, nanoparticles as it reduces water permeability. Lipases (glycerol ester hydrolases E.C.3.1.1.3) are wide spread in nature and have been shown to catalyze hydrolysis/synthesis of wide range of soluble and insoluble carboxylic acid esters and amides, fats and oils. One of the major advantages for popularity of lipases is the high regio and stereo-specificity that they can display in organic synthesis [14,15]. Lipases are

the most used and studied biocatalyst in a wide range of applications, as food technology, biodiesel production or in fine chemistry [16].

Candida antarctica lipase B (CalB) has drawn much attention due to wide industrial application, furthermore has a high stability, stereo selectivity, broad substrate specificity and enantio preference properties that make it superior to other lipases in biotransformation [17,18]. The structure appears to an open conformation with a rather restricted entrance to the active site, that can justify the high specify activity. That way, the open form of the lipases exposes a very hydrophobic pocket to the medium that is very unstable in homogenous aqueous medium, favoring the closed structure. However, this open form is readily adsorbed on the hydrophobic surface of the oil drops even at very low ionic strength (interfacial activation) [16].

The use of nanostructured materials may improve the efficiency of enzyme because small particles provide a larger surface area, leading to an increased amount of enzyme per particles [15,19]. The use of miniemulsion to obtain nanostructured system for enzyme immobilization is relatively new and still lacks a better understanding about the effect of reaction conditions on enzyme activity. Thus, in this paper, it is presented a study of immobilization of the *Candida antarctica* lipase B (CalB) on PEGlated poly(urea-urethane) nanoparticles by step miniemulsion polymerization. The effect of different operation conditions, such as ultrasound power intensity and sonication time, and the presence of ultra-hydrophobe, crodamol, in the PU nanoparticles, was evaluated based on PU particle size and enzymatic activity. The results reported in this work shown a promising immobilization method in the biocatalysis area.

5.2. MATERIAL AND METHODS

5.2.1. MATERIALS

Isophorone diisocyanate (IPDI, 98%, Mw 222 g/mol) and cyclohexane (97%) were purchased from Alfa Aesar. Poly(ethylene glycol) diol with nominal molar mass of 400 Da (PEG400) was purchased from Fluka. Polycaprolactone diol with molar mass 530 Da (PCL530) was purchased from Sigma-Aldrich. Surfactant sodium dodecyl sulfate (SDS) obtained from Aldrich Chemicals Ltd. Crodamol GTCC, a fully saturated triglyceride used as hydrophobic agent, was

purchased from Alfa Aesar. Free *Candida antarctica* B was kindly donated by Novozymes Brazil (Araucária, PR, Brazil).

5.2.2 METHODS

5.2.2.1 *Candida antarctica* lipase B purification

The enzyme CalB was dissolved in sorbitol, making it necessary to their prior purification. The purification was performed by dialysis in a phosphate buffer 50 mmol.L-1 (pH 7.0). After purification, the enzyme was lyophilized for 24h and stored under refrigeration for further analysis.

5.2.2.2 Immobilization of CalB in PEGylated poly(urea-urethane) nanoparticles

PEG-PU nanoparticles were synthesized by miniemulsion polymerization based on the procedure previously described by Valerio et al. [2]. The organic phase was composed by IPDI and PCL530 solution (2.5 NCO:OH molar ratio) and crodamol (20 wt% in relation to the organic phase). The aqueous phase was prepared with 10 wt% of surfactant (SDS), 10 wt% of PEG400 and 10 wt% of free enzyme. All of the quantities were related to the organic phase. The total holdup (wt. of organic phase / wt. of reaction medium) was 20%. IPDI was dissolved in 2 mL of cyclohexane and pre-reacted with PCL under magnetic stirring for 10 min at room temperature. The aqueous phase was then added slowly to the organic phase under magnetic stirring and kept for 5 min forming an unstable emulsion. The miniemulsion was prepared by sonication of the previous emulsion in a jacketed flask with an ultrasonic probe (Fisher- Scientific – Ultrasonic Dismembrator 500, 400 W) set to different power intensity (50, 70 and 90 %), for a period of 1, 2 or 3 min. Polymerizations were conducted at constant temperature 70°C during 3 hours in a jacketed flask (50 mL). To purify the Pegylated PU – enzyme nanoparticles, the latex was centrifuged and washed with DI water. The nanoparticles were ressuspended in DI water and subsequently analyzed.

5.2.2.3 Characterization of immobilized CalB in PEGylated poly(urea-urethane) nanoparticles

The completion of the step polymerization was verified by Fourier transform infrared spectroscopy (FTIR) in the attenuated total reflectance (ATR) mode. The absorption band with peak location at 2272 cm–1, due to N = C = O stretching vibration of the isocyanate groups, was used to identify IPDI. The absorption band with peak location between 1680-1650 cm–1 of N-H group of urea and the absorption band between 1740-1700 cm–1 due to stretching vibration of C = O group of urethane, were used to identify the PU, after the reactions had taken place [3]. Average diameters (intensity averages – Dpz) of polymer particles were measured using dynamic light scattering equipment (DLS, Zetasizer Nano S, from Malvern).

5.2.2.4 Morphology of immobilized CalB in PEGylated poly(urea-urethane)

The morphology of immobilized CalB was determined using fluorescence optical microscopy (wide-field, Leica DM5500). The samples were prepared by dropping droplets of diluted aqueous suspension (1:10, latex: DI water) containing blue methylene on a microscope glass slide at room temperature.

5.2.2.5 Determination of lipase activity

Free and immobilized lipase activity was analyzed by esterification reactions according adaptation of Oliveira et al. [20]. The esterification reactions were conducted between lauric acid and propanol at molar ratio 3:1, temperature of 60 °C and 5 wt% of enzyme concentrate ion in relation to the substrates. The lauric acid content was determined by titration with NaOH 0.01 N. One unit of activity (U) was defined as the amount of enzyme necessary to consume 1 μmol of lauric acid/min at the established experimental conditions presented previously.

5.2.2.6 Enzyme thermal stability

The thermal stability of free and immobilized enzyme were determined by incubation at 40, 50 and 60 °C. The samples were taken

out in pre-determinate interval of 1, 3, 5, and 8 hours. After incubation the relative activity of free and immobilized enzyme were measured by the residual esterification activity as previously described above.

5.3. RESULTS AND DISCUSSION

FTIR spectra confirmed the urethane formation through the absorption band with peak location between 1680-1650 cm-1 for urea and NH absorption band between 1740-1700 cm^{-1} due to stretching vibration of C = O group. From Figure 5.1 (curve c) it is possible to observe that the absorption band with peak location between 1750 and 1700 cm^{-1}, characteristic of the N-H bond of urethane, was intense for the latex obtained by miniemulsion polymerization step using PCL530/PEG400 as monomers, confirming the formation of poly(urea-urethane).

Free lipase (Figure 5.1, curve a) shows a typical spectrum with proteins absorption bands associated with amino group (CONH), primary, and secondary amino groups between 1580-1650 cm^{-1} [21]. After immobilization, the enzyme characteristic peaks were changed, possibly due to the reaction of the amino groups of enzymes with the isocyanate group of IPDI during the incorporation of free enzyme to the polymer particle surface, changing the absorptions bands. In order to verify the enzyme immobilization, the FTIR spectrum of free enzyme was compared with the FTIR spectrum of PEG-PU-enzyme. The interaction between enzyme CalB and PU nanoparticles can be confirmed by the overlap between spectra a and b (Figure 5.1) by the absorption band with peak location between 2400 and 2350 cm^{-1}.

5.3.1 Effect of crodamol content

Due to the high sonication intensity delivered by the ultrasound probe used in the miniemulsification step the enzyme structure can be damaged. Thus, in the first step of this work, the use of crodamol as a possible agent of protection during the ultrasonification time using different power intensity was studied. From Table 5.1 one can see that the use of crodamol, for all tested ultrasound power intensity and time led to an enhancement on enzyme activity. The enzyme activity (EA) with and without crodamol under different ultrasound probe intensity is presented in Table 5.1. According to the results in Table 5.1, the highest value for enzymatic activity (21.01 U/mg) was obtained when crodamol was submitted to a intensity of the ultrasound probe of 70% by 2

minutes. It can be seen that the use of ultrasound probe can increase the enzyme activity, and crodamol showed a protective effect on the enzyme, preventing or reducing the denaturation by comparing the results for the same probe conditions (70% for 2 minutes) without (10.01 U/g) and with (21.01 U/g) crodamol. One possible explanation for the this protective effect can be the increase on the viscosity in the dispersed phase due the presence of crodamol, with increasing the viscosity of the dispersed phase is possible increase the mechanical resistance on the nanoparticles. Therefore, crodamol can promote the enzyme structure protection by attenuating the intensity of sonication.

Figure 5.1 – Fourier transform infrared spectra (FTIR) of PEG-PUU nanoparticle synthesized by step miniemulsion polymerization. (a) Free enzyme, (b) Immobilized enzyme in PEGylated poly(urea-urethane) nanoparticle, and (c) PEGylated poly(urea-urethane) nanoparticle.

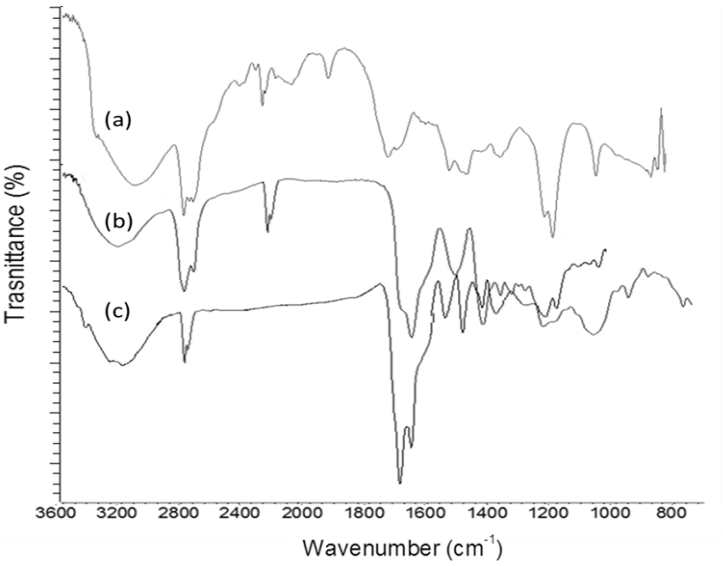


Table 5.1 – Effect of crodamol on the enzyme activity under different ultrasound power intensity and sonication time.

Intensity (%)	Time (min)	Enzyme activity (U/mg)	
		No crodamol	Crodamol
70	1	11.10 ± 0.37^{b}	20.31 ± 0.19^{a}
	2	10.01 ± 0.45^{b}	21.01 ± 0.70^{a}
	3	13.66 ± 0.68^{a}	16.06 ± 0.55^{b}
90	1	5.63 ± 0.31^{b}	14.90 ± 2.5^{b}
	2	4.27 ± 0.04^{c}	19.47 ± 0.58^{ab}
	3	7.82 ± 0.67^{a}	19.98 ± 1.01^{a}

Values are expressed as means ± SD (n=3). Different letters in the same column means significant difference at 95% confidence.

5.3.2 Effect of probe conditions: influence of power intensity on the free enzyme

In order to verify the influence of ultrasound power intensity on the enzyme activity and enzyme size, some experiments were performed using a water free enzyme(10 wt%) solution keeping the ultrasound power intensity constant at 70% at different sonication times (see Table 4.2). The direct ultrasonification in the free enzyme also affects the performance of the biocatalyst increasing the activity 8 times, from 1.81 to 14.49 U/mg in the first minute 70% of ultrasound power. On the other hand, when the sonification time was increased to 3 minutes a reduction on the enzyme activity to 8.45 U/mg was observed.

During the sonification, the ultrasound waves can stimulated the enzyme active center, resulting in conformational changes and consequently increasing lipase activity, obviously, the influence of sonication on the activity and enzyme stability also depends on the sonication probe parameters and the specific enzyme preparation [22].

Table 5.2 – Effect of sonication time (ultrasound power intensity of probe) on enzyme activity.

Time (min)	AE (U/mg)
0	1.81 ± 0.08
1	14.49 ± 0.22
2	14.62 ± 0.73
3	8.45 ± 0.09

5.3.3 Effect of probe conditions: influence of enzyme content and ultrasound operation conditions in the size of PEGylated poly(urea-urethane) nanoparticles

In order to investigate the effect of sonication time, ultrasound power intensity, and presence of enzyme on the particle size of PEG-PUU nanoparticles, different miniemulsion polymerizations were performed (see Table 5.3). The polydispersity index (PdI) higher than 0.1 shows that the particle size distributions were broad, although stable miniemulsions were obtained. Results also show that the average particle diameter could be manipulated by changing ultrasound power intensity and sonication time with and without enzyme. According to the literature there are many reports showing that the droplet size decreases with sonication time and ultrasound power intensity [2,3,23,24]. In this work, when the sonication time was increased from 1 to 3 min PEG-PUU particles size decreased. The results indicate that the shear energy provided by the ultrasonic probe was effective in breaking down large droplets to nanodroplets, and suggests that there are optimum sonication conditions where miniemulsion droplets with minimum size and minimum sonication time could be produced.

The introduction of enzyme in the reaction system increased the size of PEGPUU nanoparticles compared with the same reactions without enzyme. For the reactions without enzymes, the average particle size was less than 200 nm, on the other hand, when enzyme was used in concentration of 10 wt%, in relation to the organic phase, an average particle size higher than 200 nm was observed, except for the conditions at 70 % of power intensity when the Dp was 169 and 158 nm, respectively for 1 and 2 minutes. In this case, the increase of PEG-PUU nanoparticles size can be associated with the enzyme immobilization.

The authors decided to use a mild probe condition (70% of power intensity), with less enzyme damage, but effective on the Pegylated PU nanoparticles formation. The nanoparticles obtained by using 90% of power intensity showed lower Dp, but agglomerates formation, which leads to higher Dp after polymerization time.

Table 5.3 – Effect of sonication time and ultrasound power intensity on PEG-PU nanoparticles size obtained by step miniemulsion polymerization.

No enzyme						
	1 min		2 min		3 min	
Intensity (%)	Dp (nm)	PdI	Dp (nm)	PdI	Dp (nm)	PdI
70%	172±1.8	0.331±0.009	159±2.5	0.312±0.007	161±2.3	0.401±0.011
90%	258±2.1	0.231±0.011	256±1.9	0.277±0.008	251±4.3	0.618±0.009
10 wt% enzyme						
	1 min		2 min		3 min	
Intensity (%)	Dp (nm)	PdI	Dp (nm)	PdI	Dp (nm)	PdI
70	200±1.4	0.336±0.021	158±2.8	0.269±0.002	169±1.4	0.292±0.010
90	280±1.4	0.252±0.015	267±7.8	0.321±0.001	272±4.9	0.294±0.018

5.3.4 Effect of enzyme content in the PEGylated poly(urea-urethane) nanoparticles

The enzyme content used in the immobilization process was studied in a range from 5 to 20 wt%, and the results in terms of enzyme activity are shown in Table 5.4. As we can see from this table when the highest enzyme concentration (20 wt%) was used a significant reduction on the enzyme activity was demonstrated. The enzyme concentration increase from 10 to 20 wt% could be lead to saturation on the PEG-PUU nanoparticle surface letting the enzyme more susceptible to denaturation, reducing the enzyme activity. On the other hand, when the enzyme content was increased from 5 to 10 wt% an increase on enzyme activity from 6.23 to 21.09 U/mg was observed. The results suggest that

there is a maximum enzyme concentration in the PEG-PU nanoparticles immobilization where the enzyme can be protected against denaturation.

The enzyme immobilization yield (enzyme amount immobilized in a known PEG-PU content) and the activity retention were determined for the best polymerization condition (70%, 2 min as probe condition and 3 h of reaction time). For the enzyme immobilized in PEGylated poly(urea-urethane) nanoparticles using 10 wt% of crodamol, an immobilization yield of 82.4% was verified, with 57% of activity retention.

Table 5.4 – Effect of enzyme content in the PEGylated poly(urea-urethane) nanoparticles, using 70% ultrasound power intensity for 2 minutes and 20 wt% of crodamol related to monomers.

Enzyme content (wt%[a])	EA (U/mg)
5	6.23 ± 0.29
10	21.01 ± 0.70
20	0.38 ± 0.09

[a] wt% related to monomers.

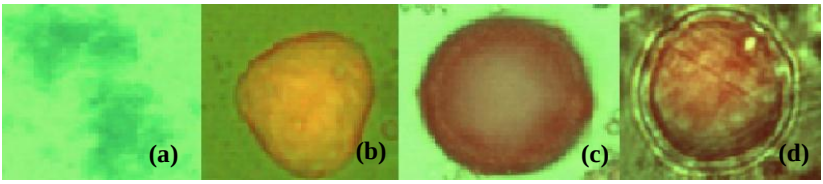
5.3.5 Morphology of immobilized PEGylated poly(urea-urethane)

The images obtained using fluorescence optical microscopy are shown in Figure 5.2, where the morphological difference between free enzyme, lipid content (crodamol), PEG-PUU nanoparticles and the immobilized lipase in PEGylated poly(urea-urethane) nanoparticles synthesized by step miniemulsion polymerization are reported. Figure 5.2a and 5.2b shows free enzyme, and the lipid content, from crodamol, previously to the polymerization, respectively. The morphology of free enzyme PEGylated poly(ureaurethane) nanoparticles with crodamol is shown in Figure 5.2c, in this case, it is possible to observe a lipid core (crodamol) and a smooth and thin polymeric shell (poly(ureaurethane)).

Finally, at the end of the step miniemulsion polymerization it is possible to observe the enzyme immobilized (core) in the PEGylated poly(urea-urethane) nanoparticles (Figure 5.2d). From Figure 5.2 it is also possible to confirm the enzyme immobilization in the core of PEGylated-poly(urea-urethane) particles, being possible to see the presence of enzyme in the particles surface (Figure 5.2d). The image

showed that the linkage between enzyme and the poly(urea-urethane) can also occurs, since the amino groups from enzyme and NCO group from IPDI present in the interface water/oil can react. Thus, also in Figure 5.2d, it is possible to see an incorporation of the enzyme in the PEG-PU particles surface.

Figure 5.2 – Different morphologies during the enzyme immobilization by step miniemulsion polymerization. Free enzyme (a), lipid content (crodamol) (b), PEGylated poly(urea-urethane) particles (c), and immobilized enzyme in PEGylated poly(urea-urethane) particles (d).



5.3.6 Thermal stability of immobilized lipase calb in PEGylated poly(ureaurethane)

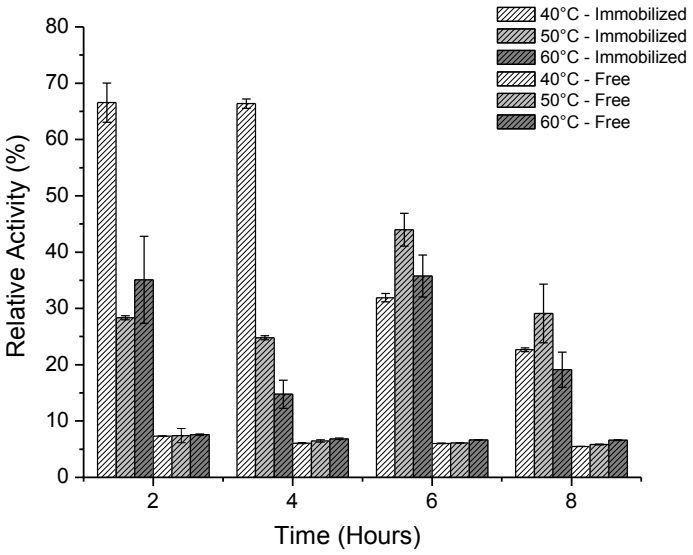
The stability of the immobilized enzyme catalyst under increasing temperature must be understood to determine optimal conditions for the reusable solid-supported catalyst. Understanding the dynamics of the enzyme/solid support interface under varied reaction environments help in the development and control of reaction rates using immobilized enzyme [25]. The results in terms of relative enzyme activity with time are shown in Figure 5.3. The influence of temperature on the catalytic activity of free and immobilized enzyme in PEGylated poly(urea-urethane) at 40, 50 and 60 °C is presented in Figure 5.3. The results suggest that the interaction between enzyme and poly(urea-urethane) as support directly influence the relative enzyme activity.

The activity of the immobilized enzyme was higher than that observed for the free enzyme, for all times and temperatures studied in this work. It was possibly due to the immobilization process used, which, although no reports were presented yet of the use of the methodology proposed in this paper, what is known about the miniemulsions is its characteristic by a great stability in suspension due to its very small size, essentially the consequence of significant steric stabilization between droplets. The first three columns in the graph

(Figure 5.3) show the immobilized enzyme stability at 40, 50, and 60 ºC, respectively. After 4 hour of incubation, the value of relative activity to immobilized enzymes was 67.0, 25.0 and 14.8% at 40, 50, and 60 °C, respectively. On the other hand, the relative activity for the free enzyme obtained was less than 10% at all temperatures (also 4 hours of incubation). A slight change in the enzyme conformation caused by temperature increase may result in enzyme activation, possibly what was observed at 6 h of incubation at 50 and 60°C (44 and 36%, respectively), whereas at 40 °C obtained value the relative activity was 31.9%.

Matsuura et al. [26] studied the encapsulation of *Phycomyces nitens* lipase in mesoporous silica monoliths (HOMs) with different pore structures (2D and 3D mesostructures). They reported that at 35°C the relative hydrolytic activity after 30 minutes of incubation was 30, 75 and 70% for the free, 3D and 2D mesostructures immobilized lipases, respectively. Uyanik et al. [27] researched the chemically immobilization of lipase from *Candida rugosa* (CRL) within a chemically inert sol–gel support in the presence of calix(aza)crowns as the new additives. The catalytic activity of the encapsulated lipases was evaluated both in the hydrolysis of p-nitrophenyl palmitate (p-NPP) and the enantioselective hydrolysis of racemic Naproxen methyl ester. The hydrolysis activity using p-nitrophenyl palmitate as substrate showed that the free enzyme loses its initial activity after 100 min at 60 ºC, while the immobilized lipases retained their initial actives in 62 % after 120 minutes of incubation at the same temperature.

Figure 5.3 - Thermal stability at 40, 50 and 60 ºC of free and immobilized CalB lipase in PEGylated poly(urea-urethane) nanoparticle prepared with SDS (10 wt%), crodamol (20 wt%), enzyme (10 wt%), IPDI : castor oil (2.5:1) and PEG400.



As reported by Cui et al. [28] polyurethane foam (PUF) was used for immobilization of *Yarrowia lipolytica* lipase Lip2 via polyethyleneimine (PEI) coating and glutaraldehyde (GA) coupling. The thermal stability for the immobilization preparations was enhanced compared with that for free preparations. At 50 ºC, the free enzyme lost most of its initial activity after a 30 min of heat treatment, while the immobilized on PEI-coated PUF retaining about 70 % of its initial activity at the same conditions, the relative activity was 40 % when the incubation time increased to 1 hour.

5.4. CONCLUSIONS

In this work PEGylated poly(urea-urethane) nanoparticles as a new alternative to conventional methods of encapsulation was used as support to *Candida antarctica* lipase immobilization. From the enzyme immobilization using IPDI, PCL 530 and PEG 400 as monomers, with 70% and 2 min as probe condition it was possible to obtain the higher

esterification activity of 21 U/mg, resulting in a 158 nm nanoparticle size. The results obtained by fluorescence microscopy proved the immobilization of lipase onto PEGylated poly(urea-urethane) nanoparticles. Moreover, the immobilization process retains h 67% of enzyme activity after 4 h of incubation at 40°C, showing that it is possible to use CalB enzymes as biocatalyst for application in the chemistry and food industry. In view of the promising results obtained in this work, one can conclude that much remains to be explored about lipase immobilization on poly(urea-urethane) nanoparticles obtained by step miniemulsion polymerization.

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RESULTADOS II

Kinetic Study of *Candida antarctica* Lipase B Immobilization Using Poly(Methyl Methacrylate) Nanoparticles Obtained by Miniemulsion Polymerization as Support

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Kinetic Study of *Candida antarctica* Lipase B Immobilization Using Poly(Methyl Methacrylate) Nanoparticles Obtained by Miniemulsion Polymerization as Support

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Abstract

With the objective to obtain immobilized *Candida antarctica* lipase B (CalB) with good activity and improved utilization rate, this study evaluated the influence of enzyme and crodamol concentrations and initiator type on the CalB enzyme immobilization in nanoparticles consisting of poly(methyl methacrylate) (PMMA) obtained by miniemulsion polymerization. The kinetic study of immobilized CalB enzyme in PMMA nanoparticles was evaluated in terms of monomer conversion, particle size, zeta potential, and relative activity. The optimum immobilization condition for CalB was compared with free enzyme in the p-NPL hydrolysis activity measurement. Results showed a higher CalB enzyme stability after 20 hydrolysis cycles compared with free CalB enzyme; in particular, the relative immobilized enzyme activity was maintained up to 40 %. In conclusion, PMMA nanoparticles proved to be a good support for the CalB enzyme immobilization and may be used as a feasible alternative catalyst in industrial processes.

Keywords: Poly(methylmethacrylate). Miniemulsion. Nanoparticles. CalB enzyme. Lipase.

6.1. INTRODUCTION

Among enzymes of industrial interest, standout lipases, which are hydrolases acting on ester bonds of triacylglycerol, have the lipid compounds as natural substrate [1–3]. Lipases can be used in a wide range of industrial processes, such as reduce fermentation time in the brewing industry, softening bread, improve crumb structure and control the non-enzymatic browning, enhance the aroma and hydrolysis of fat milk in the dairy industry, and synthesis of short-chain fatty acids esters and alcohols [2–6].

Candida antarctica lipase B (CalB) has drawn much attention due to the wide industrial application; furthermore, it has a high stability, stereo selectivity, broad substrate specificity, and enantio preference properties thatmake it superior to other lipases in biotransformation [2, 6–8]. The structure appears to an open conformation with a rather restricted entrance to the active site that can justify the high specified activity. That way, the open formof the lipases exposes a very hydrophobic pocket to the medium that is very unstable in homogenous aqueous medium, favoring the closed structure. However, this open form is readily adsorbed on the hydrophobic surface of the oil drops even at very low ionic strength (interfacial activation) [9].

The use of nanoparticle materials may improve the efficiency of enzyme because small particles provide a larger surface area, leading to an increased amount of enzyme per particle [5, 10–12]. Nanoparticles have attracted great attention due to their nanosize range situated between bulk materials and molecules and structures at an atomic level that allowed to observe, transporting photochemical and catalytic properties, due to their reduced size (below 1000 nm) that might increase the absorption and bioavailability of the released drug [13–15].
The synthesis of the nanoparticles by miniemulsion polymerization has many applications in the nanoscience as nanomedicine, drug delivery, and cosmetics and shows as an advantage in relation to the other methods the possibility of obtaining nanocapsules in one single-reaction step and is based on the differences of the interfacial tension and on the phase separation process during polymerization [6, 13, 16]. Miniemulsion are also frequently known as nanoemulsions, fine-dispersed emulsions, sub-micron emulsions, and so forth but are all characterized by a great colloidal stability in suspension due to their very small size (typically exhibiting diameters of up to 500 nm) [13, 17].

Polymers used for encapsulation can either be natural or synthetic. Despite the versatility of the miniemulsion polymerization reactions for the preparation of nanoparticles, this approach is still poorly explored to enzyme immobilization. The polymers used for this application can be a biocompatible synthetic polymer such as poly(methyl methacrylate) (PMMA). This polymer can be considered as a suitable matrix for enzyme immobilization and an adequate polymer for most biomedical and biotechnological applications [6, 15, 18]. In the previous works of the group [15, 16, 19], stable nanocapsules were obtained via methyl methacrylate (MMA) miniemulsion polymerization using Neobee M-5 or Miglyol 812 as co-stabilizer, lecithin as surfactant, and either organo-soluble 2,2′-azobisisobutyronitrile (AIBN) or aqueous phase redox pair hydrogen peroxide and ascorbic acid as initiator. The possibility of encapsulating an antioxidant as quercetin via free radical miniemulsion polymerization was evaluated [19].

Furthermore, immobilization potentially enhances the thermostability, catalytic activity, and selectivity of the enzyme catalyst. It also facilitates easy separation of the enzyme from the product which can minimize or eliminate protein contamination of the product. By permitting catalyst reuse over multiple reaction cycles, the cost of catalyst can become sufficiently low that it may be used to produce commodity chemicals. Therefore, this work aims to study the immobilization of CalB lipase in PMMA, evaluating the variable concentrations of crodamol and enzyme and initiator type (azobisisobutyronitrile-AIBN and potassium persulfate-KPS) on the kinetics of methyl methacrylate miniemulsion polymerization, particle size distribution, and zeta potential and in terms of enzyme activity.

6.2. MATERIAL AND METHODS

6.2.1 Materials

Methyl methacrylate (MMA, 99 %) was purchased from Vetec Química. 2,2′ Azobisisobutyronitrile (AIBN, 98 %) from DuPont™ and potassium persulfate (KPS, P.A.) from Vetec Química were tested as initiators. Surfactant sodium dodecyl sulfate (SDS) was obtained from Aldrich Chemicals Ltd. Crodamol GTCC, a fully saturated triglyceride used as hydrophobic agent, was purchased from Alfa Aesar. Free *Candida antarctica* B lipase was kindly donated by Novozymes Brazil (Araucária, PR, Brazil). p-Nitrophenyl palmitate (p-NPP) was from

Sigma-Aldrich. All materials were used as received except for CalB enzyme,and distilled water was used in all experiments.

6.2.2 Determination of enzyme activity

The enzymatic activity was measured by hydrolysis of p-nitrophenyl palmitate (p-NPP) 0.5 % ethanol. The increase in a peak of absorbance at 410 nm caused by the release of p-nitrophenol in the hydrolysis of p-NPP was measured by spectroscopy UV-vis [20].

6.2.3 Immobilization of CalB enzyme in PMMA nanoparticles by miniemulsion polymerization

Methyl methacrylate miniemulsion polymerizations were carried out aiming the formation of polymeric nanocapsules (Fig. 6.1). The organic phase composed of MMA, crodamol, and, if used, AIBN was prepared according to the formulations shown in Table 6.1 and magnetically stirred for 20 min in an ice bath. In sequence, the organic phase was added to the aqueous phase composed of water, SDS as surfactant, CalB enzyme, and KPS, when mentioned. The dispersion was stirred for 10 min with a magnetic stirrer before sonication with an ultrasound probe (Fisher Scientific, Sonic Dismembrator Model 500) for 2 min with an amplitude of 70 % to prepare the miniemulsion. To avoid the early onset of polymerization, the miniemulsion was cooled in an ice bath during sonication. Batch polymerization reactions were carried out in 20 mL ampoules immersed in a thermostatic bath at constant temperature (70°C). Samples were removed periodically, and reaction was short stopped with the addition of a 1 wt.% hydroquinone solution.

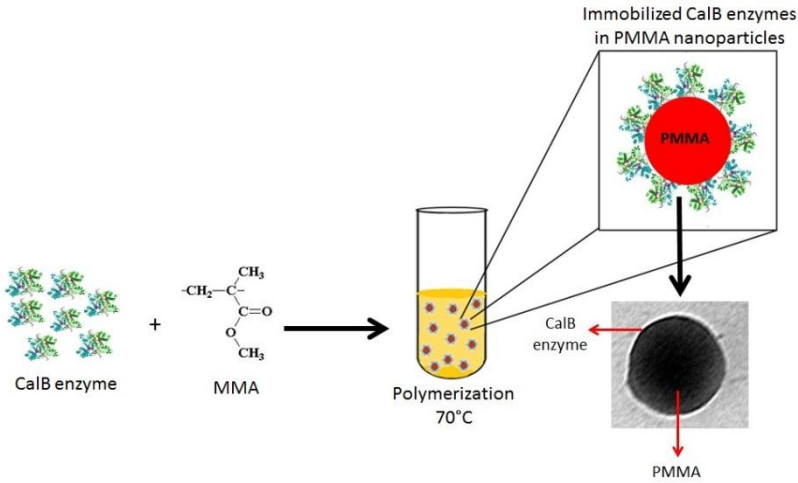
At the end of immobilization processe, the immobilization yield was calculated using equation $(1-EA_S/EA_I)\times 100$ %, where EA_S=supernatant lipolytic activity (U/g) and EA_I= lipolytic activity of the immobilized derivate (U/g).

6.2.4 Polymer characterization

Conversion of MMA in PMMA was calculated based on gravimetric data. For these measurements, latex samples taken at different time intervals were put in pre-weighted aluminum capsules that already contained around 0.2 g of a 1 wt.% hydroquinone aqueous solution. Capsules were dried at 60 °C until constant weight was

obtained. Conversion was determined as the ratio between experimental and theoretical polymer content, and in this calculation, the fraction of "nonvolatile components" (emulsifier, enzyme, crodamol, and hydroquinone added to stop polymerization) was discounted to take only the polymer content into account. Intensity average diameters (Dp) and zeta potential of polymer nanoparticles were measured by dynamic light scattering (DLS—Malvern Instruments, Zeta Sizer Nano S). For the average diameter measurements, samples were diluted in distilled water saturated with MMA monomer in order to prevent monomer diffusion from the droplets to the continuous phase. Transmission electron microscopy (TEM 100 kV—JEM-1011) was used to investigate nanoparticle morphology. Samples were prepared by latexes dilution (1:10) in deionized water. In sequence, several drops of the diluted nanoparticle suspension were placed on a carbon-coated copper grid, dried overnight, and analyzed at 80 kV.

Figure 6.1 - Schematic representation of immobilization of CalB enzyme in PMMA particles



6.2.5 Immobilized CalB enzyme in PMMA: recycling experiments

Schematic diagram of the method used for enzyme recycling is shown in Fig. 6.2, which is described in detail in the following. CalB enzyme recycling was performed by separately collecting the residual

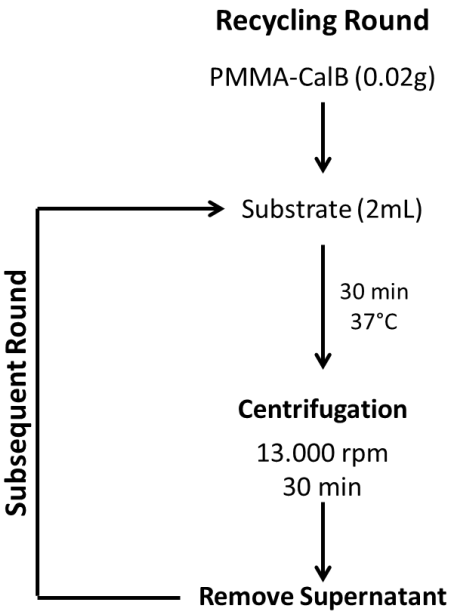
substrates (supernatant) and the reaction suspension (PMMA-CalB) after 30 min of hydrolysis at 37°C by centrifugation at 13.000 rpm for 30 min. After centrifugation, the supernatant was removed and fresh substrate p-NPP was mixed with suspension and then centrifuged. PMMA-CalB recycling experiments were performed until the enzyme activity of hydrolysis decreased to less than 50 %. All experiments were performed in triplicate, and the average values were reported.

Table 6.1 - Formulations of MMA miniemulsion polymerizations.

	Reactants (g)			
Experiment	AIBN	KPS	Crodamol**	CalB enzyme**
1	-	0.01	2.7	-
2	0.015	-	2.7	-
3	-	0.01	2.7	0.15
4	-	0.01	2.7	0.3
5	-	0.01	2.7	0.6
6	0.015	-	2.7	0.15
7	0.015	-	2.7	0.3
8	0.015	-	2.7	0.6
9	0.015	-	1.35	0.3
10	-	0.01	1.35	0.3

All reactions were carried out with 80 wt% of water, 3.0 g of MMA and 0.06 g of SDS. * in relation to disperse phase, ** in relation to organic phase.

Figure 6.2 - Schematic diagrams of enzyme recycling procedure.



6.3. RESULTS AND DISCUSSION

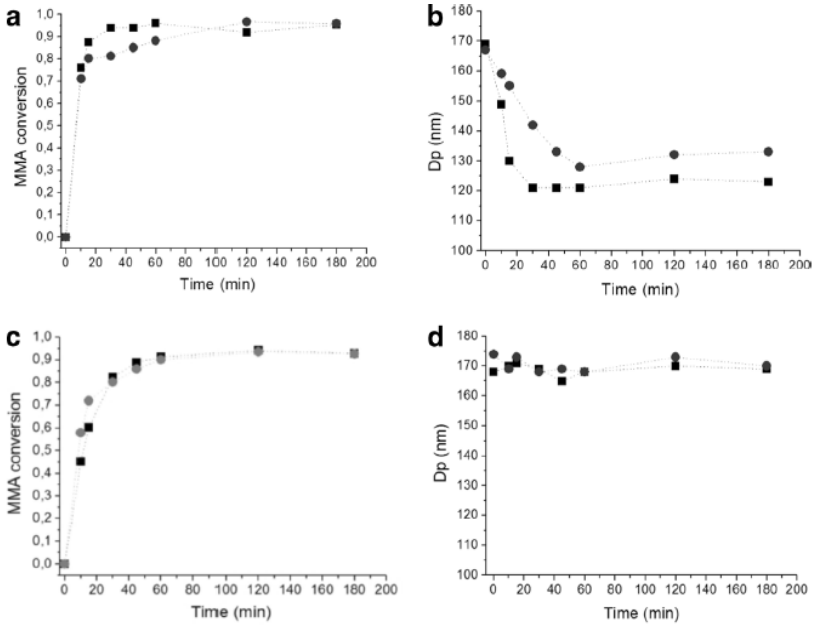
6.3.1 Influence of CalB enzyme on PMMA synthesis

In order to verify the influence of CalB enzyme on MMA miniemulsion polymerization in terms of MMA conversion and nanoparticle diameter, two experiments were performed. Figure 6.3 shows the kinetic results obtained during the MMA polymerization with and without CalB enzyme using KPS and AIBN as initiator.

Figure 6.3a, c compares the evolution of methyl methacrylate conversion during the miniemulsion polymerization using SDS as surfactant, crodamol as co-stabilizer, and different types of initiators. It might be observed that in all reactions, after 30 min reactions, 80 % of monomer conversion was achieved. It might be also observed that while 10 wt.% of CalB enzyme had no effect on kinetics of the reactions with oil soluble initiator AIBN (Fig. 6.3c), when the water-soluble initiator

KPS was used, MMA polymerization was slightly slower in the presence of 10 wt.% of CalB.

Figure 6.3 - Kinetic study of MMA miniemulsion polymerization using KPS (a, b) and AIBN(c, d) as initiator. Black circle MMA reactions using 10 wt.% CalB (exp. 5 and 8) and black square MMA reactions without CalB (exp. 1 and 2).



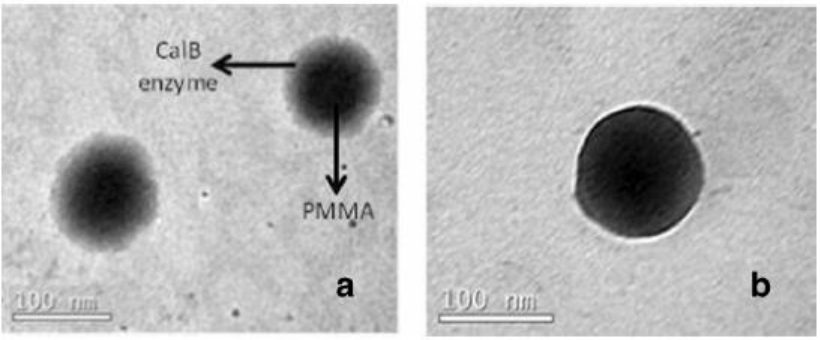
The use of hydrophobic initiators as AIBN, which are dissolved within the organic phase, favors droplet nucleation, while with aqueous phase initiators, as KPS, it becomes more difficult to avoid the coexistence of other undesired particle nucleation mechanism, especially when monomers with a certain water solubility are used [21, 22].

Reactions performed with the water-soluble initiator KPS presented a decrease in particle size during polymerization due to homogeneous particle nucleation, and this decrease was faster and more pronounced in the reaction without CalB (Fig. 6.3b): from 169 to 123 nm and from 167 to 133 nm, respectively, to MMA reactions without and using CalB enzyme in 3 h of reaction. On the other hand, reactions with the organic phase initiator (AIBN) resulted in quite similar average

particle size evolutions, maintaining the initial monomer droplet size (Fig. 6.3d).

Figure 6.4 displays TEM images of the PMMA-CalB enzyme nanoparticles where the PMMA core (darker region) and CalB enzyme surface (brighter region) are observed. TEM images of PMMA-CalB enzyme nanoparticles shown in Fig. 6.4 indicate that nanoparticles present a broad particle size distribution, as already observed by the PdI results obtained by DLS.

Figure 6.4 - TEM images of PMMA-CalB enzyme nanoparticles synthetized using 5 wt.% crodamol, 10 wt.% CalB enzyme; a AIBN and b KPS as initiator.



6.3.2 Effect of CalB enzyme content

Figure 6.5 presents the comparison of the evolutions of average particle diameter and zeta potential during methyl methacrylate miniemulsion polymerizations using SDS as surfactant, crodamol as co-stabilizer, different CalB enzyme concentrations, and different initiators types. It was possible to observe that in increasing the CalB enzyme content, the zeta potential value became less negative. When CalB enzyme content was increased from 1 to 10 wt.%, the zeta potential values becomes less negative from −62 to −41 mV using KPS as initiator and from −62 to −37 mV using AIBN as initiator, respectively. The results suggest that the presence of CalB enzyme (−25 mV) in the PMMA nanoparticles surface reaches the less negative zeta potential values compared with pure PMMA nanoparticles (−51 mV).

In terms of average particle diameter evolutions when the enzyme content was increased from 1 to 10 wt.% for the both initiators,

the particle diameter increased (Fig. 6.5). The results suggest that increasing the CalB content, more enzymes can be present in the PMMA nanoparticles surface. These results agree with the relative CalB enzyme activity, which increases with the amount of immobilized CalB enzyme (Fig. 6.6). For the next steps of this work, CalB enzyme content was fixed in 5 wt.% once the results in terms of zeta potential, particle size, and relative enzyme activity were considered acceptable and similar to those obtained using 10 wt.% of CalB enzyme.

Figure 6.5 - Influence of different CalB enzyme contents on theMMA miniemulsion polymerization using KPS (a, b) and AIBN (c, d) as initiator.

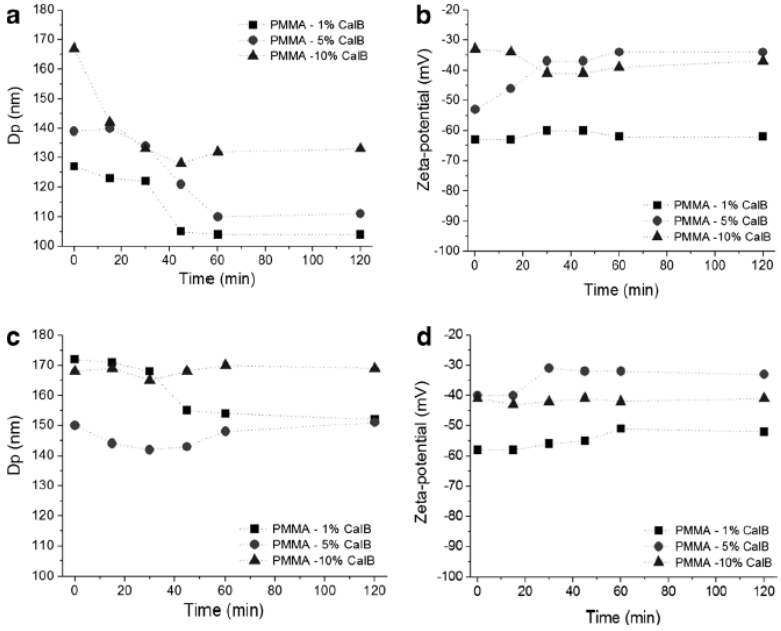
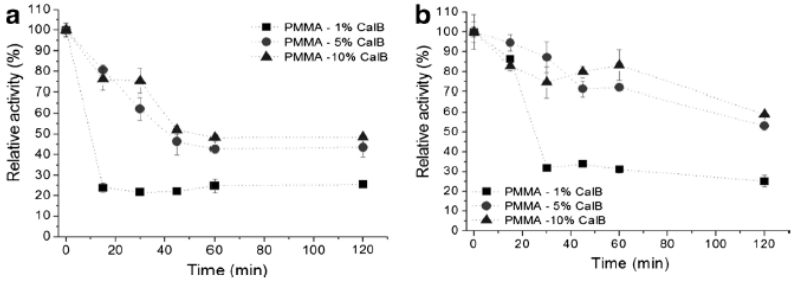


Figure 6.6 - Influence of different CalB enzyme contents in the relative CalB enzyme activity during MMA miniemulsion polymerization using KPS (a) and AIBN (b) as initiator.



6.3.3 Study of crodamol content in the PMMA-CalB synthesis using AIBN and KPS as initiator

In order to verify further co-stabilizer effect on the CalB immobilization during MMA miniemulsion polymerization, the experiments were conducted using KPS and AIBN as initiators and two different co-stabilizer (crodamol) concentrations (reactions 4, 7, 9, and 10 in Table 6.1). Results shown in Table 6.2 suggest that with a higher crodamol concentration, it is possible to increase nanoparticles stability, as the zeta potential is related to the magnitude of electrostatic interaction between colloidal particles. In addition, results show that in increasing crodamol concentrations from 5 to 10 wt.%, the zeta potential of PMMA-CalB enzyme nanoparticles becomes less negative in 120 min of reaction, reaching values from −52 to −34 mV for reactions using SDS as surfactant , and from −49 to −33 mV for reactions using AIBN as initiator, respectively.

In terms of particle size, it was possible to observe an increase from 111 to 151 nm when the crodamol content was increased from 5 to 10 wt.% (Table 6.2) using AIBN as initiator, and an increase from 99 to 129 nm, with 5 and 10 wt.% of crodamol, respectively, using KPS as initiator. The results appoint to a higher content of crodamol in the core of PMMA nanoparticles for both cases, using KPS and AIBN as initiator, due to the increase of crodamol in the PMMA-CalB enzyme nanoparticles.

Table 6.2 – Influence of crodamol content on the PMMA-CalB synthesis using SDS as surfactant and 5 wt% of CalB enzyme (in relation to the organic phase) by miniemulsion polymerization.

AIBN				
	5 wt% Crodamol (Exp. 9)		10 wt% Crodamol (Exp. 7)	
Time	Dp (nm)	Zeta-potential (mV)	Dp (nm)	Zeta-potential (mV)
0	110	-44	150	-40
15	111	-43	144	-40
30	114	-47	142	-31
45	113	-48	143	-32
60	116	-48	148	-32
120	111	-49	151	-33
KPS				
	5 wt% Crodamol (Exp. 10)		10 wt% Crodamol (Exp. 4)	
Time	Dp (nm)	Zeta-potential (mV)	Dp (nm)	Zeta-potential (mV)
0	116	-50	139	-53
15	118	-49	151	-46
30	105	-50	134	-37
45	101	-48	131	-37
60	100	-48	129	-34
120	99	-52	129	-34

6.3.4 Immobilized CalB enzyme: recycling experiments

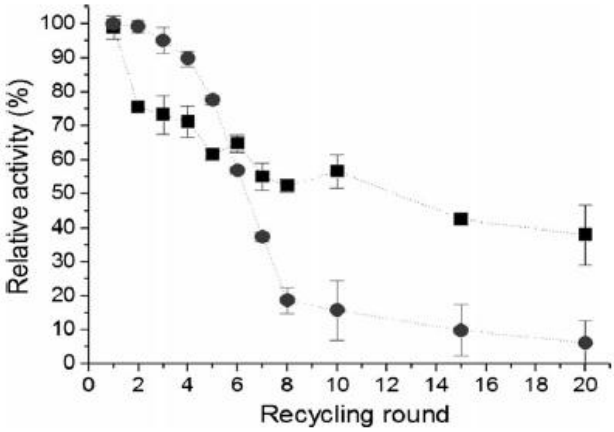
To determine the reuse efficiency of the CalB enzyme immobilization in PMMA nanoparticles, the activity of immobilized CalB enzyme was tested through the hydrolysis of p-NPP repeatedly and then the activity of the supernatant was measured, allowing the calculation of the relative remaining activity. As expected, from the results shown in Fig. 6.7, the CalB enzyme hydrolysis efficiency

decreased in each recycling step. PMMA as CalB enzyme support showed a high-enzyme immobilization yield reaching 75 %. The immobilized enzyme showed a relative activity of 40 % after 20 recycle rounds, while the free enzyme showed a relative enzyme activity of 5 % after 20 recycle rounds.

The use of immobilized enzyme in industrial processes has many advantages, such as reduced operating costs and the possibility of continuous use. Colloidal polymeric aqueous dispersions with the enzyme covalently bonded to the particle surface have the advantage of a very high superficial area emulating free water-soluble enzyme. The hydrated enzyme at the polymer particle surface will mediate the desired reaction, thus overcoming the steric hindrances and mass transfer limitations presented by insoluble matrices [22].

CalB enzyme encapsulation in PMMA as support obtained by miniemulsion polymerization using SDS as surfactant, crodamol as co-stabilizer, and different initiator types was studied. The use of different crodamol contents as co-stabilizer makes it possible to obtain PMMA-CalB enzyme nanoparticles with different sizes in a different zeta potential range. The study of CalB enzyme content enabled to observe that in all cases, relative activity decreased in 120 min of reaction time and showed that in increasing CalB content, it was possible to increase the relative activity. From the experimental results presented in the present work, it could be concluded that CalB enzyme immobilized in PMMA support when used in a repeated hydrolyzing recycling showed high recycling stability compared with free CalB enzyme.

Figure 6.7 - Recycling study of CalB enzyme. Black square PMMA-CalB enzyme (5 wt.%) and black circle free CalB enzyme.



6.4. CONCLUSIONS

CalB enzyme encapsulation in PMMA as support obtained by miniemulsion polymerization using SDS as surfactant, crodamol as co-stabilizer, and different initiator types was studied. The use of different crodamol contents as co-stabilizer makes it possible to obtain PMMA CalB enzyme nanoparticles with different sizes in a different zeta potential range. The study of CalB enzyme content enabled to observe that in the all cases, relative activity decreased in 120 min of reaction time and showed that in increasing CalB content, it was possible to increase the relative activity. From the experimental results presented in the present work, it could be concluded that CalB enzyme immobilized in PMMA support when used in a repeated hydrolyzing recycling showed high recycling stability compared with free CalB enzyme.

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RESULTADOS III

Synthesis and modification of polyurethane for immobilization of *Thermomyces lanuginosus* (TLL) lipase for ethanolysis of fish oil in solvent free system

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Synthesis and modification of polyurethane for immobilization of *Thermomyces lanuginosus* (TLL) lipase for ethanolysis of fish oil in solvent free system

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Abstract

We report the synthesis of polyurethane (PU) with polyethylene glycol (PEG) by miniemulsion polymerization as technique to produce a support for enzyme immobilization and stabilization. In order to study the influence of molar weight and concentration of PEG in the enzyme–support interaction, different PEG (400, 4000 and 6000 Da) was used during the synthesis of PU–PEG support to immobilize *Thermomyces lanuginosus* lipase (TLL). The enzymatic derivatives were effectively used in the production of polyunsaturated fatty acids (PUFAs), particularly derivative TLL–PU–PEG6000. This derivative showed the best results in terms of Km (0.183 mM) and Vmax (45.79 mmol/min/mL), calculated by Lineweaver–Burk plot (double reciprocal plot) and catalytic efficient (250.22), 100% of yield immobilization and recovered activity, reaching the highest values to the ethyl esters production (260 mM ethyl esters. U^{-1}) making this derivative a potential low cost catalyst for ethanolysis reactions in solvent free system.

Keywords: Lipase, Miniemulsion, Immobilization, Ethanolysis, Ethyl esters

7.1. INTRODUCTION

Immobilized enzymes in industrial processes provide many important advantages as to control product formation and process efficiency is enhanced [1–3]. PU foam is widely used in the enzymes immobilization [4], and few relates using PU synthesized by miniemulsion as support are reported in the literature. Recently, Cipolatti et al. reported the synthesis of PU nanoparticlesby miniemulsion to the purpose of immobilizing *Candida antarctica* lipase (CalB) using isophorone diisocyanate (IPDI) and polycaprolactone diol (PCL530) as monomers [5]. Miniemulsions are defined as aqueous dispersions of relatively stable oil droplets within a size range of 50–500 nm prepared by shearing a system containing oil, water, a surfactant, and a so-called "cosurfactant"[6], and can be used in the synthesis process of a polymeric support for the enzyme immobilization.

Polyurethanes (PU) are obtained by reaction between isocyanate and polyol to yield polymers with urethane bonds (NH COO) in their main chain. PU is a comparably well-studied biomaterial that has been used in biomedical applications such as heart valves, artificial veins, and cardiovascular catheters [7] and also for controlled release of drugs and biotherapeutics delivery [8,9]. Miniemulsion polymerization allows the production of very small PU particles, and also has the possibility of modifying particles properties, by making it more hydrophilic or hydrophobic byusing ligands agents, such as polyethylene glycol (PEG) [9]. PEG is FDA approved, nontoxic, biocompatible, no immunogenic and soluble in a variety of solvents, including water. These characteristics of PEGylated nanoparticles make then acceptable as drug delivery and medical application [10].

The lipase from *Thermomyces lanuginosus* (TLL) is a thermostable enzyme, and has several industrial applications, like as modification of fats and oils and production of biodiesel [11]. TLL immobilized on PU–PEG particles also appears as an alternative to the production of polyunsaturated fatty acids (PUFA) since the enzymatic derivative can increase the process efficiency [5]. The beneficial of n-3 PUFAs on the human health are well known, mainly eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) including the suitable development of the nervous system and the prevention of cardiovascular diseases [12,13].

Much is said about enzyme immobilization [14–18] and several supports have been developed for this purpose [5,18–20]. Considering the stability of polymer latexes synthesized by miniemulsion

polymerization [21], this work proposes the use of PU particles using PEG hydrophilic agent synthesized by miniemulsion polymerization for TLL lipase immobilization, and a study of PEG molar mass (400, 400 and 6000 Da) influence in the enzyme–support interaction.

7.2. MATERIAL AND METHODS

7.2.1. Materials

Isophorone diisocyanate (IPDI, 98%, Mw 222 g/mol) and cyclo-hexane (97%) were purchased from Alfa Aesar (USA). Poly(ethyleneglycol) diol with molar mass of 400 Da (PEG400), 4000 (PEG4000) and 6000 (PEG6000), polycaprolactone diol with molar mass 530 Da(PCL530), surfactant sodium dodecyl sulfate (SDS), *p*-nitrophenylbutyrate (p-NPB), ethanol and cyclohexane were obtained from Sigma Chemical Co. (St. Louis, Mo). Free *Thermomyces lanuginosus*(TLL) was generously donated by Novo Nordisk (Denmark). Sardineoil was obtained from Biotec BTSA (Spain), containing 18% EPA and12% DHA. All others reagents and solvents used were of analytical or HPLC grade.

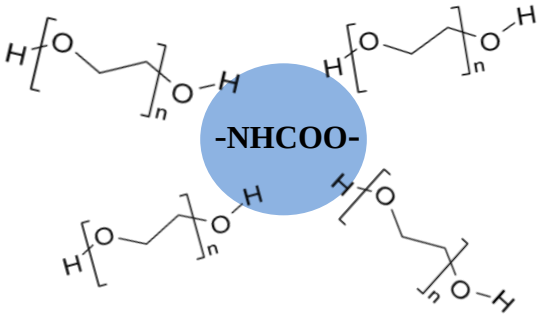
7.2.2. Methods

7.2.2.1. Synthesis of PU–PEG nanoparticles by miniemulsion polymerization

PU–PEG nanoparticles (Fig. 7.1) were synthesized by miniemulsion polymerization based on the procedure previously described by Valério et al. [22]. The organic phase was composed by IPDI and PCL530 solution (2.5 NCO:OH molar ratio). The aqueous phase was prepared with 10 wt% of surfactant (SDS) and 10 wt% of different PEG were used (400, 4000 or 6000). All of the quantities were related to the organic phase. The IPDI and PCL were dissolved in 2 mL of cyclohexane under magnetic stirring for 10 min at room temperature (25°C). The aqueous phase was added in the organic phase and kept for 2 min forming an unstable emulsion. The miniemulsion was prepared by sonication of the previous emulsion with an ultrasonic probe (Fisher-Scientific-Ultrasonic Dismembrator Ultrasonic Dismembrator 500, 400 W) set to 70% of power intensity for 2 min. Polymerization was

conducted at 70°C during 3 h in a jacketed flask (50 mL). PU–PEG support was lyophilized for further use.

Figure 7.1 - PU–PEG particles.



7.2.2.2. Immobilization of *Thermomyces lanuginosus* lipase (TLL) on PU–PEG particles and characterization of the derivatives

TLL lipase (previously dialyzed for 24 h) was diluted fold using a 5 mM phosphate buffer pH 7.0 (5 mL of enzyme and 5 mL of buffer were used in dialysis). PU–PEG particles (1 g) were used in the process of immobilization, the enzymatic solution offered in the process of immobilization was 175 U/g support. The mixture was stirred at room temperature (25°C) until maximum immobilization efficiency. Samples were taken periodically for the measurement of the enzymatic activity, according to Section 7.2.2.3, and the immobilization efficiency was monitored by measuring the enzyme activity in the suspension and in the supernatant [23]. The recovered activity was calculated by equation: Recovered activity = A/B×C×100, where A is the activity of immobilized derivative, B is the activity of the enzymatic solution offered for immobilization and C is the immobilization yield.

PU–PEG particles and the TLL immobilized derivative were analyzed by Fourier transform infrared spectroscopy (FTIR) in the attenuated total reflectance (ATR) mode. FTIR analyses were performed in the Laboratório de Controle de Processos (LCP) at Federal University of Santa Catarina (UFSC).

Derivatives TLL-PU–PEG morphology were evaluated by scanning electron microscope (SEM, JEOL JSM-6390LV), the derivatives were fixed with face duple tape, in stub and coated with

gold, the magnification used was 500 times. SEM analyzes were performed in the Laboratório Central de Microscopia Eletrônica (LCME) at Federal University of Santa Catarina (UFSC).

7.2.2.3. Loading capacity and enzyme activity determination

Different TLL enzyme solutions were prepared, at protein concentrations range from 60 to 170 mg/g support. The protein concentration was determined spectrophotometrically according to Bradford [24]. Bovine serum albumin was used as standard protein for calibration curves.

The enzymatic activity was measured by the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) using a spectrophotometer with thermostatic cell and continuous magnetic stirring (500 rpm) for 2.5 min. The increase in the absorbance at 348 nm produced by p-nitrophenol released in the hydrolysis of 0.4 mM of *p*-NPB in 25 mM sodium phosphate at pH 7 and 25°C was measured. The activity was calculated using $\varepsilon = 5.150\ M^{-1}cm^{-1}$. Enzymatic activity (U) was defined as μmol of hydrolyzed *p*-NPB per minute per mg of enzyme under the conditions described [25].

7.2.2.4. Free and immobilized enzyme TLL in PU–PEG particles: stability at different pH and temperatures

Free and immobilized enzyme TLL in PU–PEG particles were kept at room temperature for 2 h in buffer sodium citrate 50 mM at different pH (3–5), buffer phosphate 50 mM (pH 6–8), and buffer sodium bicarbonate 50 mM (pH 9 and 10). TLL enzyme activity was measured according to the methodology described previously (Section 7.2.2.3). For thermal stability free and immobilized TLL in PU–PEG particles (0.1 g) were diluted in 1 mL of buffer phosphate 25 mM, pH 7, then incubated at 50°C for different times. The enzyme activity was evaluated according to Section 7.2.2.3.

7.2.2.5. Determination of kinetic parameters of free and immobilized TLL on PU–PEG particles

The kinetic constants were determined using *p*-NPB as substrate in different concentrations (12.5, 25, 50, 75, 100 and 150 mM). The apparent Km and Vmax values for free and immobilized enzyme

TLL in PU–PEG particles were calculated from Lineweaver–Burk plots (as shown in Supplementary material).

7.2.2.6. Desorption of TLL lipase from PU–PEG particles

The derivatives were dissolved in a volume equivalent to that which the adsorption was carried out, in 5 mM sodium phosphate buffer pH 7.0 and 25°C. Then, NaCl was added progressively increasing its concentration, and samples were taken from supernatant after 30 min of magnetic stirring at room temperature. Proteins desorbed from matrices were measured by Bradford's method [24] and/or enzyme activity assays [26].

7.2.2.7. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

In order to determine the type of linking enzyme–support, an electrophoresis analysis was carried out, using gels of 12% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. Gels were stained with Coomassie brilliant blue. High and low molecular weight markers from GE Healthcare were used (14–270 kDa). Samples (TLL in PU–PEG particles) were boiled in the presence of SDS and β-mercaptoethanol [27].

7.2.2.8. Synthesis of ethyl esters (EE) from Omega-3 fatty acids

Free and immobilized enzyme TLL in PU–PEG particles were used to synthetize ethyl esters from Omega-3 fatty acids. The immobilized TLL in PU–PEG particles (0.1 g) was added to a substrate solution with 0.701 mmol of sardine oil and 5.77 mmol of ethanol, without organic solvents, and 0.2 g of molecular sieve (3 Å).The reaction was carried out at 28 and 37°C for 72 h under magnetic stirring. The synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was verified by HPLC technique [25].

The esters production was done by RP-HPLC (Spectra Physic SP 100 coupled with UV detector Spectra Physic SP 8450) using a reversed-phase column (Ultrabase-C8, 150 × 4.6 mm, 5 μm).The flow rate was 1.5 mL/min with acetonitrile/water/acetic acid(80:20:0.1, v:v), and pH 3. The UV detection was performed at 215 nm. The synthetic yields were calculated according peak corresponding at the pure

compounds, EE-EPA (retention time of 10 min) and EE-DHA (retention time of 13 min).

7.3. RESULTS AND DISCUSSION

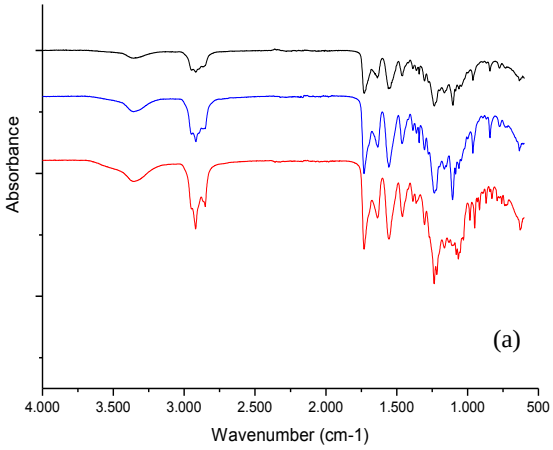
7.3.1. Characterization of PU–PEG nanoparticles

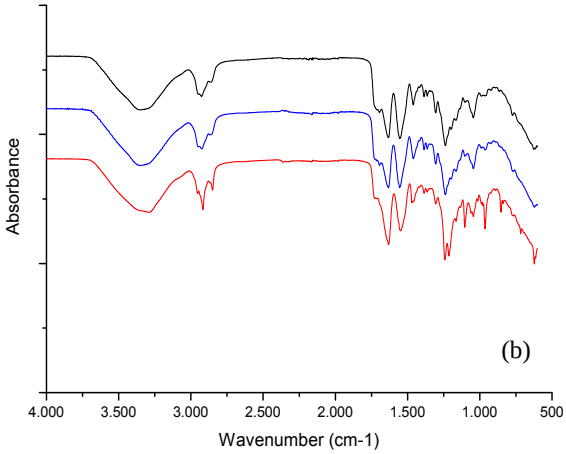
We noted that the studied PEG supports had a similar behavior for different size, confirmed by FTIR, through the absorption band with peak location at 1680–1650 $cm^{-1}$for urea and NH absorption band 1740–1700 cm^{-1} stretching vibration of C O group (Fig. 7.2a and b). Stretching of OH bond (3450 cm^{-1}) in the immobilized TLL (Fig. 7.2b) indicates a probable TLL enzyme-PEG linkage region. The same region at 1750–1700 cm^{-1} can be attributed to the bond linkage between enzyme and the functional group (NH) from polyurethane. Free lipase shows a typical spectrum with proteins absorption bands associated with amino group (CONH), primary, and secondary amino groups between 1580 and 1650 cm^{-1}[28](Fig. 7.2b). So, this fact confirmed that TLL lipase showed interaction with PU supports, by the presence of forces such as van der Waals and hydrophobic linkage between protein chains and the groups of the support.

The specific surface area was measured by nitrogen adsorption; it is the exposed area of the particles, once properly dispersed in the polymer matrix, interacts with it. The smaller size of the particles is greater specific surface area and generated greater inter-face between the enzyme and the polymer. The results obtained were 0.3057, 0.2549 and 0.2364 m^2/g for PU–PEG400, 4000 and 6000; for the immobilized TLL the results were 0.2301, 0.4746 and0.9112 m^2/g for TLL–PU–PEG400, 4000 and 6000.

The authors believe that as the support is synthesized from a technique that allows obtaining very small particles (150–300 nm)[5], it is causing agglomeration of these particles when lyophilized. It is shown in the following, when the derivatives were put in the reaction medium, for production of ethyl esters, the immobilized enzyme is dispersed in the medium, and it is possible interaction thereof with the substrate.

Figure 7.2 - Fourier transform infrared spectra (FTIR) of PEG–PU particle synthesized by miniemulsion polymerization. (a) PU–PEG supports and (b) immobilized enzyme in PU–PEG particles. The lines: red = PEG400, blue = PEG4000 and black = PEG6000 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

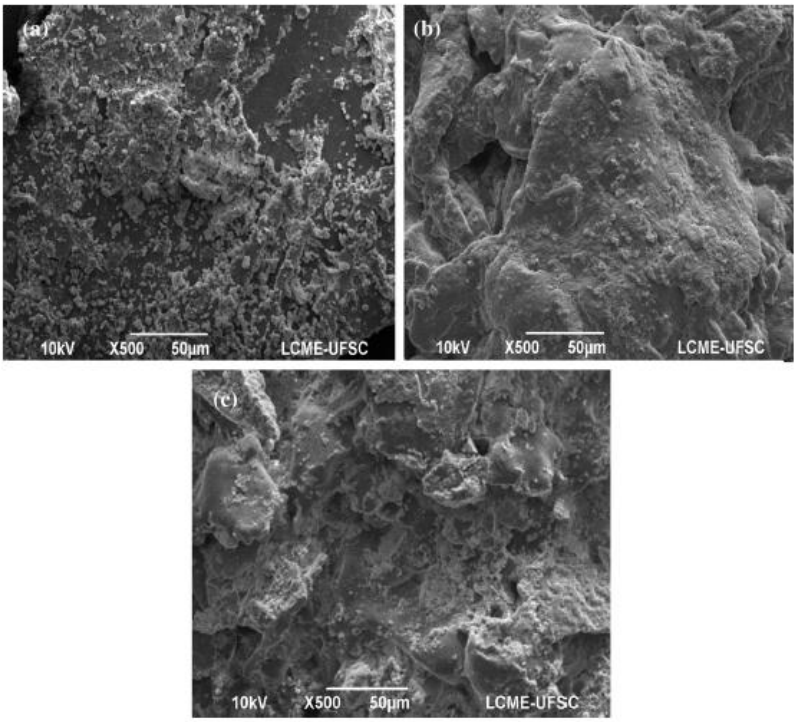




It appears that the interaction of the enzyme with the support plays a key role, because if you analyze only the supports, in the absence of the enzyme support has almost the same size (regardless of

the type and size of PEG). There is also the possibility, when the enzyme is linked in the support, that a new role motivated by the novel protein-support interaction (creating a new structure, increasing the size of matrix) takes place. These agglomerates can be observed by scanning electron microscope shown in Fig. 7.3.

Figure 7.3 - SEM microscopy of TLL immobilized on PU–PEG particles, using PEG400 (a), PEG4000 (b) and PEG6000 (c).



7.3.2. Immobilization of *Thermomyces lanuginosus* lipase in PU–PEG particles

Different size of PEG was used during the synthesis of PU–PEG particles for TLL immobilization, as shown in Table 7.1. The main function of PEG is to increase the polymer particle mechanic resistance to the supports. The magnitude of the end-group (hydroxyl) of different

PEGs used can contribute to the behavior of the enzymatic derivative. Hence, PEG400 would display a significant contribution from interactions arising from its hydroxyl groups, whereas for PEG4000 and PEG6000 only (CH_2CH_2O) units play an important role [29]. Fritzen-Gracia et al. [30] studied the peroxidase adsorption on PEGylated polyurethane (PU–PEG) nanoparticles using a purified horseradish peroxidase (HRP) for application in a biosensor for dopamine detection. These authors did not dry the proposed derivatives. They suggested that the PEG400 do not resist protein adsorption as effectively as long chains of PEG. The protein adsorption was 22.5 mg/g support.

Specific activity, immobilization yields and recovered activity obtained for each TLL derivative are presented in Table 7.1. All derivatives prepared with PU–PEG particles as support showed a high yield of immobilization. The highest enzyme activity per gram of support was obtained for derivatives synthesized using longest PEG chain (6000 Da). Although the derivative TLL-PU-PEG400 showed good immobilization yield, a possible agglomeration of small particles or a conformational deformation may explain the low results in terms of recovered activity.

Table 7.1- Effect of PEG molar mass in the TLL activity, immobilization yield and recovered activity.

Enzyme	Specific activity (*p*-NPB) [U/g support]	Immobilization yield (%)	Recovered activity (%)
Free TLL	175[a]	-	-
TLL-PU-PEG400	67	98.2	37.6
TLL-PU-PEG4000	180	100	100
TLL-PU-PEG6000	177	100	100

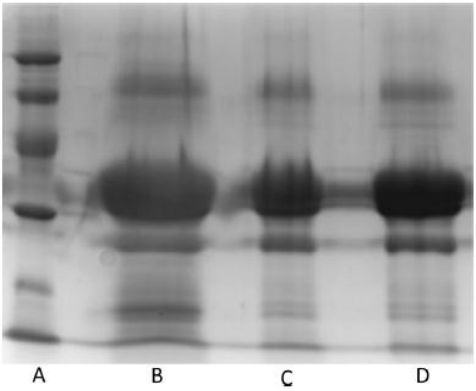
[a]Offered activity per gram of support.

With the aim to confirm the interaction enzyme–support, electrophoresis was done. The enzyme was completely desorbed in the presence of β-mercaptoethanol and SDS, confirming that this binding is non-covalent (Fig. 7.4). The derivative in the presence of Triton 0.5% in buffer phosphate 5 mM, pH 7, using the same process of

immobilization, led to 100% of immobilization yield. Therefore, when the derivative was put in the presence of CTAB, a more powerful detergent, the enzyme did not bid to the support, confirming the hydrophobic characteristic of the support.

The force of immobilization is a parameter that analyzes the condition in which the enzyme is absorbed in the support [1]. For this purpose, different NaCl concentrations were kept in contact with the derivative and the enzyme activity was measured. Using a concentration of 80 mM NaCl, the TLL enzyme desorption was possible, which also shows the presence of possible ionic interactions between enzyme/support. The maximum amount of protein immobilized when using PU-PEG particles was also determined. Different TLL enzyme solutions were prepared, at protein concentrations range from 60 to 170 mg/g support. The loading capacity was 78 mg/g support.

Figure 7.4 - SDS-Page (12% polyacrylamide). Lane A: protein molecular mass marker,Lane B: derivative TLL–PU–PEG400, Lane C: derivative TLL–PU–PEG4000, Lane D:derivative TLL–PU–PEG6000.



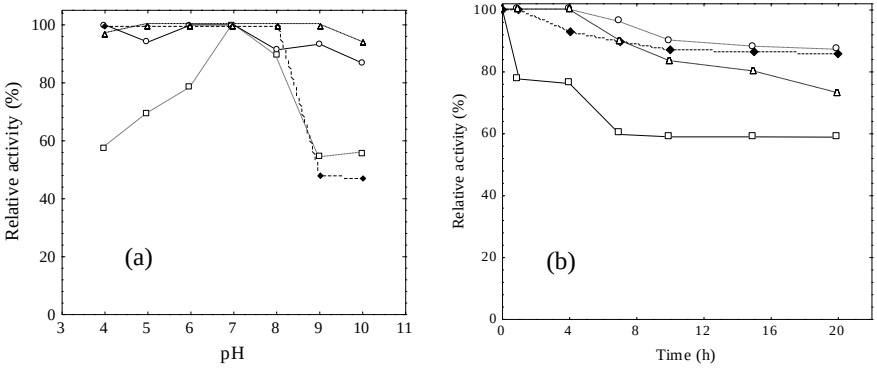
7.3.3. Stability of free and immobilized TLL IN PU–PEG particles at different pH and temperatures

Polyurethane synthesized via miniemulsion polymerization is still few used to enzyme immobilization, making it necessary to study the stability against different environmental conditions such as pH (Fig. 7.5a). The relative activity was calculated based on the difference of values obtained for the initial enzymatic activity and after 2 h in the

corresponding pH value. The support obtained using PEG 6000 showed the best values in terms of relative activity, near to 100%, throughout the studied range of pH, as it is possible to see in Fig. 7.5a.

TLL is a quite stable enzyme, so any improvement in its stability may be interesting to further enlarge its range of applications [11].Based on that, the thermal stability was also studied. Fig. 7b shows the residual activity of free and immobilized TLL in PU-PEG400, PU-PEG-4000 and PU-PEG6000 at 50°C for 20 h. At the end of the incubation time, all results obtained for the immobilized TLL in terms of residual activity showed higher values than that obtained for free enzyme. These results suggested that the thermostability of immobilized lipase increased due to the linkage between TLL enzyme and polymer matrix.

Figure 7.5 - Relative activity for 2 h at different pH values (a) and thermal stability at 50°C (b), of free (□) and immobilized TLL in the supports PU-PEG400 (○), PU-PEG4000 (♦), and PU-PEG6000 (Δ).



7.3.4. Kinetic study of free and immobilized *Thermomyces lanuginosus* lipase

The kinetic constants were determined using *p*-NPB as substrate. The activities of free and immobilized TLL in different concentrations of substrate (12.5, 25, 50, 75, 100 and 150 mM) were plotted using Lineweaver–Burk plots (Supplementary material). Table 7.2 shows that TLL immobilized in different supports had different Km

and Vmax values. Several reasons can explain the lipase Vmax and Km values variations after immobilization. The formation of very small polymer particles may cause agglomeration, which may difficult the interaction between immobilized enzyme with the substrate, and may explain the high Km and low maximum rate (Vmax) found for the immobilized derivative TLL-PU-PEG400 (Table 7.2).

The increase in the support specific surface area is directly related to the enzyme–substrate interaction, then, the specific surface area was increased to verify changes in the values of Km and Vmax. The derivative TLL-PU-PEG6000, with higher specific surface area, showed Km values close to free enzyme, and highest rate compared to other supports studied. The catalytic efficiency (Vmax/Km) was also higher, showing values of 37.7 and 15.5 times higher than TLL-PU-PEG400 and TLL-PU-PEG4000, confirming the affinity of the biocatalyst to PU-PEG6000.

Table 7.2 - Kinetic parameters of free and immobilized TLL in PU-PEG nanoparticles.

Enzyme	Km (mM)	Vmax (μmol/min/mg)	Vmax/Km
Free TLL	0.211	292.85	1387.91
TLL-PU-PEG400	1.194	7.92	6.63
TLL-PU-PEG4000	1.379	22.33	16.19
TLL-PU-PEG6000	0.183	45.79	250.22

7.3.5. Ethanolysis of fish oil without organic solvents by immobilized TLL in PU–PEG particles.

Fatty acid composition of the sardine oil used is EPA 18%, DHA 12%, docosapentaenoic acid (0.95%), myristic acid (7.3%), palmitic acid (17.5%), stearic acid (7%), palmitoleic acid (14%), oleicacid (20%), linoleic acid (1%), linolenic acid (2%), arachidonic acid (0.25%). The molecular weight is 356.55 and density of 900 kg/m^3.

TLL is known as a lipase with strict sn-1,3-regiospecificity [11,31]. Free and immobilized TLL in PU–PEG particles were used for enzymatic synthesis of ethyl esters (Fig. 7.6).

These supports showed different behaviors in the reaction, resulting in different selectivity (Table 7.3). Two different temperatures

(28 and 37°C) were studied in the reactions. The results were expressed as mM ethyl esters produced divided to enzymatic activity (U) of support used in the same time. In general, 37°C allowed for higher production of ethyl esters, but lower selectivity (Table 7.3), probably due to the higher temperature leads to higher production of DHA.

The selectivity is defined as the relationship between the initial rate of release EE-EPA and EE-DHA [25]. The EPA/DHA selectivity of the derivatives was different, and it was possible to obtain high selectivities in all derivatives studied, especially for the TLL-PUPEG6000 (31.8) at 28°C.

Considering the stability of these PU-PEG supports, after 4 days in fish oil, the residual activity was 2.40, 51.2 and 83.1% for TLL-PU-PEG400, TLL-PU-PEG4000 and TLL-PU-PEG6000, respectively.

Figure 7.6 – Ethanolysis of omega-3 fatty acids without solvent catalyzed by free TLL (a), and TLL immobilized in different supports: PU-PEG400 (b), PU-PEG4000 (c), and PU-PEG6000 (d). (◌) 28°C EPA, (□) 28°C DHA, (♦) 37°C EPA, (▲) 37°C DHA.

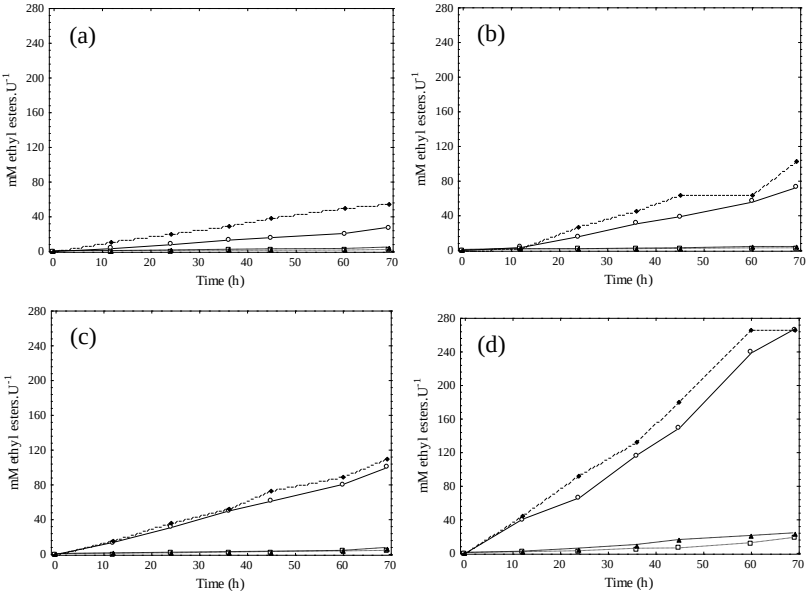


Table 7.3 - Selectivity of immobilized TLL in different PU-PEG as support.

Enzyme	Selectivity*	
	28 ºC	37 ºC
Free TLL	23.4	17.4
TLL-PU-PEG400	25.4	12.4
TLL-PU-PEG4000	28.8	15.8
TLL-PU-PEG6000	31.8	21.3

*Selectivity expressed as molar ratio between synthesized ethyl esters of EPA and DHA, measured at 15% of conversion to total ethyl esters produced (EPA+DHA).

7.4. CONCLUSIONS

Results obtained in this work show PU–PEG particles as an efficient support for TLL immobilization. The three derivatives studied exhibited different functional properties for the ethanolysis of sardine oil. The derivative TLL-PU-PEG6000 although not show greater stability at 50°C, was more stable in a wide range of pHs (4–10) and led to a higher production of ethyl esters, with consequent higher selectivity in both temperatures tested (28 and 37°C) with EE-PA/EE-DHA= 31.8 and 21.3, respectively.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.09.006

Figure 7.7 - Calculation of kinetic parameters for free enzyme

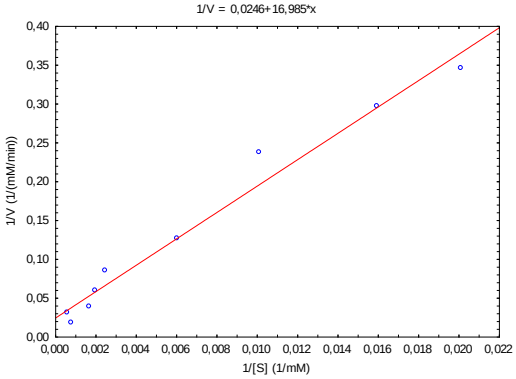


Figure 7.8 - Calculation of kinetic parameters for TLL-PU-PEG400

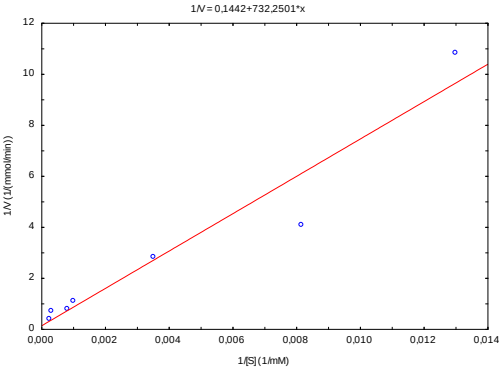


Figure 7.9 - Calculation of kinetic parameters for TLL-PU-PEG4000

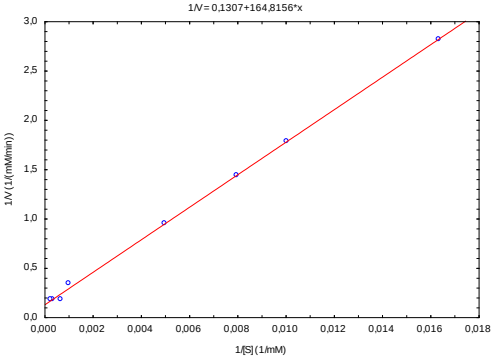
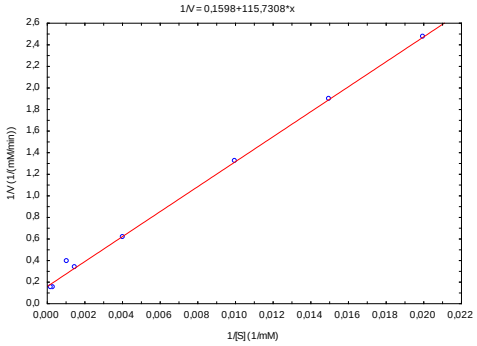


Figure 7.10 - Calculation of kinetic parameters for TLL-PU-PEG6000



7.5. REFERENCES

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RESULTADOS IV

Stabilization of lipase from *Thermomyces lanuginosus* by crosslinking in PEGylated polyurethane particles by polymerization: Application on fish oil ethanolysis

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Abstract

The adsorption of *Thermomyces lanuginosus* lipase (TLL) on PEGylated polyurethane particles as support permitted the development of several strategies to improve the properties of this commercial low-cost enzyme. The supports were synthesized by miniemulsion technique using isophoronediisocyanate (IPDI) and poly(ε-caprolactone) diol (PCL530) as monomers. The aqueous phase was composed of distilled water, surfactant sodium dodecyl sulfate (SDS), and poly(ethylene glycol) with different molar mass (PEG 400, 4000 or 6000). Polyethyleneimine (PEI) and trehalose were used to coat the PEGylated polyurethane particles in order to increase the stability. PEGylated polyurethane particles coated with 20% of PEI led to the best results in terms of temperature (100% of relative activity at 50°C during 8 h). TLL immobilized on PEGylated polyurethane particles as support improved the production of ethyl esters from fish oil compared to the free TLL.

Keywords: lipase; miniemulsion; polymeric particles; omega-3 fatty acids; crosslinking

8.1. INTRODUCTION

Miniemulsion are classically defined as aqueous dispersions of relatively stable oil droplets within size range of 50-500 nm prepared by shearing a system containing an organic phase, water, surfactant, and "co-surfactant" [1-3] . Nanoparticles obtained by miniemulsion are used as drug carrier, such as particles of biodegradable poly(ureaurethane) (PU). Recent studies disclose the use of this technique in the synthesis of supports for immobilization of enzymes [4,5]. The use of polymers such as polyurethane as a support for the immobilization of enzymes are well studied, but the use of PU synthesized from miniemulsion is still barely investigated for this purpose. The advantage of using this technique to obtain PU particles is the one-step synthesis, by interfacial polycondensation in miniemulsion.

Lipases immobilization has been widely studied, mainly due to the industrial importance of the catalysts obtained [6-9]. Although much discuss about immobilization, there is still a search for the "ideal support", that allows obtaining a biocatalyst efficient, low cost, simple synthesis, which results in higher production of the compound of interest. Previous results (data not shown) verified that PU-pegylated particles as support obtained by the miniemulsion are promising to Thermomyces lanuginosus lipase (TLL), showing satisfactory results in fish oil esterification. However, the stability of the catalyst can compromise its application, so soluble stabilizing additives (eg polyethyleneimine and trehalose) can be used after the immobilization process in order to provide greater stability to the derivative.

TLL is an important enzyme for several industrial applications, although initially oriented toward the food industry. This enzyme is also widely used, for example, in biodiesel and fine chemicals production [10]. An important application for this enzyme is in the production process of long-chain polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3) [11-12].

The use of fish oil, rich in omega-3 fatty acids in reactions with enzymes is an attractive approach since enzymatic processes can be carried out under mild conditions without undesirable byproducts formation [13-14]. The methanolysis reaction of fish oil allows the formation of ethyl esters rich in omega-3 using mild temperatures, thereby protecting the polyunsaturated fatty acids from oxidation, and an alternative for obtaining specific structured lipids [15]. Moreover, the separation of eicosapentaenoic acid (EPA) and docosahexaenoic acid

(DHA) from enzymatic catalysis (selective hydrolysis of methanolysis) is very interesting due to the similar molecules that are hard to separate by physicochemical protocols [13,16].

This report investigated the effects of polyethyleneimine and trehalose in the stability of immobilized derivatives from TLL lipase. PEGylated polyurethane particles synthesized by miniemulsion polymerization were used as enzyme support. The derivatives were used as catalyst is the solvent-free transesterification of fish oil.

8.2. EXPERIMENTAL

8.2.1. Chemicals

Isophoronediisocyanate (IPDI, 98%, Mw 222 g/mol) and cyclohexane (97%) were purchased from Alfa Aesar (USA). Poly(ethylene glycol) diol with nominal molar mass of 400 (PEG400), 4000 (PEG4000) and 6000 Da (PEG6000), polycaprolactonediol with molar mass 530 Da (PCL530), and polyethyleneimine 25,000 Da (PEI 25,000) were purchased from Sigma-Aldrich (Germany). Surfactant sodium dodecyl sulfate (SDS) obtained from Aldrich Chemicals Ltd. D-Trehalose, 99% anhydrous was purchased from ACROS Organics. p-nitrophenyl butyrate (p-NPB), ethanol, cyclohexane were obtained from Sigma Chemical Co. (USA). Free Thermomyces lanuginosus (TLL) was generously donated by Novo Nordisk (Denmark). Sardine oil was obtained from Biotec BTSA (Spain). All others reagents and solvents used were of analytical or HPLC grade

8.2.2. Enzymatic activity determination

The enzymatic activity was measured by hydrolysis of p-nitrophenyl butyrate (pNPB) used a spectrophotometer (JASCO V-630) with thermostatic cell and continuous magnetic stirring (500 rpm) for 2.5 min. The increase in absorbance at 348 nm produced by p-nitrophenol released in the hydrolysis of 0.4 mM of p-NPB in 25 mM sodium phosphate at pH 7 and 25°C was measured. The value of activity was calculated using ε=5.150 $M^{-1}.cm^{-1}$. One unit of enzyme activity (U) was defined as μmol of hydrolyzed p-NPB per minute per mg of enzyme under the described conditions [13]. The results were obtained in triplicate.

8.2.3. Immobilization of *Thermomyces lanuginosus* lipase (TLL) in PEGYlated PU particles

The lipase (previously dialyzed on distilled water) was diluted the-fold using 5 mM phosphate buffer pH 7.0. PEGylated PU particles (1 g) were used in the immobilization process. The immobilization mixture was stirred at room temperature (25 ºC) until achieving the maximum immobilization percentage. Samples were taken periodically for enzymatic activity measurement according to item 8.2. The immobilization was monitored by the enzymatic activity in the suspension and in the supernatant [17].

8.2.4. Preparation of coated supports- PEGylated PU-PEI and PEGylated PU-trehalose

After the end of immobilization process, the derivatives were coated with polyethyleneimine (PEI) (10 or 20 %wt/v) or trehalose (10%wt/v). A solution of PEI or trehalose was prepared in sodium phosphate buffer pH 7.0 and 0.05 M. The derivatives (PEGylated PU-PEG400, 4000 and 6000 Da) were kept at stirring overnight at 25°C. Finally, the suspensions were filtered and stored at 4 °C.

8.2.5. Determination of kinetic parameters of free and immobilized *Thermomyces lanuginosus* lipase (TLL)

The kinetic constants were determined using p-NPB as substrate in different concentrations (12.5, 25, 50, 75, 100, and 150 mM). The apparent Km and Vmax values for immobilized lipase in PEGylated PU-PEG particles were calculated from Lineweaver–Burk plots. The results shown were obtained from the resulting equation of the means of the values plotted in the graph.

8.2.6 Free and immobilized enzyme TLL in coated PU-PEG particles: thermal stability and pH effect

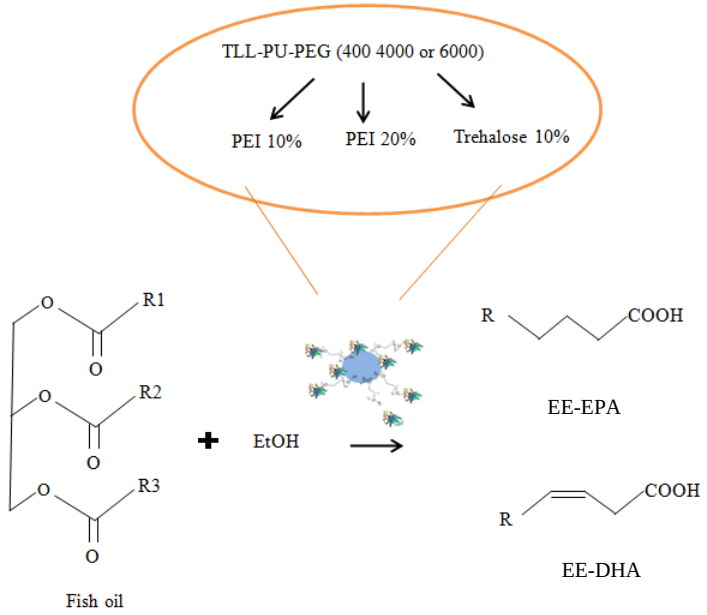
Free and immobilized enzyme TLL in PU-PEG particles were kept at room temperature for 2 h in buffer sodium citrate 50 mM (pH 5), buffer phosphate 50 mM (pH 7), and buffer sodium bicarbonate 50 mM (pH 9). TLL enzyme activity was measured according to the

methodology described previously (section 8.2.3). For thermal stability free and immobilized TLL in PU-PEG particles (0.1 g) were diluted in 1 mL of buffer phosphate 25 mM, pH 7, then incubated at 50°C for different times. The enzyme activity was evaluated according to the section 2.2. The results were obtained in triplicate.

8.2.7. Synthesis of ethyl esters of omega-3 fatty acids

The immobilized TLL was used for the synthesis of ethyl esters of Omega-3. The immobilized TLL in PEGylated PU-PEG particles (0.1 g) was added to the substrate solution with 0.701 mmol of sardine oil and 5.77 mmol of ethanol, without organic solvents, and 0.2 g of molecular sieve (3 Å) (Figure 8.1). The experiments were carried out at 37 ºC for 72 h under magnetic stirring. The synthesis of ethyl esters (EE) of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was determinate by HPLC [13]. The results were obtained in triplicate.

Figure 8.1 – Scheme of EPA and DHA production from TLL immobilized in PEGylated PU particles covered with PEI or trehalose.



8.2.8. Monitoring of esters production

The content of esters was determined by RP-HPLC (Spectra Physic SP 100 coupled with UV detector Spectra Physic SP 8450) using a reversed-phase column (Ultrabase-C8, 150 x 4.6 mm, 5 μm). The flow rate was 1.5 mL/min with acetonitrile/water/acetic acid (80:20:0.1, v:v), and pH 3. The UV detection was performed at 215 nm. The synthetic yields were calculated according peak corresponding at the pure compounds, EPA (retention time of 10 min) and DHA (retention time of 13 min).

8.3. RESULTS AND DISCUSSION

8.3.1 Effect of coating on the *Thermomyces lanuginosus* lipase (TLL) activity immobilized in PEGylated PU particles

Table 8.1 shows the specific activity and recovered activity for the TLL immobilized in PEGylated PU-PEG particles. The PEI coating derivatives with 20% showed the highest activity values. In previous experiments we found that the enzyme activities of derivatives without PEI coatings were 67, 180 and 177 U/g support for PU- PEG400, PU-PEG4000 and PU-PEG6000, respectively. The percentage of PEI has a positive influence on the interaction with the enzyme (Table 8.1), increasing considerably the activity by hyperactivation.

PEI is a polybasic aliphatic amine, used in immobilization techniques as a minor constituent, giving a hydrophilic character and greater mechanical strength to the preparations of immobilized enzymes [18]. PEI treatment can result in better retention activity, thermal stability, and support coated with amine could allow more effective proteins adsorption [8].

Table 8.1 – Effect of coating on the TLL enzyme activity using different PEGylated PU particles as support.

Enzyme	Specific activity (p-NPB) [U/g support][a]	Recovered activity (%)
Free TLL	175.0±0.1[b]	-
PU-PEG400-PEI10	46.7±0.2	54.0
PU-PEG400-PEI20	104.2±0.5	119.0
PU-PEG400-T10	26.1±0.3	29.8
PU-PEG4000-PEI10	132.3±1.0	75.6
PU-PEG4000-PEI20	229.2±1.3	130.0
PU-PEG4000-T10	69.1±0.6	39.4
PU-PEG6000-PEI10	168.8±2.0	96.6
PU-PEG6000-PEI20	193.7±0.1	110.7
PU-PEG6000-T10	146.1±0.5	83.5

[a]Means ±SD (n=3); [b]Offered activity.

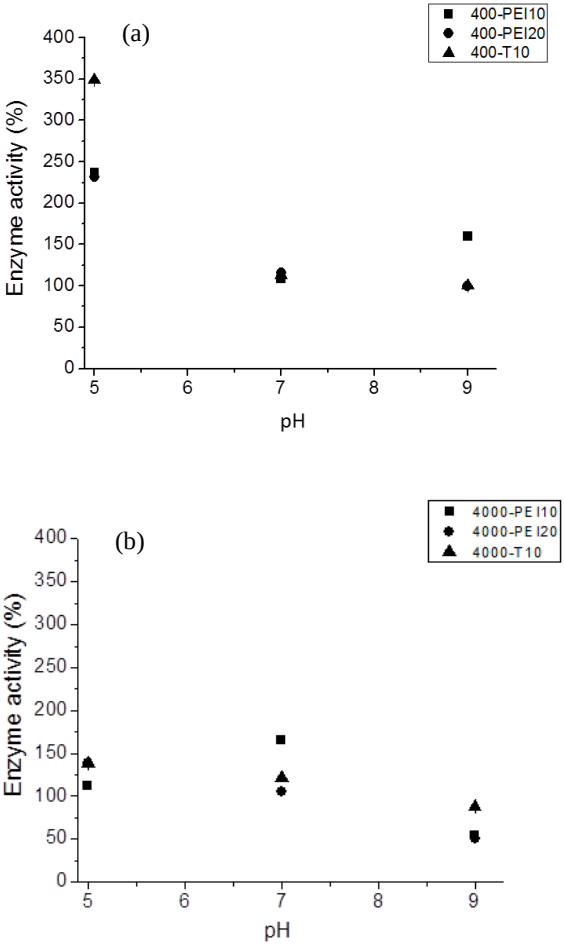
8.3.2 Study of pH in the immobilized TLL activity in coated pegylated PU particles

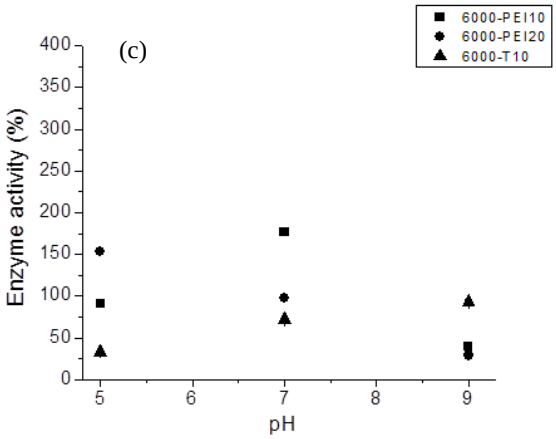
The derivatives were incubated at pH 5.0, 7.0 and 9.0 for 2 hours, and the relative activity was calculated (Figure 8.2). The coatings applied to the PU-PEG400 resulted in stable derivatives across the range studied. On the other hand, PU-PEG4000 and PU-PEG6000 showed different behavior. This should be because of congruence, first the size of PEI and PEG as well as your interaction with some important amino acids in catalytic center. Clearly, was possible to see this behavior in the study of effect of pH and in the enzymatic activity. In both cases, the increase in the size of the polymer (PEG) derivatives tends to destabilize it more due to the effects of diffusion and solvation of amino acids involved in the catalytic center of the derivative with the molecules and loads of PEI.

In our previous study (results not shown), the polymeric supports no coated showed high stability, with slight reduction in the pH, except for the PU-PEG4000 at pH 9.0 and 10.0, that showed 50% of relative activity. In general, the coatings were effective at pH 5.0 and 7.0 (Figure 8.2). The PU-PEG 6000 support with trehalose 10% (T10) covering showed opposite behavior, with 34% of relative activity at pH 5.0. The trehalose, in this case, could be playing a role deactivator at

lower pH values. Due to the fact that this enzyme present isoelectric point below 7, and in these conditions predominate positive charges on the surface of the enzyme, it may be playing an opposite role (destabilizing) with aminoacid structures in protein.

Figure 8.2 – Effect of pH in the enzyme activity of TLL immobilized in PU-PEG400 (a), PU-PEG4000 (b), and PU-PEG6000 (c) coated with PEI10 (■), PEI20 (●), and T10 (▲).

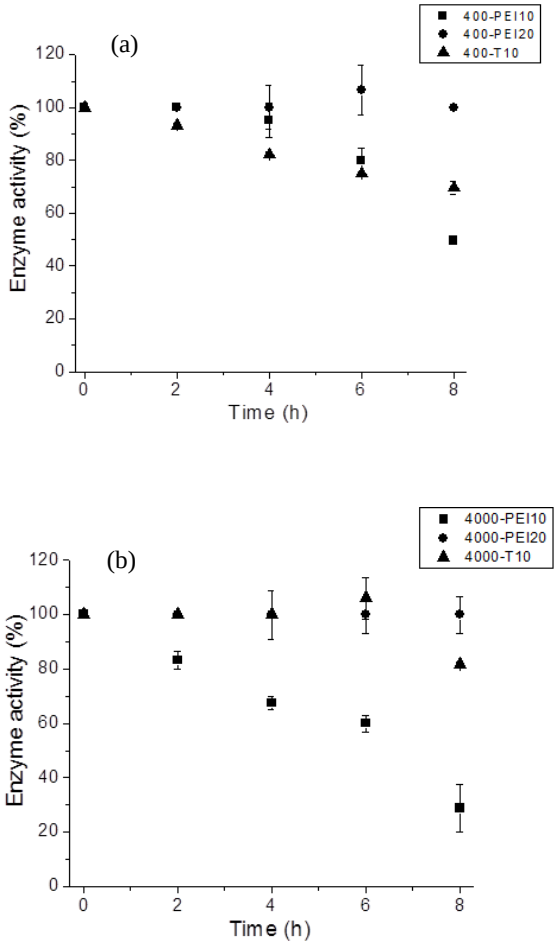


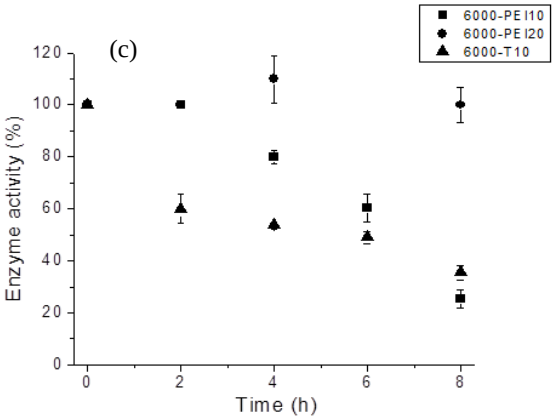


8.3.3 Thermal stability of immobilized TLL in coated PEGYlated PU particles

The thermal stability study is very important for further applications. Figure 8.3 shows the relative activity of PEGylated PU particles evaluated at 50°C for 8h. The support coated with 20% of PEI (PEI 20) showed higher stability and 100% of relative activity in all studied range. The coating with PEI20 influenced positively all supports studied, PU-PEG400, PU-PEG4000 and PU-PEG6000. The results suggested that TLL lipase adsorption in the PEGylated PU particles coated with PEI20 increased the thermal stability of the biocatalyst. This is due to the presence of numerous charges of the amino groups in the PEI, promoting a many intense interactions between enzyme and the support with PEI, increasing considerably stability.

Figure 8.3 – Thermal stability of TLL immobilized in PU-PEG400 (a), PU-PEG4000 (b), and PU-PEG6000 (c),coated with PEI 10 (■), PEI 20 (●), and T10 (▲) at 50°C.





8.3.4 Kinetic properties of immobilized lipases

The kinetic constants were determined using p-NPB as substrate. The activities of free and immobilized TLL enzyme at different concentrations (12.5, 25, 50, 75, 100 and 150 mM) were calculated (Table 8.2). Km is a measurement of enzyme-substrate affinity. Free TLL showed value of 0.212 mM, and the enzyme derivative that showed approximate value to free TLL enzyme was the PU-PEG400-PEI10 with 0.539 mM. The Vmax value for free TLL enzyme was determined as 292.9μmol/min/mg, and the best results for the enzyme derivative were obtained when PU-PEG4000-PEI20 was used (20.0 μmol/min/mg). The catalytic efficiency, given by Vmax/Km, was also calculated, and in this work we can highlight the value found for PU-PEG400-PEI10 (29.50 μmol/min/mg) as support.

8.3.5 Ethanolysis of fish oil in solvent free system catalyzed by immobilized tll enzyme in coated PEGylated PU particles

TLL immobilized in coated PEGylated PU particles was used as catalyst for enzymatic ethanolysis of sardine oil in solvent free system. All derivatives were used for this purpose. Figure 8.4 shows the kinetic of ethyl esters production. The results are promising and at a minimum 4 times higher than the values obtained in previous work using PU-PEG particles without coatings [19]. The derivatives coated with PEI showed the best results. Trehalose probably caused a

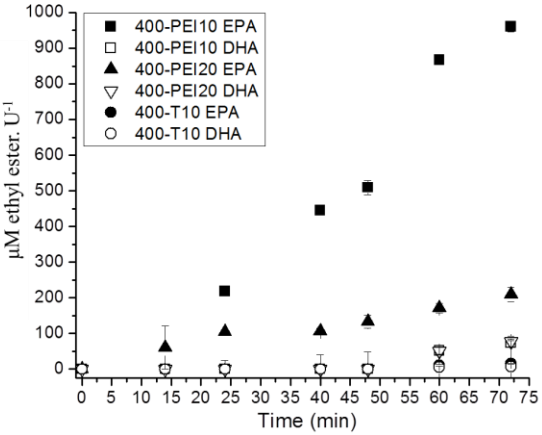
destabilization in the derivative during the reaction of synthesis of esters, hindering the formation of the desired product.

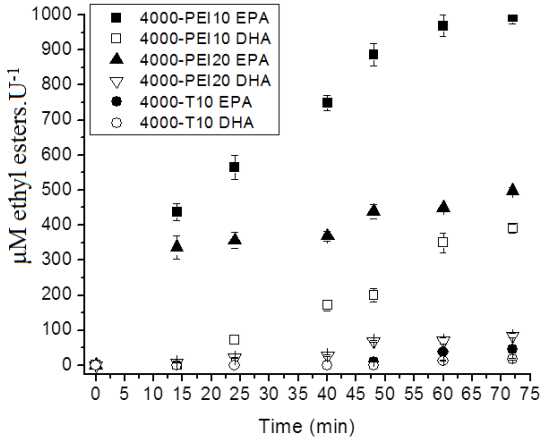
The selectivity EPA/DHA of TLL varied from 0.9 to 45.8. In all cases the derivative coated with 20% PEI (PEI20) plays a very important role in both stabilizing and selectivity (Table 8.3).

Table 8.2 - Kinetic parameters of immobilized lipase in PEGylated PU particles.

Enzyme	Km (mM)	Vmax (μmol/min/mg)	Vmax/Km
Free TLL	0.212	292.9	1381.37
PU-PEG400-PEI10	0.539	15.9	29.50
PU-PEG400-PEI20	1.127	9.4	8.34
PU-PEG400-T10	1.426	4.0	2.81
PU-PEG4000-PEI10	1.912	17.4	9.10
PU-PEG4000-PEI20	2.239	20.0	8.93
PU-PEG4000-T10	5.908	4.7	0.80
TLL-PU-PEG6000-PEI10	5.247	13.5	2.57
TLL-PU-PEG6000-PEI20	3.210	12.0	3.74
TLL-PU-PEG6000-T10	0.921	13.2	14.33

Figure 8.4 – Ethanolysis of omega-3 fatty acids in solvent free system catalyzed by TLL immobilized in polymeric particles: PU-PEG400 (a), PU-PEG4000 (b), and PU-PEG6000 (c), coated with PEI10 EPA (■), PEI10 DHA (□), PEI20 EPA (▲), PEI20 DHA (Δ),T10 EPA (●), andT10 DHA (○).




4000-PEI10 EPA
4000-PEI10 DHA
4000-PEI20 EPA
4000-PEI20 DHA
4000-T10 EPA
4000-T10 DHA
µM ethyl esters.U-1
Time (min)

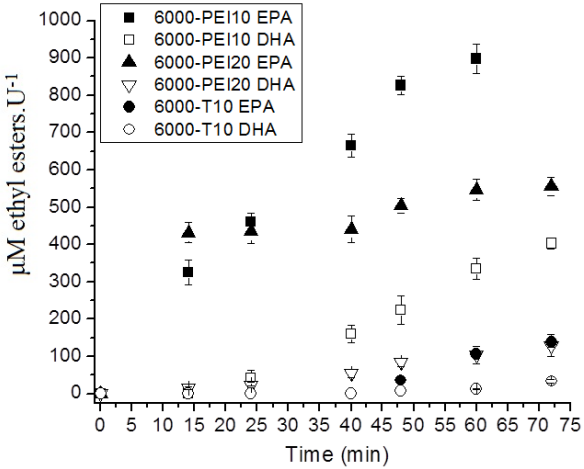

6000-PEI10 EPA
6000-PEI10 DHA
6000-PEI20 EPA
6000-PEI20 DHA
6000-T10 EPA
6000-T10 DHA
µM ethyl esters.U-1
Time (min)

Table 8.3 –Selectivity of immobilized TLL in PEGylated PU nanoparticles

Enzyme	Selectivity*
Free TLL	17.4
PU-PEG400-PEI10	4.2
PU-PEG400-PEI20	21.8
PU-PEG400-T10	0.9
PU-PEG4000-PEI10	22.5
PU-PEG4000-PEI20	45.8
PU-PEG4000-T10	2.0
TLL-PU-PEG6000-PEI10	23.4
TLL-PU-PEG6000-PEI20	29.4
TLL-PU-PEG6000-T10	2.2

*Measured at 15% of conversion total ethyl esters produced (EPA+DHA). Selectivity expressed as molar ratio between synthesized EPA and DHA.

8.4. CONCLUSIONS

Based on the results obtained in this study, the proposed support, synthesized from PEGylated PU particles coated with polyethyleneimine and trehalose, exhibited promising results, high percentages of immobilization and recovered activities. The derivatives were effective in the production of ethyl esters, having a production at least 4 times higher compared to PU particles without coating.

Acknowledgments

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RESULTADOS V

Synthesis of ethyl esters of Omega-3 fatty acids and enzymatic hydrolysis of (R,S)-mandelic acid ethyl ester by *Candida antarctica* lipase B immobilized in a biodegradable matrix

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Abstract

In this study, PU-PEGylated particles were synthetized by minimulsion with differents PEG molar mass (400, 4000 or 6000 Da) for immobilization of *Candida antarctica* lipase B. The derivatives were evaluated with and without crosslinking agents (polyethyleneimine or trehalose). The enzymatic activity was determined by hydrolysis of p-PNB. Until now, it was not found in the literature the use of PEGylated PU nanoparticles synthesized by miniemulsion polymerization in the immobilization of lipase B from *C. antarctica* (CALB) for ethyl esters production from EPA and DHA and for enantiomeric resolution of mandelic acid. The derivative PU-PEG6000 highlighted in relation to kinetic parameters with Km of 0.815 and Vmax of 41.15 μmol/min/mg. The coating with trehalose allowed greater thermal stability of the derivatives at 50 °C. PEG 400 showed the highest values of production of ethyl esters, 43.72 and 16.83 mM.U-1 EE-EPA and EE-DHA, respectively. CalB immobilized on PEGylated particles with different chain sizes of PEG were also applied in enantiomeric resolution of mandelic acid with satisfactory enantiomeric ratios (E>20). The results obtained can be considered promising in the area of catalysis and enzyme immobilization.

Keywords: polyurethane, lipase, crosslinking, ethyl esters, enantioselectivity.

9.1. INTRODUCTION

Immobilization processes can allow enzyme to become more stable and resistant to abrupt conditions, as a wide range of pH and temperature. The enzyme immobilization is an alternative process for application of these biocatalysts in a food, pharmaceutical and chemistry industry. For the use of immobilized enzymes become a process economically viable, it must consider the type of the support employed as support as well the immobilization method, which will greatly influence the subsequent activity and biocatalyst reuse (CIPOLATTI et al., 2014b; GUISÁN, 1988; MILETIĆ; NASTASOVIĆ; LOOS, 2012; PESSELA et al., 2006). There are different immobilization techniques and different supports described in the literature, the choice of the technique and support will depend of the peculiarities and specific biocatalyst applications (CIPOLATTI et al., 2014a; DALLA-VECCHIA; NASCIMENTO; SOLDI, 2004; GUISÁN, 1988; HANEFELD; CAO; MAGNER, 2013; VALÉRIO et al., 2015a).

Polyurethanes (PU) has received increasing attention due to its versatility, featuring unique mechanical and biological properties, and because it is considered one of the most biocompatible materials (VALÉRIO et al., 2014, 2015a). Noteworthy also for its durability, flexibility and biostability, which makes it interesting in the application as a carrier of drugs, tissue engineering, medical device development and enzyme immobilization (CIPOLATTI et al., 2014b; VALÉRIO et al., 2015b). Different from the other polymers, polyurethanes do not contain repeating units in the regular way, PU chain can contain besides urethane groups, aliphatic and aromatic hydrocarbons, ester groups, ether, amide and urea. Segments of PU can be represented in the general form by two basic components, polyol source of hydroxyl (OH) and diisocyanate (NCO) (Wegener et al., 2001; Randall and Lee, 2002). An important advantage of polyurethanes is that PU can be synthesized in order to become biodegradable and biocompatible, important characteristics and increasingly exploited by researchers concerned with health and the environment (Gaudin and Sintes-Zydowicz, 2008; Valério et al., 2014; Zanetti-Ramos et al., 2009, 2008).

The literature reports some works with polyurethane synthesized via miniemulsion for biomedical applications (DING et al., 2011; VALÉRIO; ARAÚJO; SAYER, 2013; WANG; GRAYSON, 2012), and recently the immobilization of lipase B from *Candida antarctica* (CIPOLATTI et al., 2014b). PU synthesis by miniemulsion allows obtaining small support particles in one step. The use of poly

(ethylene glycol) (PEG) is also very interesting, besides to conferring greater resistance to the polymer synthesized via miniemulsion, it is also non-toxic, biodegradable and approved from Food and Drug Administration (FDA) for its internal consumption (Pereira-Cipolatti et al., 2015; Askinadze et al., 2013; Zanetti-Ramos et al., 2009).

Considering the technological importance of lipase B from *C. antarctica* B (CalB), is of great value the immobilization of CalB enzyme in a biodegradable and low cost support, adding value to this biocatalyst, and thus improving its stability and performance in the reaction. This paper reports the initial modifications in the PU polymer proposed by Cipolatti et al. (2014), which was modified (used in the lyophilized form) by Pereira-Cipolatti et al. (2015) for immobilization of *Thermomyces lanuginosus lipase* (TLL). The use of the proposed method with other lipase allows us to study the support-enzyme interaction, and the difference between these lipases, also considering the differences in their active centers, will influence the immobilization.

In this work, CalB immobilized on PU-PEGylated particles using different PEG molar mass (400, 400 and 6000 Da) was studied. Furthermore, the crosslinking agents (polyethyleneimine and trehalose) were also evaluated. The obtained derivatives were applied in the synthesis of ethyl esters. The beneficial effects of free n-3 PUFAs (eicosapentaenoic acid - EPA and docosahexaenoic acid - DHA) are well known and described in the literature (AKANBI; ADCOCK; BARROW, 2013; CASAS-GODOY et al., 2014; CASTRO-GONZÁLEZ, 2002). As they can be easily oxidized, commonly are stored in the triglycerides or ethyl esters form, being more easily manipulated. Numerous papers with PUFAs transformations in triglycerides can be found in the literature, but there is little reported about the preparation of PUFAs ethyl ester by direct esterification (MORRONE et al., 2012).

Another important application of lipases was in the enantiomeric separation of chiral compounds. The chemical chosen was the mandelic acid, this has a chiral carbon atom, leading to two optically active forms. The mandelic acid provides important medical applications such as bacteriostatic properties, and is employed for the treatment of urinary tract infections. The pure form of the (R)-mandelic acid is appointed as a precursor for the synthesis of cephalosporin and penicillin (CAO et al., 2014; TULASHIE; LORENZ; SEIDEL-MORGENSTERN, 2010).

Until now, it was not found in the literature the use of PEGylated PU nanoparticles synthesized by miniemulsion

polymerization in the immobilization of lipase B from *C. antarctica* B (CALB) for ethyl esters production from EPA and DHA and for enantioselectivity. Furthermore, the use of a solvent-free system also contributes to the process proposed in this work.

9.2. MATERIAL AND METHODS

9.2.1 Materials

Isophorone diisocyanate (IPDI, 98%, Mw 222 g/mol) and cyclohexane (97%) were purchased from Alfa Aesar. Poly(ethylene glycol) diol with molar mass of 400 Da (PEG400), 4000 (PEG4000) and 6000 (PEG6000) purchased Sigma Aldrich. Polycaprolactone diol with molar mass 530 Da (PCL530) was purchased from Sigma-Aldrich. (R,S)-mandelic acid ethyl ester was from Sigma Chem. Co. (St. Louis, USA). Surfactant sodium dodecyl sulfate (SDS) obtained from Aldrich Chemicals Ltd. p-nitrophenyl butyrate (p-NPB), ethanol, cyclohexane were obtained from Sigma Chemical Co. (St. Louis, Mo). Free *Candida antarctica* lipase B (CalB) was generously donated by Novo Nordisk (Denmark). Sardine oil was obtained from Biotec BTSA. All others reagents and solvents used were of analytical or HPLC grade.

9.2.2 Methods

9.2.2.1 Synthesis of particles of PU-PEG

PU-PEG particles were synthesized by miniemulsion polymerization based on the procedure previously described by Valerio et al. (2014). The organic phase was composed by IPDI and PCL530 solution (2.5 NCO: OH molar ratio). The aqueous phase was prepared with 10 wt% of surfactant (SDS) and 10 wt% of different PEG were used (400, 4000 or 6000). All of the quantities were related to the organic phase. The IPDI and PCL were dissolved in 2 mL of cyclohexane under magnetic stirring for 10 min at room temperature (25 ºC). The aqueous phase was added in the organic phase and keep for 2 min forming an unstable emulsion. The miniemulsion was prepared by sonication of the previous emulsion with an ultrasonic probe (Fisher-Scientific – Ultrasonic Dismembrator 500, 400 W) set to 70% of power intensity for 2 min. Polymerization was conducted at constant temperature of 70ºC during 3 h in a jacketed flask (50 mL)

(CIPOLATTI et al., 2014b). The support of PU-PEG was lyophilized for 24 h, for further use.

9.2.2.2. Determination of enzyme activity

The enzymatic activity was measured by hydrolysis of p-nitrophenyl butyrate (p-NPB) used a spectrophotometer with thermostatic cell and continuous magnetic stirring (500 rpm) for 2.5 min. The increase in absorbance at 348 nm produced by p-nitrophenol released in the hydrolysis of 0.4 mM of p-NPB in 25 mM sodium phosphate at pH 7 and 25 ºC was measured. The value of activity was calculated using ε=5.150 M-1.cm-1. Enzymatic activity (U) was defined as μmol of hydrolyzed p-NPB per minute per mg of enzyme under the conditions described (Moreno-Pérez et al., 2014).

9.2.2.3. Immobilization of *Candida antarctica* lipase B on PU-PEG particles

The lipase (previously dialyzed) was diluted the-fold using 5 mM phosphate buffer pH 7.0. PU-PEG particles (1 g) were used in the process of immobilization. The immobilization mixture was stirring at room temperature (25 °C) until the maximum percentage of immobilization. Samples were taken periodically and measured the enzymatic activity according to item 9.2.2.2. Immobilization was monitored by measuring the enzyme activity in the suspension and in the supernatant (Urrutia et al., 2013).

9.2.2.4. Preparation of coated supports- PU-PEG-PEI, PU-PEG-Trehalose

After the end of the immobilization process, the derivatives were coated with polyethyleneimine (PEI) (10 or 20% w/v) or trehalose (10% w/v). A solution of PEI or thehalose was prepared in sodium phosphate buffer pH 7, 0.05 M. The derivatives (PU-PEG400, 4000 and 6000) were kept at stirring overnight at 25 °C with the previously mentioned solutions. Finally, the suspensions were filtered and stored at 4°C.

9.2.2.5. Determination of the kinetic parameters of free and immobilized *Candida antarctica* lipase B

The kinetic constants were determined using p-NPB as substrate in different concentrations (12.5, 25, 50, and 75, 100 and 150 mM). The apparent Km and Vmax values for the immobilized lipase in PU-PEG particles were calculated from Lineweaver–Burk plots.

9.2.2.6. Synthesis of ethyl esters of Omega-3 fatty acids

The immobilized CalB was used for the synthesis of ethyl esters of Omega-3. The immobilized CalB in PU-PEG particles (0.1 g) was added to the substrate solution with 0.701 mmol of sardine oil and 5.77 mmol of ethanol, without organic solvents, and 0.2 g of molecular sieve (3 Å) (Figure 9.1). The reaction was carried out at 37 ºC for 72 h under stirring. The synthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was carried out by HPLC.

9.2.2.7. Monitoring of esters production

The analysis of esters production was done by RP-HPLC (Spectra Physic SP 100 coupled with UV detector Spectra Physic SP 8450) using a reversed-phase column (Ultrabase-C8, 150 x 4.6 mm, 5 μm). The flow rate was 1.5 ml/min with acetonitrile/water/acetic acid (80:20:0.1, v: v), pH 3. The UV detection was performed at 215 nm. The synthetic yields were calculated according peak corresponding at the pure compounds, EPA (retention time of 10 min) and DHA (retention time of 13 min).

9.2.2.8 Enzymatic hydrolysis of (R,S)-mandelic acid ethyl ester

The activities of PU-PEG400-CalB, PU-PEG4000-CalB and PU-PEG6000-CalB on the hydrolysis of (R,S)-mandelic acid ethyl ester were investigated by adding the enzyme derivatives (0.1 g) to a 5mL of 10 mM (R,S)-mandelic acid ethyl ester at 37 °C and pH 7.0 (25 mM) under constant mechanical stirring. At different times, the degree of hydrolysis was confirmed by reverse-phase HPLC (Spectra Physic SP 100 coupled with an UVdetector Spectra Physic SP 8450) on a Kromasil C18 (25 cm×0.4 cm) column supplied by Analisis Vinicos (Spain) with a mobile phase of acetonitrile (30%) and 10 mM ammonium phosphate buffer (70%) at pH 2.95 (adjusted with acetic acid) with a flow rate of

1.5 mL/min. The elution was monitored by recording the absorbance at 254 nm (TORRES et al., 2006b). Triplicates of each assay were made and experimental error was never higher than 5%.The acid presented a retention time of 2.5 minutes while the ester had a retention time of 6.5 minutes.

9.2.2.9 Determination of enantiomeric excess (ee)

The elution was performed using a mobile phase composed of acetonitrile (30%) and MilliQ water (70%) at a final pH of 2.3, and a flow of 0.5 mL/min detected by UV at 225 nm (PALOMO et al., 2003b). The column was a Chiracel OD-R (Spectra Physic SP 100 coupled with an UVdetector Spectra Physic SP 8450).

9.2.2.10 Statistical analysis of data

The data generated from this study were subjected to a one-way analysis of variance (ANOVA) at 5% level of significance. Means were compared by Tukey test by Software Statistica 7. All the determinations were carried out in triplicates.

9. 3. RESULTS AND DISCUSSION

9.3.1 Effect of coating on the *Candida antarctica* lipase B (CALB) activity immobilized in PEGylated PU particles with differents PEGs

Table 9.1 shows the enzymatic activity of the PEGylated polymer derivatives, using PEGs with different molecular weights (400, 4000 and 6000 Da). During the synthesis of the proposed polymer it was necessary to add a "co-agent" (PEG) with the function to confer greater resistance to the formed particles it also exerted interaction with the enzyme, resulting in a greater adsorption with increasing chain (Table 9.1). PEG is commonly used in enzyme immobilization, it can be used as a spacer to couple bioactive molecules on surfaces, and may carry "inert" or "active" function (LI et al., 2009).

After the synthesis of the support PEGylated PU and the immobilization of CALB, the derivatives were coated with polyethyleneimine (PEI) at concentrations of 10% (PEI10) or 20% (PEI20) or trehalose at a concentration of 10% (T10). The enzymatic

activity results are shown in Table 9.2, where one can note that the derivatives coated with PEI, at the two concentrations studied, allowed an increase in the enzymatic activity, possibly caused by conformational changes in the enzyme due to interactions with the aminated surface of the PEI. PEI is a synthetic and non-toxic polymer widely used in biological systems, and has been shown to be effective in enzyme immobilization by adsorption, with positive effect on activity (ANDERSSON; HATTI-KAUL, 1999; GULTEKINOGLU et al., 2015).

Table 9.1 – Effect of different immobilized method on the activity, yield and recovered activity of derivatives from CalB. (The enzymatic activity was carried out as described on the Materials and Methods section).

Enzyme/ Derivative	Specific activity [U/g support]**	Immobilization yield (%)	Recovered activity (%)
Free	154.0±1.0[a*]	-	-
CNBr	70.3±7.7[d]	60.5	75.5
PU-PEG400	55.6±2.1[e]	57.0	63.3
PU-PEG4000	141.0±3.6[b]	70.0	100.0
PU-PEG6000	114.0±3.4[c]	60.0	100.0

* Offered activity per gram of support.
**The results are expressed as means ± SD.

Table 9.2 – Effect of coating on the CalB enzyme activity using different PEGylated PU particles as support (The enzymatic activity was carried out as described on the Materials and Methods section).

Enzyme/ Derivative	Specific activity [U/g support]*
PU-PEG400-PEI10	62.7±0.3
PU-PEG400-PEI20	268.5±5.0
PU-PEG400-T10	68.3±0.4
PU-PEG4000-PEI10	91.5±5.3
PU-PEG4000-PEI20	135.6±13.8
PU-PEG4000-T10	86.4±1.9
PU-PEG6000-PEI10	84.0±0.1
PU-PEG6000-PEI20	297.4±28.8
PU-PEG6000-T10	91.3±2.7

*The results are expressed as means ± SD.

9.3.2 Kinetic parameters of CalB immobilized on PEGylated PU particles

The kinetic parameters were calculated by different concentrations of pNPB (12.5, 25, 50, 75, 100 and 150 mM). The Lineweaver-Burk plots were used. The results were present in Table 8.3. The derivative PU-PEG6000 showed a greater affinity for the enzyme, and higher values of Vmax (41.15 μmol/min/mg) and catalytic coefficient (50.49). The same behavior observed when TLL was immobilized on PEGylated PU particles (Pereira-Cipolatti et al., 2015).

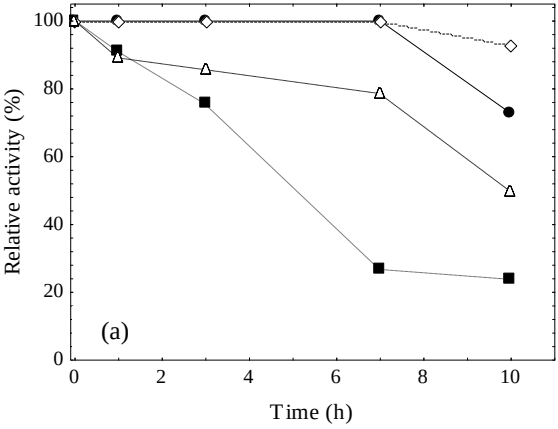
Table 9.3 – Kinetic parameters of immobilized enzyme on *PEGylated* PU particles.

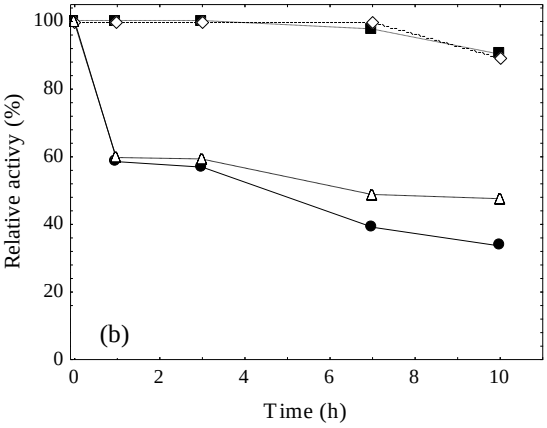
Enzyme	Km (mM)	Vmax (μmol/min/mg)	Vmax/Km
PU-PEG400	4.632	7.85	1.69
PU-PEG4000	8.417	17.95	2.13
PU-PEG6000	0.815	41.15	50.49

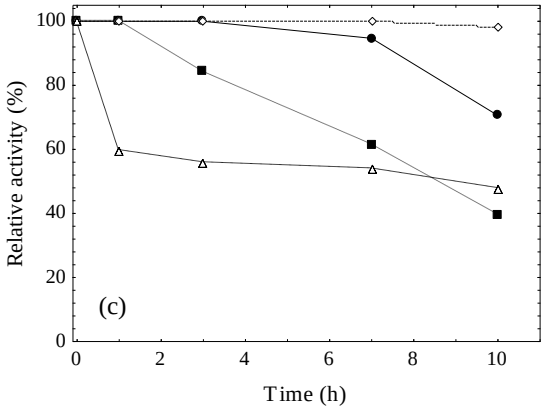
9.3.3 Thermal stability of immobilized CalB in coated PEGylated PU particles

The main objective of coating derivatives is to provide greater temperature stability, but some coatings can destabilize the derivative, causing the release of the enzyme, and/or its possible denaturation. This behavior will depend on the used polymer and the immobilized enzyme. According to the results, trehalose (T10) was more effective in terms of the thermal stability at 50°C (Figure 9.1), behavior contrary to that found when immobilize the enzyme TLL on the same coated support with T10 (Pereira-Cipolatti et al., 2015).

Figure 9.1 – Thermal stability of CalB immobilized in PU-PEG400 (a), 4000 (b) and 6000 (c) without coating (Δ), and coated with PEI10 (●), PEI20 (■),T10(◊) at 50 ºC (The enzymatic activity was carried out as described in Materials and Methods section).







9.3.4 Ethanolysis of fish oil in solvent free system catalyzed by immobilized CalB enzyme in coated PEGylated PU particles

Lipases from TLL and RMN are seen as more selective than CALB in ethanolysis reaction in fish oil catalyzed in cyclohexane (Moreno-Pérez et al., 2014). The same study indicates a selectivity of 3 using CalB adsorbed on Sepabeads C18 support in cyclohexane. Table 9.4 shows the synthesis of ethyl esters of EPA and DHA.

In general, the derivatives coated with trehalose and without coatings present the best results in relation to the synthesis and selectivity of ethyl esters of EPA and DHA. The low production obtained by others coatings were not expected, since the use of coatings agents was used in order to also improve the stability in the reaction. Comparing the derivatives with each other, those with greater PEG chain (4000 e 6000) showed better results compared to the polymeric support with PEG400. PEG4000-T10 showed values of EE-EPA and EE-DHA of 3.8 and 5.7 times, respectively, higher than derivative without coating. For derivative PEG6000-T10, the increase was 1.6 and 2.5 times for EPA and DHA, respectively. CNBr derivative was used for comparison and resulted in a best value of selectivity (9.6).

Table 9.4 – Synthesis and selectivity of ethyl esters (EE) of EPA and DHA of immobilized CalB in different PEGylated PU particles as support.

Enzyme/Derivative	mM EE-EPA.U^{-1}	mM EE-DHA.U^{-1}	Selectivity*
Free	43.72	16.83	2.6
CNBr	0.90	0.10	8.6
PEG400	81.20	36.90	2.2
PEG400-PEI10	1.31	0.74	1.8
PEG400-PEI20	0.61	0.50	1.2
PEG400-T10	35.16	11.03	3.2
PEG4000	17.77	5.46	3.3
PEG4000-PEI10	1.88	0.52	3.6
PEG4000-PEI20	4.26	0.57	7.4
PEG4000-T10	68.32	28.24	2.4
PEG6000	41.22	9.29	3.9
PEG6000-PEI10	1.25	0.44	2.8
PEG6000-PEI20	1.06	0.37	2.9
PEG6000-T10	65.77	23.30	5.0

The results of EPA and DHA (mM.U^{-1}) were obtained in 4 h of reaction. *Selectivity expressed as molar ratio between synthesized EPA and DHA, measured at 15% of conversion to total ethyl esters produced (EPA+DHA).

9.3.5 Enantioselective hydrolysis of (R,S)-mandelic acid ethyl ester of CalB immobilized on PU-PEG particles

The enzymatic derivatives of CalB were also used in the enantioselective hydrolysis of (R,S)-mandelic acid ethyl ester. The high value of yield was obtained with the CalB-PU-PEG400. All enantiomeric ratios was satisfactory (E>20), but the enantiomeric excess, especially for application to medical application, was unsatisfactory (Table 9.5).

The purpose of this stage of the work was to investigate the possibility of applying the polymer derivatives of CalB in the enantioselective hydrolysis. The authors believed that if an optimization was done can be possible to obtain very interesting results.

Table 9.5 – Effect of immobilization process on the enantioselectivity of CalB-derivatives.

Enzyme/ Derivative	Activity ($U/mg_{support}$)	Yield (%)	E(R/S)[a]	ee[b]
CalB-PU-PEG400	0.06	61.2	28.3	93.2
CalB-PU-PEG4000	0.14	50.4	38.7	95.0
CalB-PU-PEG6000	0.11	59.0	23.3	91.8

Experiments were performed at pH 7, 25°C after 7 h of reaction.
[a]Enantiomeric ratio (specificity).
[b]Enantiomeric excess.

9.4. CONCLUSIONS

Based on the results obtained in this study, supports from polyurethane PEGylated obtained by miniemulsion can be efficiently used for the immobilization of lipase B from *Candida antarctica*. Differents PEG (400, 4000 and 6000 Da) was used in the synthesis of polymeric support and the use of coating agents (polyethyleneimine or trehalose). The derivatives with more large chain showed the best results for enzymatic activity, immobilization yield and recovered activity. In addition, this derivative also showed the best results for the kinetic parameters. The same was observed for the synthesis of ethyl esters of EPA and DHA. The derivative coated with trehalose showed greater temperature stability and better results in the synthesis of ethyl esters. It can be promoted for the configuration and disposition of the enzyme on the new layer of the sugar in the PEG. TLL immobilized on PEGylated supports with different chain sizes of PEG were also applied in enantiomeric resolution of mandelic acid with satisfactory enantiomeric ratios (E>20).

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10. CONCLUSÕES E SUGESTÕES PARA TRABALHOS FUTUROS

10.1 CONCLUSÃO GERAL

Neste trabalho foi possível imobilizar as lipases de *Candida antarctica* fração B (CalB) e de *Thermomyces lanuginosus* (TLL) em suportes poliméricos via miniemulsão. Primeiramente foi realizado estudo da imobilização de CalB em nanopartículas de poliuretano PEGuilado diretamente na miniemulsão. A maior atividade enzimática obtido por esterificação foi 21 U/mg na condição de 70% de sonda de ultrassom por 2 mim, resultando em partículas com diâmetro médio de 158 nm. Foi verificado também que o uso de crodamol durante o processo de imobilização funcionou como um "agente de proteção" da enzima. A imobilização foi verificada também pelas técnicas de espectroscopia de infravermelho (FTIR- do inglês - *Fourier transform infrared spectroscopy*) e microscopia ótima de fluorescência, confirmando o derivado enzimático obtido.

CalB foi imobilizada também em nanopartículas de PMMA via miniemulsão. O uso de diferentes quantidades de crodamol como co-estabilizador possibilitou a obtenção de derivados com diferentes diâmetros e diferentes faixas de potencial zeta. Foi possível observar que em todos os casos estudados, houve um decréscimo na atividade relativa em 120 min de reação. Foi possível observar que ao utilizar a CalB em ciclos repetidos de hidrólise esta apresentou elevada estabilidade em relação ao mesmo procedimento aplicada à enzima livre.

A segunda etapa do trabalho foi realizada com a enzima TLL imobilizada em suportes de poliuretano via miniemulsão. Optou por estudar mais variáveis relacionadas à síntese do suporte de PU à estudar o suporte PMMA em paralelo. A enzima TLL foi escolhida como modelo por apresentar um menor custo comparado com a CalB.

TLL foi imobilizada nos suportes de PU-PEGuilados. A enzima foi imobilizada após reação de síntese e liofilização das partículas. Diferentes cadeias de PEG foram utilizadas (400, 4000 e 6000 Da). Embora todos os derivados tenham apresentado potencial para aplicação na produção de ésteres etílicos de ácidos graxos, o derivado com maior cadeia polimérica se destacou. TLL–PU–PEG6000 apresentou melhor afinidade pelo substrato pNPB (Km de 0,183 mM) e melhores resultados para Vmax de 45.79 mmol/min/mL calculado pelo gráfico do duplo recíproco (Lineweaver–Burk). Possibilitou 100% de imobilização

e de atividade recuperada. Elevada produção de EE de ácidos graxos foram alcançadas (260 mM $EE.U^{-1}$).

Os derivados de TLL com diferentes PEGs foram recobertos com polietilenoimina e trealose, exibindo elevadas porcentagens de imobilização e atividade recuperada. De forma geral, os recobrimentos com PEI foram mais eficientes. Os derivados recobertos apresentaram elevada produção de EE, com no mínimo 4 vezes mais em comparação aos derivados sem recobrimento.

CalB também foi imobilizada em PU-PEGuilado com diferentes tamanhos de cadeia do PEG. Os melhores resultados foram obtidos com o uso de cadeias maiores (4000 e 6000 Da). CalB-PU-PEG6000 apresentou os melhores valores em relação aos parâmetros cinéticos (Km de 0,815 mM e Vmax de 41,15 μmol/min/mg). Os derivados recobertos com trealose se mostraram mais estáveis. CalB-PU-PEG400 apresentou maior produção de EE-EPA e EE-DHA (43,72 e 16,83 $mM.U^{-1}$). CalB imobilizada foi utilizada na hidrólise do éster etílico (R,S) de ácido mandélico com satisfatórios valores de razão enantiomérica (E>20).

Os resultados obtidos ao longo deste trabalho levam à conclusão de que, embora ainda pouco explorada para este fim, a síntese de polímeros por miniemulsão pode ser uma alternativa eficiente para a produção de suportes para imobilização de lipases. Enzimas com comprovada importância, como é o caso da CalB e TLL, imobilizadas nos suportes propostos, podem se tornar ainda mais interessantes do ponto de vista científico e tecnológico.

10.2 SUGESTÕES PARA TRABALHOS FUTUROS

Devido à escassez de trabalhos que utilizam o processo de miniemulsão na síntese de suportes poliméricos, e os resultados favoráveis encontrados neste trabalho, seria interessante a imobilização de outras lipases bem como de outras classes de enzimas.

Considerando que o PMMA, embora promissor, não foi completamente explorado durante este trabalho, é fortemente sugerido a aplicação deste suporte em importantes reações de catálise, como a de produção de ésteres etílicos de ácidos graxos.

As partículas de PU sintetizadas foram aplicadas eficazmente em reações de catálise, seria interessante o estudo da reutilização do suporte na reação.

O uso de CalB imobilizada em suporte de PU-PEGuilado foi brevemente estudada em relação à sua aplicação na hidrólise seletiva de enantiômeros. Sugere-se o estudo mais aprofundado da reação com ácido mandélico, como variação de pH, temperatura e cinética da reação.

11. PUBLICAÇÃO CIENTÍFICA RELACIONADA

Neste capítulo estão apresentadas as publicações científicas advindas da realização deste trabalho. Pode ser observado que, ao longo do desenvolvimento da tese, alguns trabalhos foram apresentados em congressos científicos de importância relevante na área de biotecnologia enzimática. Alguns trabalhos citados aqui não fazem parte do corpo do documento de tese, mas foram realizados em paralelo e têm fundamental importância no desenvolvimento do trabalho.

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Review

Current status and trends in enzymatic nanoimmobilization

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ORIGINAL PAPER

Evaluation of different methods for immobilization of *Candida antarctica* lipase B (CalB lipase) in polyurethane foam and its application in the production of geranyl propionate

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Alexsandra Valério; Eliane Cipolatti; Daniela Remonatto; J. Vladimir Oliveira; Débora Oliveira. Transesterification synthesis of geranyl cinnamate ester by a new commercial immobilized lipase. Submetido à Food Chemistry em 22 de outubro de 2015.