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SÍNTESE DE BIODIESEL ATRAVÉS DE TRANSESTERIFICAÇÃO ENZIMÁTICA DE ÓLEOS VEGETAIS CATALISADA POR LIPASE IMOBILIZADA POR LIGAÇÃO COVALENTE MULTIPONTUAL

Tese submetida ao Programa de Pós Graduação em Engenharia Química como requisito parcial para obtenção do grau de Doutor em Engenharia Química

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RESUMO

Biodiesel consiste de ésteres alquílicos de ácidos graxos produzidos pela transesterificação de triglicerídeos com álcoois de cadeia curta. Tradicionalmente, a reação ocorre na presença de catalisadores químicos, como álcalis ou ácidos. A utilização alternativa de lipases como biocatalisadores na reação de síntese do biodiesel não gera materiais residuais tóxicos, e o glicerol pode ser facilmente recuperado sem um processamento complexo. Neste trabalho estudou-se a síntese de biodiesel através da transesterificação enzimática de óleos vegetais e álcoois de cadeia curta catalisada pela lipase de *Thermomyces lanuginosus* (TLL). Primeiramente, as condições da reação de transesterificação, entre óleo de soja e etanol catalisada pela TLL em sua forma livre foram otimizadas através de planejamento experimental e da metodologia de superfície de resposta. A seguir, foram estudadas as condições para a reação catalisada pela TLL imobilizada comercial (Lipozyme TL-IM). Na etapa seguinte foram avaliados diferentes óleos vegetais (óleo de soja, óleo de girassol e óleo de arroz) e álcoois (metanol, etanol, propanol e butanol), na reação de transesterificação catalisada por três derivados enzimáticos comerciais de lipases de diferentes fontes (Lipozyme TL-IM, Novozym 435, Lipozyme RM-IM), onde se verificou que cada lipase apresentou uma especificidade diferente em relação ao álcool preferente para a reação de transesterificação. Além disso, a reação utilizando diferentes óleos vegetais apresentou resultados semelhantes, mostrando ser este um fator mais dependente do ponto de vista econômico que processual. Também se estudou diferentes tratamentos para melhorar o reuso da lipase imobilizada, onde a lavagem do derivado com n-hexano, após cada ciclo, manteve a atividade da lipase em torno de 90% da atividade inicial após sete ciclos. Prosseguindo, foi estudada a imobilização e estabilização da TLL através da ligação covalente multipontual em suporte glioxil-agarose. Para obter isto se procedeu a aminação química da superfície da enzima, o que permitiu a obtenção de um derivado com alto grau de imobilização e alta estabilidade quanto à inativação por temperatura e solventes orgânicos. A seguir avaliou-se a possibilidade de reativação de derivados submetidos a processos de inativação. Em um primeiro momento os estudos foram realizados com derivados de TLL imobilizada unipontualmente em agarose ativada com brometo de cianogênio, onde foi possível obter a reativação completa do derivado inativado por solventes orgânicos através de incubação em meio aquoso. Verificou-se também que um dos principais pontos que dificultam a reativação de lipases é a recuperação do mecanismo de abertura do *lid*, que é facilitado com a presença de detergentes. Logo, estudos de reativação com derivados imobilizados por ligação covalente multipontual, mostraram que este tipo de imobilização auxilia no processo de reativação por oferecer mais pontos de referência no momento da reativação. A etapa final deste trabalho foi a aplicação do derivado de TLL imobilizado multipontualmente em suportes glioxil na reação de síntese de biodiesel. Verificou-se que este derivado foi mais ativo que o derivado comercial (Lipozyme TL-IM) na reação de transesterificação entre etanol e óleo de soja, obtendo 100% de conversão nas condições previamente otimizadas com presença de n-hexano no meio de reação. Porém, quando se realizou a reação em dois passos, isto é a adição do etanol em duas etapas, foi possível obter 100% de conversão em 10 h de reação em um meio sem solvente.

Palavras-chaves: biodiesel, lipase, etanólise, óleos vegetais, planejamento experimental, imobilização multipontual, estabilidade enzimática

ABSTRACT

Biodiesel consists of fatty acids alkyl esters, produced by transesterification of triglycerides with short-chain alcohols. Traditionally, the reaction occurs in the presence of chemical catalysts, such as acid or alkali. The alternative use of lipases as biocatalysts in the reaction of synthesis of biodiesel does not generate toxic waste material, and the glycerol can easily be recovered without complex processing. In this work it was studied the synthesis of biodiesel by transesterification of vegetable oils and short-chain alcohols catalyzed by lipase from *Thermomyces lanuginosus* (TLL). First, the conditions of transesterification reaction from soybean oil and ethanol catalyzed by TLL in its free form were optimized by experimental design and response surface methodology. Next, we studied the conditions for the reaction catalyzed by commercial immobilized TLL (Lipozyme TL-IM). In the next stage were evaluated some vegetable oils (soybean oil, sunflower oil and rice bran oil) and alcohols (methanol, ethanol, propanol and butanol) in the transesterification reaction catalyzed by three commercial enzyme preparations of lipases from different sources (Lipozyme TL-IM, Novozymes 435, Lipozyme RM-IM), where it was found that each lipase presented a different specificity in relation to preferred alcohol for the reaction of transesterification. In addition, the reaction using different vegetable oils presented similar results, showing that it is more dependent on the economic than technical aspects. It was also studied different treatments to improve the reuse of immobilized lipase, where washing the derivative with n-hexane after each batch, showed the high stability of the system, with activities of 90 % still remaining after seven cycles. Thus, it was studied the immobilization and stabilization of TLL through covalent multipoint immobilization in glyoxyl-agarose support. For this procedure, a chemical amination of the enzyme surface, allowed obtaining a derivative with a high degree of immobilization and high stability for inactivation by temperature and organic solvents. Then, it was evaluated the possibility of reactivation of derivatives previously inactivated. At first, the studies were carried out for TLL mild immobilized in cyanogen bromide activated agarose, where it was able to fully reactivate the derivative inactivated by organic solvents through incubation in aqueous medium. It was also noted that one of the main points that difficult the reactivation of lipases is the recovery of the mechanism for lid opening, which is helped by the presence of detergents. So, studies of reactivation with derivatives immobilized by multipoint covalent attachment showed that this type of immobilization helps the reactivation process by offering more reference points at the moment of reactivation. The final step of this study was the application of the TLL derivative multipointly immobilized on glyoxyl supports in the reaction of synthesis of biodiesel. It was found that this derivative was more active than the commercial derivative (Lipozyme TL-IM) in the transesterification reaction between ethanol and soybean oil, reaching 100% of yield conversion under the previously optimized conditions with the presence of n-hexane in the reaction medium. However, in the two stepwise ethanolysis it was possible to obtain 100% conversion in 10 h of reaction in a solvent-free system.

Key-words: biodiesel, lipase, ethanolysis, vegetable oils, experimental design, multipoint immobilization, enzyme stability.

INTRODUÇÃO

Combustíveis alternativos para motores a diesel estão tornando-se cada vez mais importantes devido à diminuição das reservas de petróleo e as conseqüências ambientais da exaustão de gases dos motores abastecidos com combustíveis petroquímicos. Com a crescente demanda por energia, e como o fornecimento de combustíveis fósseis é limitado, as pesquisas estão cada vez mais se direcionando para produção de combustíveis renováveis.

Óleos vegetais, ou seus triglicerídeos, têm se tornado alvo destas pesquisas, onde esforços têm sido feitos no desenvolvimento de derivados de óleos vegetais que aproxime as propriedades e desempenho do diesel derivado de hidrocarbonetos. Os problemas encontrados em substituir o diesel por triglicerídeos são principalmente associados à sua viscosidade, baixa volatilidade e caráter poliinsaturado dos ácidos graxos.

Neste contexto encaixa-se o biodiesel. Para que biodiesel seja produzido, óleos vegetais ou gorduras animais são submetidos a uma reação química denominada transesterificação. Nesta reação, óleos vegetais e gordura animal reagem com um álcool de cadeia curta (usualmente metanol), na presença de um catalisador (usualmente uma base) para produzir ésteres alquílicos, correspondentes da mistura de ácidos graxos que é encontrada no óleo vegetal ou gordura de origem animal, e glicerol. Estes ésteres alquílicos possuem características que se aproximam do diesel tradicional e solucionam os problemas encontrados para a aplicação de óleos vegetais como combustíveis.

O biodiesel pode ser produzido de uma grande variedade de matérias primas. Além dos diversos óleos vegetais (soja, girassol, canola, mamona, algodão, entre outros), também podem ser usadas gorduras de origem animal (como sebos), óleos de descartes (como óleos usados em frituras), e algas.

A reação de transesterificação pode ser realizada de diversas formas, como o uso de catalisadores alcalinos, catalisadores ácidos, biocatalisadores, catalisadores heterogêneos ou usando álcoois nos seus estados supercríticos. Entre todos os métodos mencionados para produção de biodiesel, apenas o processo alcalino é utilizado em escala industrial. É um processo com custo efetivo e altamente eficiente. Porém surgem problemas nas operações de *downstream* incluindo a separação do catalisador e o álcool não reagido do biodiesel. A remoção do catalisador envolve muitas complicações e o biodiesel requer repetidas lavagens para atingir a pureza necessária.

A produção de biodiesel usando um biocatalisador elimina as desvantagens do processo alcalino através da síntese de um produto com alto grau de pureza com pouca ou nenhuma operação *downstream*. Entre as vantagens do uso de biocatalisadores estão: a inexistência de rejeito aquoso alcalino; menor produção de outros contaminantes; maior seletividade e bons rendimentos. Estas vantagens motivam a realização de pesquisas que visem diminuir a principal desvantagem da metodologia, i. e., o alto custo das enzimas puras.

O custo das lipases ainda é o principal obstáculo em relação à exploração total de seu potencial. Para isso, o reuso da lipase é essencial do ponto de vista econômico, o que pode ser atingido através do uso de lipases imobilizadas. A escolha de uma metodologia adequada de imobilização onde se obtenha um derivado imobilizado altamente estável, ativo e seletivo, e que permita o seu reuso, é uma das principais chaves para a aplicação de enzimas a nível industrial.

Este trabalho tem como **objetivo principal** estudar a síntese de biodiesel, através da reação de transesterificação enzimática a partir de óleos vegetais e álcoois de cadeia curta.

Os objetivos específicos são:

- Estudar as variáveis que influenciam na reação de transesterificação, e otimizá-las para obter maiores rendimentos;
- Avaliar diferentes tipos de óleos vegetais e álcoois na síntese do biodiesel, assim como lipases microbianas de diferentes fontes;
- Estudar estratégias de re-uso da lipase imobilizada que aumentem a vida útil do biocatalisador;
- Estudar metodologias de imobilização da lipase para obter derivados altamente estáveis e ativos na reação de síntese do biodiesel, através de ligação covalente multipontual;
- Avaliar possibilidades de reativação de derivados imobilizados de lipase previamente inativados;
- Estudar metodologias para a reação de síntese de biodiesel que melhorem o desempenho do biocatalisador.

A presente Tese de Doutorado foi desenvolvida principalmente no Laboratório de Biotecnologia do Instituto de Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul, com uma parte realizada no Laboratório de Ingeniería Enzimática, do Instituto de Catálisis y Petroleoquímica do Consejo Superior de Investigaciones Científicas em Madri na Espanha, durante um estágio de doutorado de oito meses.

O trabalho será apresentado na forma de artigos científicos de acordo com as normas estabelecidas pelo Programa de Pós-Graduação em Engenharia Química. No Capítulo I uma revisão bibliográfica abordando os principais pontos do tema proposto. A seguir nos Capítulos de II a VIII os resultados, na forma como foram submetidos à publicação em periódicos internacionais. Por fim, as considerações finais, com as principais conclusões obtidas e as perspectivas para trabalhos futuros.

CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

1.1. Biodiesel

Segundo resolução da Agência Nacional de Petróleo n^{o}. 42/2004, Biodiesel é definido como (BRASIL, 2004):

> "*Combustível composto de alquil estéres de ácidos graxos oriundos de óleos vegetais ou gorduras animais, designado B100 observando atendimento ao Regulamento Técnico ANP n° 4/2004*."

A Figura 1 apresenta a reação de transesterificação, para síntese de biodiesel. O catalisador utilizado pode ser de origem química, ou biocatalisadores, como as enzimas.

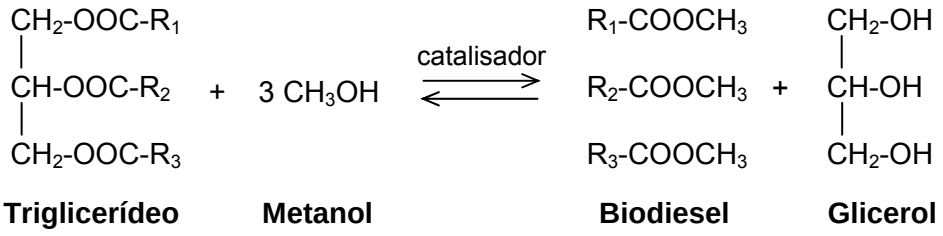


Figura 1. Reação de síntese do Biodiesel

O biodiesel é miscível com o diesel de petróleo em qualquer proporção. Em muitos países esta propriedade levou ao uso de misturas binárias diesel/biodiesel, ao invés do biodiesel puro. Neste sentido, é importante salientar que estas misturas binárias não podem ser caracterizadas como biodiesel. Muitas misturas deste tipo são designadas por acrônimos como B20, que representa a mistura de 20% de biodiesel no diesel de petróleo. Obviamente, óleos vegetais e gorduras de origem animal não transesterificadas também não podem ser denominadas "biodiesel" (KNOTHE *et al.*, 2006).

A Lei nº 11.097, de 13 de janeiro de 2005 (BRASIL, 2005), estabelece a obrigatoriedade da adição de um percentual mínimo de biodiesel ao óleo diesel comercializado ao consumidor, em qualquer parte do território nacional. Esse percentual obrigatório será de 5% oito anos após a publicação da referida lei, havendo um percentual obrigatório intermediário de 2% três anos após a publicação da mesma.

Além de ser totalmente compatível com o diesel de petróleo em praticamente todas as suas propriedades, o biodiesel ainda apresenta algumas vantagens em comparação com o combustível fóssil:

- É derivado de matérias-primas renováveis de ocorrência natural, reduzindo assim a atual dependência sobre os derivados do petróleo e preservando as suas últimas reservas;

- O gás carbônico liberado é absorvido pelas oleaginosas durante o crescimento, o que equilibra o balanço negativo gerado pela emissão na atmosfera;

- É biodegradável;

- Gera redução nas principais emissões presentes nos gases de exaustão (com exceção de óxidos de nitrogênio);

- Possui um alto ponto de fulgor, o que lhe confere manuseio e armazenamento mais seguros;

- Apresenta excelente lubricidade, fato que vem ganhando importância com o advento do petrodiesel de baixo teor de enxofre, cuja lubricidade é parcialmente perdida durante o processo de produção. A lubricidade ideal deste combustível pode ser restaurada através da adição de baixos teores de biodiesel.

1.2. Lipases

Lipases (triacilglicerol éster hidrolases, EC. 3.1.1.3) são enzimas que catalisam a hidrólise de óleos e gorduras com subseqüente liberação de ácidos graxos livres, diacilgliceróis, monoacilgliceróis e glicerol. Estas enzimas são comumente encontradas na natureza, podendo ser obtidas a partir de fontes animais, vegetais e microbianas, nos quais

preenchem um papel chave na modificação biológica de lipídios. Estão envolvidas no metabolismo de lipídios intracelulares, e, portanto, na funcionalidade de membranas biológicas. Lipases têm sido extensivamente investigadas com respeito às suas propriedades bioquímicas e fisiológicas, e posteriormente sobre suas aplicações industriais (VILLENEUVE *et al.*, 2000).

A maioria das lipases utilizadas como catalisadores em sínteses orgânicas são de origem microbiana, como *Candida rugosa*, *Pseudomonas fluorescens*, *Rhizopus oryzae*, *Burkholderia cepacia*, *Aspergillus niger*, *Thermomyces lanuginosus*, e *Rhizomucor miehei* (AL-ZUHAIR, 2007).

As razões para o enorme potencial biotecnológico de lipases microbianas incluem os fatos de que elas são (1) estáveis em solventes orgânicos, (2) não necessitam de co-fatores, (3) possuem uma grande especificidade de substrato e (4) exibem uma alta enantioseletividade (JAEGER e REETZ, 1998).

O uso de lipases na bioconversão de óleos e gorduras tem muitas vantagens sobre os catalisadores químicos clássicos. Lipases operam sob condições brandas de reação em uma faixa de temperaturas e pressões que minimizam a formação de produtos laterais. Tanto em meios aquosos ou não aquosos, lipases catalisam muitas reações como hidrólise, síntese de ésteres, transesterificação e interesterificação (HOU, 2002).

1.2.1 Estrutura e mecanismo de atuação das lipases

As lipases são enzimas que apresentam um mecanismo peculiar de atuação, chamado ativação interfacial. Em 1958, Sarda e Desnuelle definiram as lipases em termos cinéticos baseados neste fenômeno de ativação interfacial, que difere do modelo cinético clássico de Michaelis-Menten.

Este mecanismo de atuação é facilmente explicado através de sua estrutura tridimensional e de seu sitio ativo. O sítio ativo da lipase é geralmente caracterizado pela tríade composta dos aminoácidos serina, histidina e ácido aspártico (ou glutâmico),

complexos acil-enzima sendo intermediários cruciais em todas as reações catalisadas por lipases (JAEGER e REETZ, 1998). Ademais, este sitio ativo é coberto por uma cadeia polipeptídica em forma de α-hélice comumente chamada de tampa, ou *lid* (CAJAL *et al.*, 2000).

Em presença de meios aquosos homogêneos, com ausência de solventes orgânicos e interfaces, estruturas obtidas através de técnicas de cristalografia mostram que o sitio ativo das lipases está totalmente isolado do meio de reação através do *lid*, o que se chama de conformação fechada, apresentando uma baixa atividade lipolítica (Figura 2a) (GROCHULSKI *et al.*, 1994). Por outro lado, quando a lipase se adsorve a uma interface, este *lid* se desloca, expondo o sítio ativo para o meio de reação, tornando-o acessível ao substrato, ao que se chama conformação aberta da enzima em interfaces lipídicas (Figura 2b) (GROCHULSKI *et al.*, 1993). Nesta conformação, o movimento do *lid*, não apenas abre o acesso ao sítio ativo como também expõe uma extensa zona hidrofóbica o que provavelmente interage favoravelmente com a interface lipídica (DEREWENDA *et al.*, 1992).

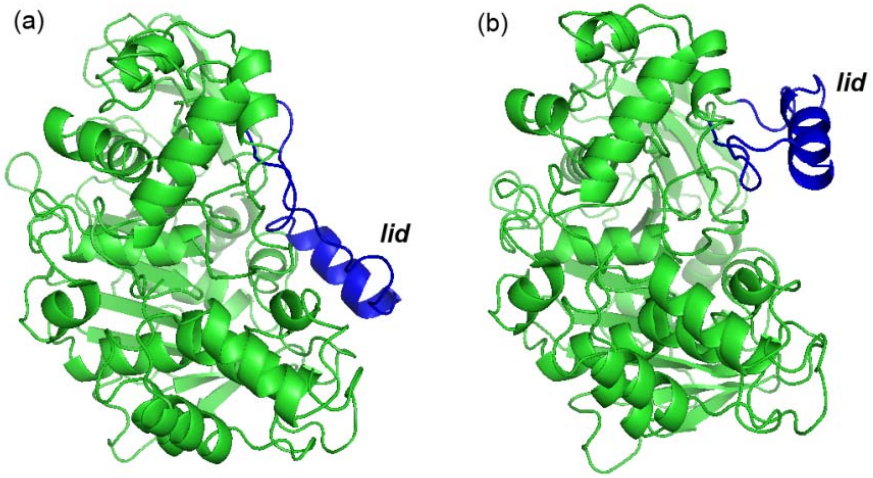


Figura 2. Distintas conformações da lipase de *Candida rugosa*. (a) Conformação fechada; (b) Conformação aberta. Estruturas obtidas através do Protein Data Bank (PDB) utilizando o software Pymol v. 0.99. Os códigos pdb para as lipases são: 1trh e 1crl, respectivamente.

Assim, estes dados permitiram estabelecer que no mecanismo catalítico das lipases o que ocorre é a ativação interfacial (SARDA e DESNUELLE, 1958; CHAPUS et al., 1976;

BRZOZOWSKI et al., 1991; GROCHULSKI et al., 1993; MINGARRO et al., 1995; BERG et al., 1998; BRZOZOWSKI et al., 2000; CAJAL et al., 2000; MILED et al., 2001). Complementarmente, dado que o substrato natural das lipases são os óleos e gorduras, esta atividade na interface é um requisito indispensável para a sua função biológica. A Figura 3 apresenta um esquema do mecanismo de atuação das lipases.

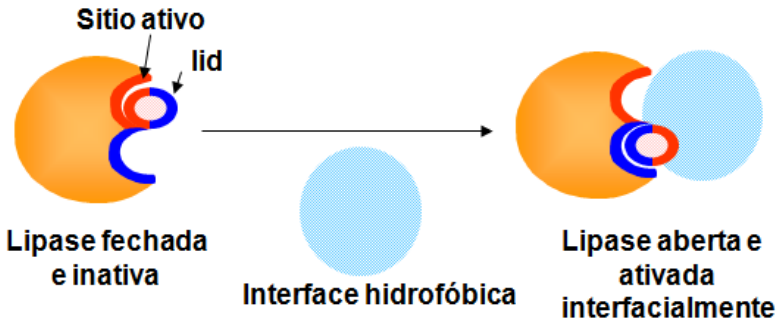


Figura 3. Mecanismo de ativação interfacial de lipases em interfaces hidrofóbicas

A ativação e a subsequente ligação das lipases a interfaces lipídicas são processos complexos. As mudanças da energia livre envolvidas nestes processos são causadas por vários fatores como pontes de hidrogênio, interações eletrostáticas e de van der Waals, interações hidrofóbicas entre outros (PETERS *et al.*, 1998). No entanto, o fato de que em meios aquosos homogêneos as lipases apresentem atividade, mesmo que baixa, sugere que as lipases encontram-se em equilíbrio entre a conformação fechada e distintas conformações abertas que permitem que elas sejam ativas na ausência de interfaces, como pode ser visualizado na Figura 4.

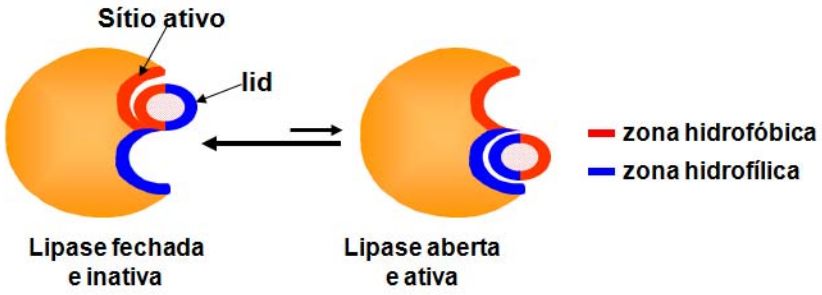


Figura 4. Equilíbrio entre as diferentes conformações de lipases em meios aquosos homogêneos

1.3. Reação de transesterificação enzimática

Entre as diversas aplicações industriais das lipases, uma em especial vem ganhando crescente atenção, a aplicação de lipases como catalisadores da reação de síntese do biodiesel. Embora não haja ainda o uso comercial para este fim, esforços vêm sendo feito para que seja possível a implementação do processo enzimático, com um custo competitivo ao processo químico. Diversos trabalhos na literatura apresentam o uso de lipases na reação de transesterificação de óleos vegetais e álcoois, principalmente metanol. Fatores como a fonte do óleo vegetal, o tipo de álcool, o meio reacional (presença ou ausência de solvente orgânico) são os principais focos das investigações (NELSON *et al.*, 1996; KAIEDA *et al.*, 1999; UOSUKAINEN *et al.*, 1999; SAMUKAWA *et al.*, 2000; WATANABE *et al.*, 2000; ISO *et al.*, 2001; KAMINI e IEFUJI, 2001; BELAFI-BAKO *et al.*, 2002; KOSE *et al.*, 2002; DENG *et al.*, 2003; DU *et al.*, 2003; HSU *et al.*, 2003; SHIEH *et al.*, 2003; SOUMANOU e BORNSCHEUER, 2003a; SOUMANOU e BORNSCHEUER, 2003b; XU *et al.*, 2003; XU *et al.*, 2004; CHANG *et al.*, 2005; DENG *et al.*, 2005; DU *et al.*, 2005; LAI *et al.*, 2005; SALIS *et al.*, 2005; DEMIRKOL *et al.*, 2006; LI *et al.*, 2006; MAHABUBUR *et al.*, 2006; NIE *et al.*, 2006; OLIVEIRA e ROSA, 2006; KUMARI *et al.*, 2007; ROYON *et al.*, 2007).

Para obtenção do biodiesel por catálise enzimática, alguns fatores devem ser levados em consideração como: origem da enzima, quantidade de enzima, razão molar entre os reagentes óleo e álcool, tipo de álcool, uso de solvente orgânico na reação, quantidade de água adicionada na mistura de reação, temperatura da reação, e reuso da enzima em operações repetidas.

Embora lipases geralmente catalisem a hidrólise de ésteres carboxílicos, elas também atuam em outra faixa de reações de bioconversões como esterificações, transesterificações, acidólises e aminólises. Um *screening* de lipases é uma excelente opção para encontrar a lipase com melhor atividade catalítica na reação de transesterificação (NOUREDDINI *et al.*, 2005). Lipases de diferentes origens seja microbiana, vegetal ou animal, apresentam diferentes atividades catalíticas quanto ao modo

de atuação com o substrato. A quantidade de enzima adicionada à reação também é importante, pois o aumento da quantidade de enzima favorece o aumento da velocidade da reação, entretanto existe um limite onde com o acréscimo de enzima a taxa de formação de produto permanece constante.

A razão molar de substrato é uma variável com grande influência na reação de síntese de biodiesel. Álcool em excesso da razão estequiométrica de 3:1 é usado para assegurar uma alta taxa de reação e minimizar as limitações de difusão. Entretanto, níveis excessivos de álcool podem inibir a atividade da enzima e assim diminuir sua atividade catalítica ao longo da reação de transesterificação (NOUREDDINI *et al.*, 2005). Contribuindo com isso, Salis *et al.* (2005) reportaram que uma alta razão álcool:triglicerídeo significa uma maior polaridade do meio, produzida pelo álcool e pela água. Uma alta polaridade é freqüentemente associada com a inativação do biocatalisador. A queda na atividade enzimática e rendimento de alquil ésteres a altas concentrações de álcool, refletem a habilidade do excesso de álcool em distorcer a camada de água essencial que estabiliza as enzimas imobilizadas (KOSE *et al.*, 2002).

Como já mencionado, metanol é o álcool mais utilizado na produção de biodiesel. Além do mais, as altas temperaturas dos processos químicos aumentam a miscibilidade entre o metanol e o óleo. Ao contrário, quando um processo biocatalítico é realizado, as baixas temperaturas não permitem uma boa mistura. Além disso, metanol está envolvido no processo de inativação da enzima (SALIS *et al.*, 2005).

O álcool utilizado para a reação de transesterificação influencia na necessidade de uso de um solvente orgânico. Álcoois de cadeia menor, como metanol e etanol, apresentam uma solubilidade menor aos óleos normalmente utilizados para produção de biodiesel. Álcoois com cadeia maior, como propanol ou butanol, apresentam melhor solubilidade ao óleo e dispensam o uso de um solvente orgânico na reação. Segundo Iso *et al.* (2001), para aplicação prática do biodiesel, não é desejável o uso de solvente. Isto porque após o

término da reação o solvente deve ser removido por destilação, extração ou outro método, que necessita de mão de obra e energia adicional.

Reações de alcoólise não envolvem água, entretanto, o controle do conteúdo de água é importante por algumas razões: a água atua como um “lubrificante” mantendo a enzima na sua conformação ativa; a água participa em muitos mecanismos que causam a inativação da enzima; a água promove a agregação das partículas de enzima; a altos conteúdos de água podem ocorrer limitações de difusão de substrato; água pode promover a hidrólise do substrato diminuindo assim o rendimento do produto (SALIS *et al.*, 2005). As lipases possuem uma característica única de atuar na interface entre uma fase aquosa e outra orgânica. A atividade das lipases geralmente depende da disponibilidade da área interfacial. Com o aumento na adição de água, a quantidade de água disponível para o óleo para formar gotículas óleo-água aumenta, assim, aumentando a disponibilidade de área interfacial. Entretanto, desde que lipases usualmente catalisam hidrólise em meio aquoso, um excesso de água pode estimular esta reação competidora do substrato. O ótimo conteúdo de água é um compromisso entre minimizar a reação de hidrólise e maximizar a atividade da enzima para a reação de transesterificação (NOUREDDINI *et al.*, 2005).

A taxa de reação aumenta com a temperatura. Este processo não continua indefinidamente. Na verdade, em torno de certa temperatura, ocorre a inativação da enzima, e a atividade catalítica diminui. A temperatura é uma grande vantagem que o processo enzimático apresenta frente o processo utilizando catalisadores químicos, visto que neste processo altas temperaturas são necessárias para a reação, enquanto que quando se usa enzimas temperaturas mais baixas são utilizadas e há um ganho econômico com energia.

Um importante parâmetro na avaliação de uma enzima imobilizada é seu tempo de vida ou meia-vida. Além disso, um maior tempo de vida útil das lipases significará um decréscimo dos custos de processo que acelerará aplicações industriais da tecnologia com lipases (DENG *et al.*, 2003). A principal vantagem da imobilização de uma enzima é que uma enzima com custo elevado pode ter uso repetido. Quando uma enzima imobilizada é

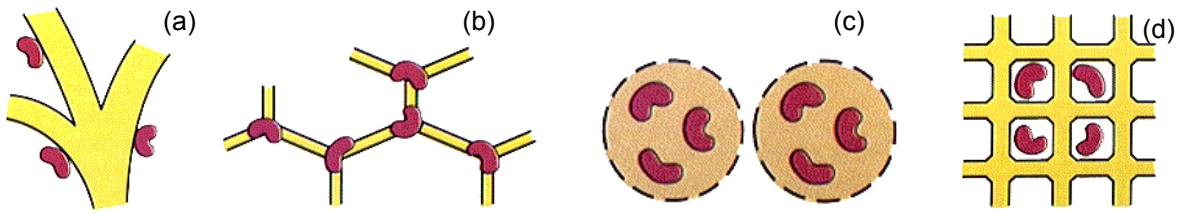
utilizada pela primeira vez, alguma quantidade de enzima é dessorvida. Entretanto, após vários usos repetidos ocorre uma diminuição do fenômeno da dessorção e há uma tendência à estabilidade da enzima. Por isso, enzimas imobilizadas podem ser usadas repetidamente. Enzimas imobilizadas podem ser separadas por processos de decantação, após o término da reação e não necessitam métodos especiais de separação (ISO *et al.*, 2001).

1.4. Imobilização de enzimas

A imobilização de enzimas tem um importante papel dentro da biotecnologia aplicada. A principal razão para imobilizar enzimas é a habilidade para isolar o biocatalisador do produto da reação e reusá-lo com o objetivo de aumentar a produtividade. Para o caso de reações catalisadas por lipase, a imobilização pode ajudar na promoção de condições não-aquosas necessárias para a síntese de ésteres e interesterificação (CHRISTENSEN *et al.*, 2003).

Entretanto, a idéia do reuso da enzima implicitamente significa que a estabilidade da preparação final da enzima deve ser alta o suficiente para permitir este reuso. Portanto, a enzima necessita ser altamente estável ou tornar-se altamente estabilizada durante a imobilização para ser um processo adequado (MATEO *et al.*, 2007).

Os métodos para imobilização de enzimas podem ser classificados em duas categorias básicas (BUCHHOLZ *et al.*, 2005): imobilização por ligação em suportes e encapsulamento. A Figura 5 apresenta uma representação esquemática dos principais métodos de imobilização de enzimas.



Fonte: http://www.agen.ufl.edu/~chyn/age4660/lect/lect_21/lect_21.htm

Figura 5. Métodos de Imobilização de enzimas: (a) Adsorção ou Ligação covalente em suporte sólido; (b) Ligação cruzada entre a enzima e o suporte; (c) Micro-cápsulas; (d) Matriz Poliméricas sintéticas e naturais.

A imobilização por ligação em suportes pode ser realizada através da ligação da enzima ao suporte por adsorção - onde a enzima é imobilizada em um suporte sólido por ligações da baixa energia, tais como interações de van der Waals ou hidrofóbicas, ligações de hidrogênio e iônicas, entre outras; ligação covalente - onde as enzimas são covalentemente ligadas ao suporte através de grupos funcionais nas enzimas, que não são essenciais para a atividade catalítica (D'SOUZA, 1999). A imobilização também pode ocorrer através de ligação cruzada, onde é realizada a ligação cruzada intermolecular de enzimas, por meio de reagentes bifuncionais ou multifuncionais.

A adsorção é o método mais comum usado para imobilização, por causa da sua facilidade e porque possui menor custo. A imobilização de lipases por adsorção é encontrada vastamente na literatura. Entre alguns suportes utilizados estão principalmente materiais argilosos ou cerâmicos, e porosos, que facilitem a fixação da enzima ao suporte, como exemplo, celita, que consiste de terra diatomácea altamente porosa composto de sílica e outros óxidos inorgânicos (CHANG *et al.*, 2007), caolinita, que é um tipo de argila (RAHMAN *et al.*, 2005), bentonita, outro material cerâmico (YESILOGLU, 2005).

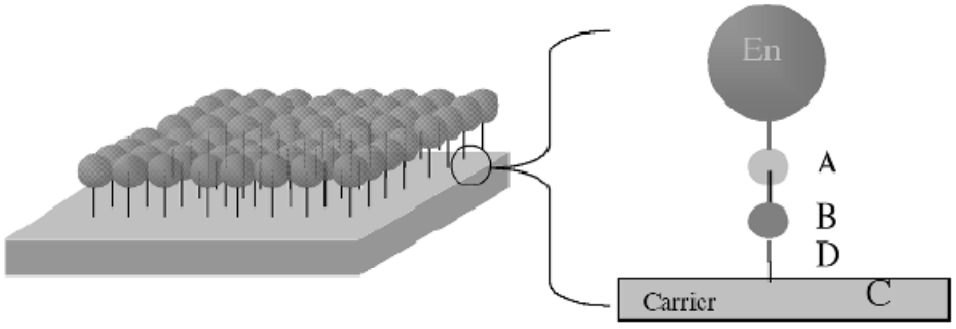
Os métodos de encapsulamento consistem em “confinar” uma enzima em um polímero insolúvel ou em uma micro-cápsula. Neste sistema cria-se uma cela artificial delimitada por uma membrana porosa. Moléculas grandes, tais como enzimas, não são capazes de se difundir através desta membrana porosa, enquanto que pequenas moléculas, como substratos e produtos, se difundem (DALLA-VECCHIA *et al.*, 2004).

Uma técnica bastante utilizada é a imobilização por encapsulamento em pérolas de polissacarídeos, como agarose, alginato e quitosana (VEMURI *et al.*, 1998; BETIGERI e NEAU, 2002; FADNAVIS *et al.*, 2003; ALSARRA *et al.*, 2004; WON *et al.*, 2005; YADAV e JADHAV, 2005; DAVE e MADAMWAR, 2006; FORESTI e FERREIRA, 2007).

1.4.1 Imobilização por ligação covalente

A ligação covalente de enzimas a suportes sólidos pré-existentes é uma das estratégias mais utilizadas devido principalmente a três importantes propriedades conferidas aos derivados enzimáticos obtidos por este sistema, além das provenientes da própria imobilização. Estes atributos característicos são o caráter covalente, e, portanto a estabilidade da ligação, a estabilização adicional que se pode obter quando a interação for multipontual, e permitir manipulações posteriores com o objetivo de modificar propriedades químicas ou catalíticas, provocar mudanças de especificidade de substratos, etc. (ALONSO, 1996). Além disso, em muitos casos é possível a seleção do suporte adequado segundo o preço, o tipo de reator ou a capacidade de carga enzimática desejados.

A metodologia da ligação covalente baseia-se na ativação de grupos químicos do suporte para que reajam com os nucleófilos das proteínas. Dentre os 20 aminoácidos diferentes que se encontram na estrutura das enzimas, os mais empregados para a formação de ligações com o suporte são principalmente lisina, cisteína, tirosina e histidina, e em menor medida metionina, triptofano, arginina e ácido aspártico e glutâmico. O restante dos aminoácidos, devido ao seu caráter hidrofóbico, não se encontram expostos para o exterior da superfície protéica, e não podem intervir na ligação covalente (ARROYO, 1998). A Figura 6 apresenta um esquema da imobilização covalente de enzimas.



Fonte: CAO, 2005.

Figura 6. Imobilização covalente de enzima em suporte sólido. (A) resíduo de aminoácido ativo; (B) funcionalidade ligante do suporte; (C) suporte; (D) braço espaçador.

Como visto na Figura 6, a imobilização covalente de enzimas pode ser considerada como uma composição dos componentes, suporte, braço espaçador, ligação e enzima. Assim, as seguintes propriedades são esperadas para afetar o desempenho da enzima imobilizada por ligação covalente (CAO, 2005):

- Natureza física do suporte (por exemplo, tamanho do poro, tamanho de partícula, porosidade, forma, etc.);
- Natureza química do suporte (composição química da estrutura principal, funcionalidade ativa, outras funcionalidades não ativas);
- Natureza da união ou ligação química;
- Conformação da enzima no momento da imobilização ou após a imobilização;
- Natureza e comprimento do braço espaçador;
- Propriedades do meio usado para a ligação das enzimas;
- O número de ligações formadas entre a enzima e o suporte;
- A distribuição da enzima na superfície ou dentro do suporte.

Entre algumas vantagens que o método apresenta, pode-se citar:

- A manipulação dos derivados imobilizados é simples;
- A carga de enzima permanece constante após a imobilização;

- Os derivados podem ser utilizados em reatores contínuos, empacotados, de leito fluidizado ou tanque agitado;
- Uma maior resistência à desativação por efeito de temperatura, solventes orgânicos ou pH, ao ter estabilizada sua estrutura terciária.

Porém, alguns inconvenientes são encontrados na imobilização por ligação covalente:

- É necessário conhecer a densidade de grupos ativos por unidade de superfície, já que condiciona o número de ligações enzima-suporte e sua geometria, já que a estrutura da enzima pode ser distorcida e conduzir a derivados inativos;
- O processo de imobilização pode alterar a estrutura do sítio ativo. Para evitar esta possível alteração, pode realizar-se a imobilização na presença de um inibidor que bloqueie o sítio ativo.
- A imobilização covalente não é aconselhável naquelas enzimas muito sensíveis a mudanças de pH, força iônica, etc.

Entre as aplicações práticas recentes da imobilização covalente de enzimas, Brigida *et al*. (2007), realizaram a imobilização covalente da lipase de *Candida antarctica* tipo B em fibras de coco verde silanizadas. Entre as variáveis utilizadas para controlar o número de ligações entre a enzima e o suporte, os autores avaliaram o tempo de contato, pH e redução final com borohidreto de sódio. Em outro trabalho, nanofibras de poliestireno foram utilizadas como suporte para imobilização de lipase (NAIR *et al.*, 2007). Uma pequena quantidade de anidrido maleico na fibra de poliestireno foi usada para ligação covalente da lipase na superfície da fibra.

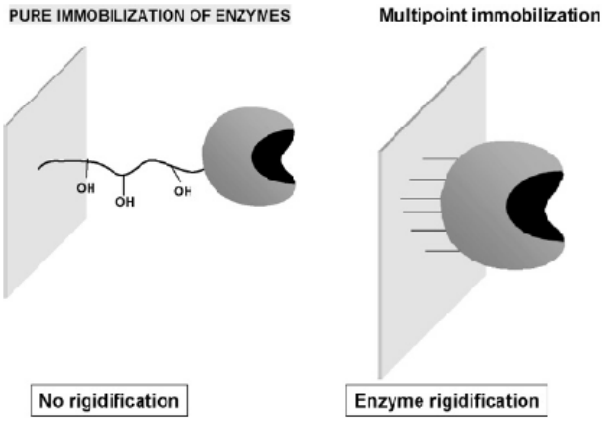
A imobilização de enzimas em suportes ativados com grupos epóxidos por ligação covalente é uma técnica bastante utilizada. Entre os principais suportes estão polímeros acrílicos particulados epóxi-ativados, Eupergit e Sepabeads. Estes suportes são desejáveis

para a imobilização de enzimas em escala industrial, devido a sua comercialização mundialmente disponível, resistência a estresse químico e mecânico, e processos específicos realizados em reatores (KNEZEVIC *et al.*, 2006). O processo de imobilização de lipases em suportes epóxi-ativados tem sido objeto de várias pesquisas científicas (MATEO *et al.*, 2000; MATEO *et al.*, 2003; BAYRAMOGLU *et al.*, 2005; GRAZU *et al.*, 2005; LEE *et al.*, 2006).

1.4.1.1 Ligação covalente multipontual

A ligação covalente multipontual de enzimas em suportes pré-existentes altamente ativados via pequenos braços espaçadores e envolvendo muitos resíduos colocados na superfície da enzima promove uma rigidificação da estrutura da enzima quando imobilizada. Esta técnica permite controlar a área da enzima envolvida no processo de imobilização, o comprimento do braço espaçador através do qual a enzima se liga ao suporte sólido, o número de ligações que se formam entre cada molécula de enzima imobilizada e o suporte sólido, entre outros fatores (PALOMO *et al.*, 2002; LÓPEZ-GALLEGO *et al.*, 2004; LÓPEZ-GALLEGO *et al.*, 2005; TORRES *et al.*, 2005; BETANCOR *et al.*, 2006; MATEO *et al.*, 2007; PEDROCHE *et al.*, 2007).

Para isso a distância relativa entre todos os resíduos envolvidos na imobilização multipontual deve ser muito próxima, para que o maior número possível de resíduos de aminoácidos da enzima reaja com o suporte e desta forma obtendo-se uma união muito intensa entre ambos, enzima e suporte. Isso reduz as mudanças conformacionais envolvidas na inativação da enzima e aumenta consideravelmente a sua estabilidade (MATEO *et al.*, 2007). A Figura 7 apresenta um esquema da imobilização multipontual da enzima em um suporte.



Fonte: MATEO *et al*., 2007.

Figura 7. Imobilização covalente multipontual de enzimas.

Os suportes para imobilização multipontual devem apresentar uma grande superfície interna para ter uma boa congruência geométrica com a superfície da enzima (MATEO *et al.*, 2006). Se o suporte é formado por uma camada muito fina, isto é, menor que a proteína, dificilmente será possível obter uma intensa interação entre a enzima e o suporte. Além disso, o suporte deve apresentar alta densidade superficial de grupos reativos e estes grupos devem reagir com os grupos localizados na superfície da enzima. Apenas desta forma uma imobilização multipontual será conseguida. E, deve ser fácil a obtenção de uma superfície final inerte no suporte após a imobilização, através da destruição ou bloqueio dos grupos reativos remanescentes no suporte, sem afetar a enzima (MATEO *et al.*, 2007).

Entre os principais suportes empregados para a imobilização multipontual de enzimas estão os suportes ativados com grupos glioxil. Esta metodologia foi desenvolvida pelo Prof. José Manuel Guisán (GUISÁN, 1988) e a imobilização neste tipo de suporte se dá através da ligação entre os grupos aldeídos (glioxil) do suporte e os grupos aminos primários presentes na superfície da enzima. Ao final da imobilização o derivado é submetido a uma redução com borohidreto de sódio, obtendo-se uma matriz inerte (BLANCO e GUISAN, 1989). O processo é bem desenvolvido e vem sendo amplamente utilizado para a imobilização e estabilização de diversas enzimas em suportes sólidos (LÓPEZ-GALLEGO *et al.*, 2005; MATEO *et al.*, 2005; BOLIVAR *et al.*, 2006; MATEO *et al.*,

2006; MATEO *et al.*, 2007; PEDROCHE *et al.*, 2007; FERNANDEZ-LORENTE *et al.*, 2008; RODRIGUES *et al.*, 2008). A Figura 8 apresenta um esquema de como ocorre a imobilização em suportes ativados com grupos glioxil.

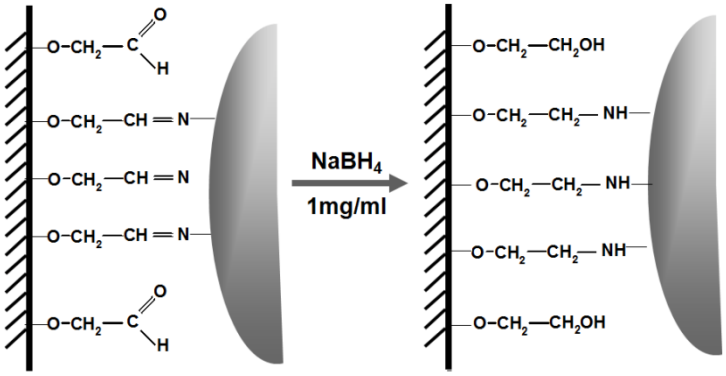


Figura 8. Imobilização de enzimas a suportes ativados com grupos glioxil

A ligação entre os grupos aldeídos e aminos ocorre através da formação de uma base de Schiff. A pH 7, o único grupo amino reativo na superfície da enzima é o amino terminal, pois outros grupos aminos estão na forma ionizada e não reagem com o suporte. A ligação formada pela base de Schiff é muito instável, portanto a pH neutro não é possível a imobilização neste tipo de suporte, pois para isso teria que haver ao menos duas ligações entre enzima e suporte para que para que a enzima ficasse imobilizada. Entretanto, em pH alcalino, as lisinas presentes nas enzimas não mais se encontram em sua forma ionizada, podendo assim reagir com o suporte. Como geralmente as enzimas apresentam várias lisinas em sua superfície, ocorre a união multipontual da enzima ao suporte. O passo final, como dito anteriormente é a redução com borohidreto de sódio. Nesta etapa, além da redução dos grupos aldeídos que não reagiram a grupos hidroxilos que são inertes, ocorre a redução da base de Schiff a uma ligação covalente simples, que é estável e desta forma a enzima estará fixa a um suporte sólido e inerte.

Outra maneira de obter derivados imobilizados mais estáveis é através da modificação química ou genética da superfície da enzima. A modificação química da proteína para aumentar a estabilidade pode ser realizada de diferentes maneiras. Entre elas

pode-se modificar quimicamente a enzima, não para obter uma enzima mais estável, mas para ter uma superfície protéica enriquecida em grupos reativos, e, portanto, com muito melhores possibilidades de atingir uma maior ligação covalente multipontual durante a imobilização (LÓPEZ-GALLEGO *et al.*, 2005). Lipases, em geral, por atuarem em substratos hidrofóbicos e principalmente meios não aquosos, apresentam poucas lisinas em sua superfície. Neste caso, a modificação de grupos carboxílicos constituintes dos aminoácidos aspárticos e glutâmicos para grupos aminos primários, através de ativação com carbodiimida e reação com etilenodiamina, permite a imobilização em suportes com grupos glioxil a pH mais baixo (ao redor de 9), pois o pK destes novos grupos aminos é menor, além de fornecer uma união mais intensa, e assim um derivado mais estável (FERNANDEZ-LORENTE *et al.*, 2008).

De maneira similar, a modificação da superfície da enzima pode ser realizada geneticamente. A inserção de resíduos de aminoácidos em determinadas áreas da superfície da enzima, irá favorecer a ligação covalente multipontual em suportes altamente reativos (ABIAN *et al.*, 2004).

1.5. Planejamento experimental aplicado a síntese de biodiesel

Conforme comentado anteriormente, diversos fatores influenciam para obtenção de biodiesel por catálise enzimática. Para otimizar estas variáveis, geralmente utiliza-se a técnica que varia um fator por vez, fixando os demais, porém está é uma técnica dispendiosa, e que não verifica a interação entre as variáveis (ISO *et al.*, 2001; HSU *et al.*, 2003; SALIS *et al.*, 2005). A metodologia de superfície de resposta (MSR) é uma técnica estatística eficiente para a investigação de processos complexos. A principal vantagem da utilização da MSR é o número reduzido de experimentos necessários para prover informação suficiente para resultados estatisticamente aceitáveis, além de ser um método mais rápido e mais barato para coleta de dados do que o método clássico. (GUNAWAN *et al.*, 2005). Alguns trabalhos utilizam a MSR para a transesterificação enzimática entre

metanol e óleos vegetais (UOSUKAINEN *et al.*, 1999; SHIEH *et al.*, 2003; CHANG *et al.*, 2005; DEMIRKOL *et al.*, 2006).

Chang et al. (2005) investigaram a alcoólise de óleo de canola pela enzima Novozym 435 usando MSR e delineamento composto central rotacional (DCCR) obtendo um conteúdo de metil ésteres de 99,4% nas condições ótimas. Shieh et al. 2003 realizaram a otimização da reação de transesterificação enzimática entre óleo de soja e metanol pela enzima Lipozyme IM-77. A máxima conversão molar obtida foi de 92,2% de metil ésteres.

Um estudo similar usando MSR em combinação com análise de componentes principais para avaliar a interdependência das variáveis do processo enzimático de transesterificação (UOSUKAINEN *et al.*, 1999). Os autores estudaram a alcoólise com óleo de colza e trimetilolpropano, encontrando que, alguns dos fatores que afetam a reação são a atividade de água e a eliminação do metanol produzido durante a reação. Dermikol et al. (2006) relataram a otimização da metanólise enzimática de óleo de soja através de MSR catalisada pela enzima Lipozyme RM-IM. Nas condições ótimas os autores obtiveram 76,9% de metil ésteres.

CAPÍTULO II - OPTIMIZATION OF THE LIPASE-CATALYZED ETHANOLYSIS OF SOYBEAN OIL IN A SOLVENT-FREE SYSTEM USING CENTRAL COMPOSITE DESIGN AND RESPONSE SURFACE METHODOLOGY

Neste trabalho foram estudadas as condições para a reação de transesterificação entre etanol e óleo de soja catalisada para lipase de *Thermomyces lanuginosus*. As variáveis avaliadas através de delineamento composto central rotacional e da metodologia de superfície de resposta foram o tempo de reação, a temperatura, a razão molar de substrato entre álcool e óleo, a quantidade de enzima e de água adicionada à reação, tendo como resposta o rendimento de conversão. Os resultados estão apresentados no manuscrito a seguir, publicado no *Journal of Chemical Technology and Biotechnology*, v. 83, p. 849-854.

Optimization of the lipase-catalyzed ethanolysis of soybean oil in a solvent-free system using central composite design and response surface methodology

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Optimization of the lipase-catalyzed ethanolysis of soybean oil in a solvent-free system using central composite design and response surface methodology

Abstract

BACKGROUND: In this work we describe the synthesis of ethyl esters, commonly known as biodiesel, using refined soy bean oil and ethanol in a solvent-free system catalyzed by lipase from *Thermomyces lanuginosus*. Central composite design (CCD) and response surface methodology (RSM) were employed to optimize the biodiesel synthesis parameters, which were: reaction time, temperature, substrate molar ratio, enzyme content, and added water, measured as percentage of yield conversion.

RESULTS: The optimal conditions obtained were: temperature, 31.5 °C; reaction time, 7 h; substrate molar ratio, 7.5:1 ethanol:soybean oil; enzyme content, 15%; added water, 4%. The experimental yield conversion obtained under these conditions was 96%, which is very close to the maximum predicted value of 94.4%. The reaction time-course at the optimal values indicated the 5 h was necessary to obtain high yield conversions.

CONCLUSION: A high yield conversion was obtained at the optimized conditions, with relative low enzyme content and short time. The comparison of predicted and experimental values showed good correspondence between them implying that empirical model derived from RSM can be used to adequately describe the relationship between the reaction parameters and the response (yield conversion) in lipase-catalyzed biodiesel synthesis.

Keywords: biodiesel, lipase, response surface methodology, optimization, solvent-free system.

Introduction

Biodiesel consists of alkyl esters produced by transesterification of triglycerides with short chain alcohols, i.e., methanol and ethanol. Biodiesel has become commercially attractive due to its environmental benefits and to the fact that it is made from renewable natural resources such as soybean and other plants[1]. This process relies on high energy consumption and the reaction product is a mixture of the desired esters, mono- and di-glycerides, glycerol, water and the alkaline catalyst (usually CH_3ONa, NaOH or KOH). Due to these drawbacks, alternative, and more sustainable routes for biodiesel production are being researched[2].

The use of lipases, which allow for mild reaction conditions and easy recovery of glycerol, largely prevent these drawbacks. Several reports describe lipase-catalyzed alcoholysis reactions in solvents and solvent-free media, especially the reaction parameters affecting the rates of lipase activities in alcoholysis reactions[2-8].

The optimization of the reaction parameters involved in lipase-catalyzed biodiesel synthesis is commonly made by varying one factor at a time and keeping the others constant[2, 8, 9]. But this method is inefficient as it fails to explain relationships between the variables and the response when there is interaction between the variables. Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results and it is a faster and less expensive method for gathering research data than the classical method[10].

The use of RSM for biodiesel enzymatic production was reported by some authors. Chang et al.[6] investigated the alcoholysis of canola oil by Novozym 435 using RSM and CCD obtaining a methyl ester content of 99.4% at the optimum conditions. Shieh et al.[5] conducted the optimization of lipase-catalyzed synthesis for biodiesel (soybean oil methyl ester) by Lipozyme IM-77. The maximum molar conversion obtained was 92.2%. Similarly, a study was realized using RSM in combination with principal-component analysis methods for

optimizing the enzymatic transesterification of rapeseed oil methyl esters[11]. Dermikol et al.[7] reported the optimization of enzymatic methanolysis of soybean oil by RSM using Lipozyme RM IM.

The objective of this work was to optimize the ethanolysis of soybean oil by lipase from *Thermomyces lanuginosus* in a solvent-free system using central composite design and response surface methodology analysis. The reaction parameters (reaction time, temperature, substrate molar ratio, enzyme concentration, and added water content) were studied to evaluate the relationship among them and the percentage yield of conversion.

Experimental

Materials

Lipase from *Thermomyces lanuginosus* (Lipolase 100L) was a gift of Novozymes Latin America (Araucária, Paraná, Brazil). Refined soybean oil was purchased in a local market. Ethanol and other chemicals were of analytical grade.

Synthesis and analysis

Different molar ratios of ethanol were added to 2.75 mmol of soybean oil into 50 mL Erlenmeyer flasks, followed by the addition of different amounts of water and enzyme. The mixtures of soybean oil, ethanol and Lipolase 100L were stirred in an orbital shaker (200 rpm) at different reaction temperatures and reaction times (Table 2). After finishing the reaction 5 mL of distilled water were added followed by a centrifugation (2,453 g, 15 min, 4 °C). The lower phase, containing glycerol, was analyzed by HPLC. In order to verify that the glycerol could be related to the liberation of esters, free fatty acids in the soybean oil and in the product reaction were periodically monitored by titration[12]. This was necessary to show that hydrolysis reaction was not favored instead of the desired transesterification.

Glycerol concentration was determined by HPLC with a refractive index (RI) detector (Perkin Elmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300 x 7.8 mm), at 80 ºC, using ultrapure water as eluent, flow of 0.6 mL.min^{-1} and sample volume of 20 µL. The percentage yield conversion was calculated as follows:

$$Conversion \quad yield = \left[\frac{mmol\ glycerol}{mmol\ initial\ soybean\ oil} \right] * 100\ \% \qquad (1)$$

Experimental design

A five-level-five-factor CCD was employed to obtain the optimum conditions for biodiesel synthesis. The variables and the coded and uncoded values of the variables at various levels are given in Table 1.

Table 1: Process variables and their levels used in CCD

Variables	Name	Coded Levels				
		-2.38	-1	0	1	2.38
X_1	Temperature (°C)	15	23.7	30	36.3	45
X_2	Time (h)	3.2	6	8	10	12.8
X_3	Substrate Molar Ratio (ethanol: soybean oil)	3	6.5	9	11.5	15
X_4	Enzyme Content (% by weight of oil)	5	9.3	12.5	15.7	20
X_5	Added Water (% by weight of oil)	0	5.8	10	14.2	20

Table 2 shows 50 treatments of the five variables, each at five levels. The design was made up of 32 factorial points, 10 axial points (two axial points on the axis of design variable) and eight replications of the central point. In each case, the percentage yield conversion was determined. Second-order polynomial equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^{\,2} \qquad (2)$$

where Y is the response variable, β_0 the constant, β_i the coefficient for the linear effect, β_{ii} the coefficient for the quadratic effect, β_{ij} the coefficient for the interaction effect, X_i and X_j the coded level of variable x_i and x_j. The above quadratic equation was used to plot surfaces for the variables.

Statistical analysis

The experimental design and results analysis were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, p(t), were determined by Student's t-test; the second order model equation significance was determined by Fisher's F-test. The variance explained by model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

Results and Discussion

Model fitting and ANOVA

Experimental data for lipase-catalyzed synthesis of biodiesel from soybean oil and ethanol in a solvent-free system are given in Table 2. Among the various treatments, the highest yield conversion (99.98%) was obtained in treatment 10 (23.7 °C, 10 h, 6.5:1 ethanol:oil, enzyme content 9.3%, added water 14.2%), and the smallest yield conversion (52.11%) was shown in treatment 39 (30 °C, 8 h, 9:1 ethanol:oil, enzyme content 5%, added water 10%). Most of the treatments presented yield conversions between 90-100%, showing that Lipolase 100L presented a good conversion rate in the ethanolysis of soybean oil. In a similar work, Chang et al.[6] found lower yield conversions with methanolysis of canola oil by immobilized lipase Novozym 435. Although the higher yield conversion value obtained by the

authors was around 96%, most values were in the range of 25-50%. Demirkol et al.[7] in the methanolysis of soybean oil found yield conversions in the range of 70-80%.

Table 2: Experimental design and results of CCD

Treatment	X_1	X_2	X_3	X_4	X_5	Yield Conversion (%)
1	-1	-1	-1	-1	-1	86.68
2	-1	-1	-1	-1	1	91.81
3	-1	-1	-1	1	-1	93.19
4	-1	-1	-1	1	1	94.16
5	-1	-1	1	-1	-1	60.97
6	-1	-1	1	-1	1	70.71
7	-1	-1	1	1	-1	90.04
8	-1	-1	1	1	1	93.93
9	-1	1	-1	-1	-1	98.96
10	-1	1	-1	-1	1	99.98
11	-1	1	-1	1	-1	97.67
12	-1	1	-1	1	1	96.49
13	-1	1	1	-1	-1	64.62
14	-1	1	1	-1	1	95.86
15	-1	1	1	1	-1	72.08
16	-1	1	1	1	1	94.53
17	1	-1	-1	-1	-1	81.84
18	1	-1	-1	-1	1	94.53
19	1	-1	-1	1	-1	88.73
20	1	-1	-1	1	1	92.88
21	1	-1	1	-1	-1	71.10
22	1	-1	1	-1	1	79.84
23	1	-1	1	1	-1	91.71
24	1	-1	1	1	1	98.25
25	1	1	-1	-1	-1	92.94
26	1	1	-1	-1	1	93.57
27	1	1	-1	1	-1	86.08
28	1	1	-1	1	1	93.44
29	1	1	1	-1	-1	75.63
30	1	1	1	-1	1	93.79
31	1	1	1	1	-1	91.92
32	1	1	1	1	1	93.70
33	-2.38	0	0	0	0	89.55
34	2.38	0	0	0	0	96.81
35	0	-2.38	0	0	0	68.83
36	0	2.38	0	0	0	81.39
37	0	0	-2.38	0	0	76.27
38	0	0	2.38	0	0	79.82
39	0	0	0	-2.38	0	52.11
40	0	0	0	2.38	0	91.93
41	0	0	0	0	-2.38	89.04
42	0	0	0	0	2.38	92.24
43 (C)	0	0	0	0	0	91.60
44 (C)	0	0	0	0	0	90.85
45 (C)	0	0	0	0	0	94.13
46 (C)	0	0	0	0	0	94.23
47 (C)	0	0	0	0	0	92.69
48 (C)	0	0	0	0	0	93.52

49 (C)	0	0	0	0	0	95.41
50 (C)	0	0	0	0	0	94.06

Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA). The computed *F*-value (5.17) was highly significant ($p<0.0001$). The goodness of a model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient ($R^2 = 0.80$) implies that the sample variation of 80% for biodiesel production is attributed to the independent variables, and can be explained by the model. The closer the value of R (correlation coefficient) to 1, better the correlation between the experimental and predicted values. Here the value of R (0.88) suggests a satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation. Linear, quadratic and interaction terms were significant at the 5% level. Therefore, the second-order polynomial model is given by:

$$Y = 93.969 + 0.820X_1 + 2.096X_2 - 3.137X_3 + 4.864X_4 + 3.253X_5 + 0.569X_1^2 - 2.625X_2^2 - 2.106X_3^2 - 3.172X_4^2 + 0.121X_5^2 - 0.517X_1X_2 + 2.755X_1X_3 - 0.282\ X_1X_4 - 0.413\ X_1X_5 - 0.305X_2X_3 - 2.965X_2X_4 + 0.926X_2X_5 + 3.479X_3X_4 + 2.242X_3X_5 - 1.293X_4X_5 \quad (3)$$

where Y is the percentage yield conversion, and X_1, X_2, X_3, X_4 and X_5 are the coded values of temperature, time, substrate molar ratio, enzyme content and added water, respectively.

Effect of parameters

The linear effects of the studied variables are presented in Table 3. The entire relationship between reaction variables and response can be better understood by examining the planned series of contour plots depicted in Fig. 1, which was generated from the predicted model by keeping constant the enzyme concentration (9.3, 12.5, and 15.7 %) and

temperature (23.7, 30 and 36.3 °C). The added water was fixed at the central point (10%) for all plots. It can be observed a similar behavior with the increase of the yield conversion from the beginning and the decrease after 8 h in all nine contour plots. This was observed by Shieh et al.[5] and Chang et al.[6], and could be probably a consequence of product inhibition of the alcoholysis reaction.

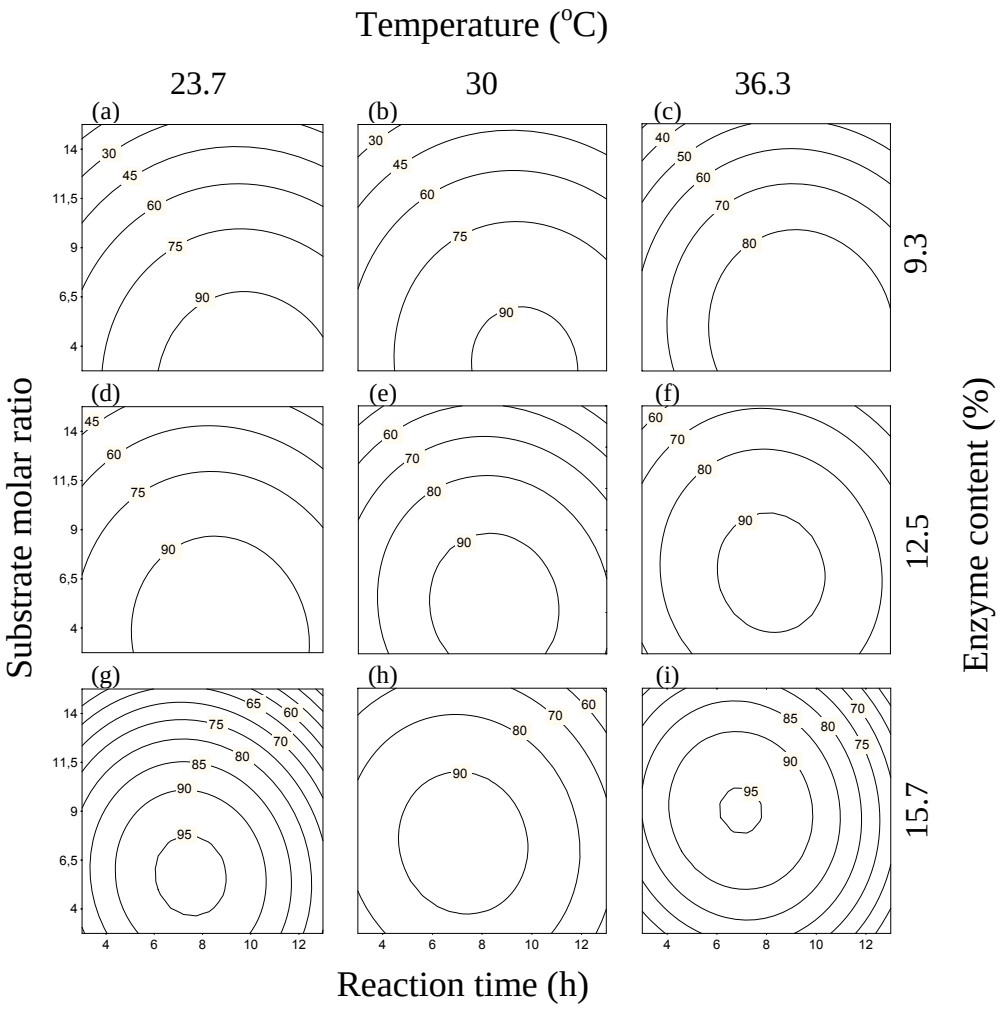

Figure 1. Contour plots of yield conversion in ethanolysis of soybean oil by Lipolase 100L in a solvent-free system. Added water fixed at 10%. The numbers inside the contour plots indicate yield conversions (%) at given reactions conditions.

Table 3: Linear effects estimated for the reaction parameters

Variables	Effect	p-value
Temperature	1.64	0.0089
Time	4.19	<0.0001
Substrate Molar Ratio	-6.27	<0.0001
Enzyme Content	9.72	<0.0001
Added Water	6.50	<0.0001

The yield conversion decreases when the substrate molar ratio increases, as can be seen by the negative effect (Table 3). The highest yield conversion occurred in between 4:1 - 6:1 ethanol:soybean oil molar ratio (Fig. 1). According to Salis et al.[2], a higher alcohol:triglyceride ratio means a higher polarity of the medium produced by both the alcohol and the water. A high polarity is often associated with the inactivation of the biocatalysts, as have been observed by Noureddini et al.[13], who has stated that the increase in alcohols concentration might inhibit the enzyme activity, thereby decreasing its catalytic activity on the transesterification reaction. However, an excess of the stoichiometric molar ratio of 3:1 was used to ensure higher reaction rates and minimize the diffusion limitations.

The effect of temperature on ethanolysis of soybean oil was the lowest compared with the other reaction parameters. A preliminary study showed that temperatures higher than 50 °C caused a thermal inactivation of the enzyme leading to low yield conversion (data not shown), thus it was defined the higher level at 45 °C. Other authors[2,5,13] observed similar behavior, with temperature presenting no significant effect in the range between 20-40 °C, indicating that, although temperature is an important parameter that affect the economic feasibility of biodiesel synthesis, its variation had low effect in the yield conversion.

Overall, all nine contour plots shown in Fig. 1 indicated that predicted yield conversion increases with higher enzyme concentrations. In practice, increasing enzyme content gave higher yield conversions[6], as observed by the positive effect of this variable (Table 3). As the interaction effect between enzyme content and substrate molar ratio was highly significant

(X_3X_4 = 6.96, p<0.0001) it should always be considered and Fig. 2 shows the effect of these parameters on the response. When the substrate molar ratio was fixed at the lower level the increase in the enzyme concentration presents no effect in the yield conversion. However, when the substrate molar ratio was fixed at its upper level, increasing the enzyme content leads to a higher yield conversion. Presence of larger amounts of substrates generally increase the probability of substrate-enzyme collision, and increasing amount of enzyme will lead to an increased yield conversion. This relationship holds when there are no limiting factors such as a low substrate concentration, presence of activators or inhibitors or mass transfer effect[10].

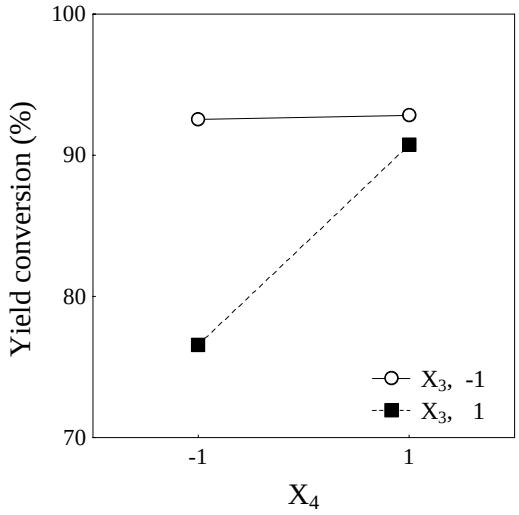

Figure 2. Effect of substrate molar ratio and enzyme content on the response

The added water presented a positive effect in the biodiesel synthesis (X_5 = 6.5). Lipase activity generally depends on the available interfacial area. With the increased addition of water, the amount of water available for oil to form oil–water droplets increases, thereby, increasing the available interfacial area[13]. Nevertheless, the control of the water content is important for several reasons: water acts as a “lubricant”, maintaining the enzyme in the active conformation; water participates in many mechanisms that cause enzyme inactivation; water can promote the hydrolysis of the substrate thus decreasing the yield of products[2].

Optimal conditions

The optimal conditions of lipase-catalyzed biodiesel synthesis were predicted using the critical values function of the Statistica 7.0 software. The coded critical values were: X_1 = 0.25; X_2 = -0.34; X_3 = -0.59; X_4 = 0.88; X_5 = -1.44. The uncoded optimal conditions for enzymatic ethanolysis of soybean oil were: Temperature = 31.5 °C; Reaction time = 7 h; Substrate Molar Ratio = 7.5:1 ethanol:soybean oil; Enzyme content = 15%; Added water = 4%. At the optimum conditions, the predicted yield conversion was 94.4%. Fig. 3 shows the contour plot of reaction time and substrate molar ratio, keeping fixed the other variables at the optimum level. The dashed lines represent the optimal levels for the parameters represented in the axis.

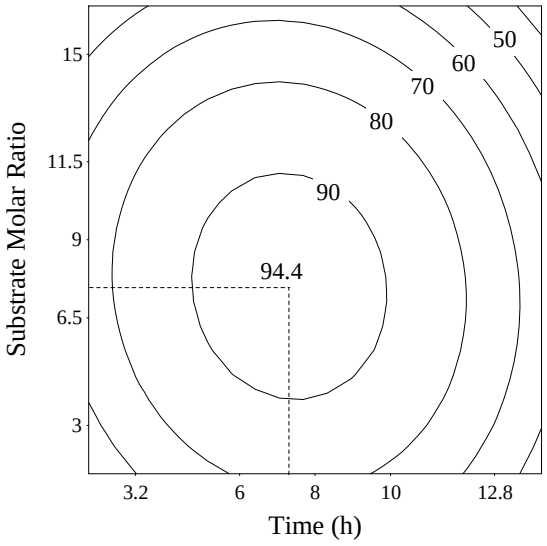

Figure 3. Contour plot of substrate molar ratio versus reaction time. The other variables were fixed at the optimal level.

Time course and model validation

In order to validate the model, experiments were run at the optima conditions realized and Fig. 4 presents the time course of lipase-catalyzed biodiesel synthesis. Samples were taken along the time to accompany the ethyl ester synthesis. It was observed that 5 h is enough to obtain high levels of yield conversion. This is relevant because the reaction time is

considered an important parameter for lipase-catalyzed biodiesel production as indicator of effectiveness and economical performance[6]. At 7 h, optimal condition predicted by the model, the yield conversion was 96 ± 2%, showing a good correlation of the experimental results with the statistical predicted by the model (94.4%).

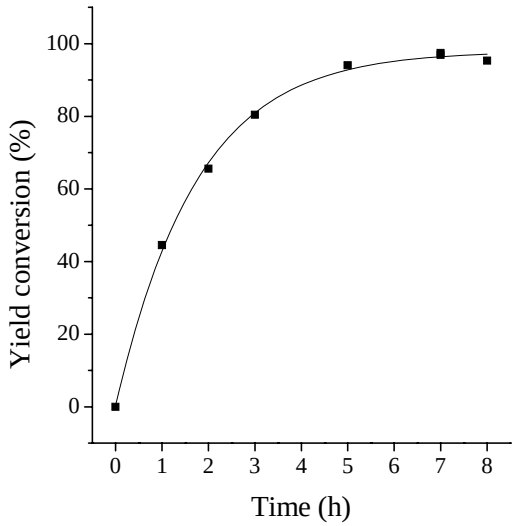

Figure 4. Time course for lipase-catalyzed biodiesel synthesis at the optimum conditions.

Conclusions

Central Composite Design and Response Surface Methodology analysis were successfully employed to optimize the ethanolysis of soybean oil by lipase from *Thermomyces lanuginosus* in a solvent-free system. A high yield conversion was obtained at the optimized conditions, with relative low enzyme content and short time. The comparison of predicted and experimental values showed good correspondence between them implying that empirical model derived from RSM can be used to adequately describe the relationship between the reaction parameters and the response (yield conversion) in lipase-catalyzed biodiesel synthesis.

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CAPÍTULO III - IMPROVED ENZYME STABILITY IN LIPASE-CATALYZED SYNTHESIS OF FATTY ACID ETHYL ESTER FROM SOYBEAN OIL

Neste trabalho estudou-se a otimização das condições da reação enzimática de síntese de biodiesel catalisada pela lipase imobilizada de *Thermomyces lanuginosus* (Lipozyme TL-IM). Aqui, o tempo da reação foi fixado, e foram avaliados a temperatura, a razão molar de substrato e a quantidade de enzima e água adicionadas. Primeiramente foi feita uma triagem nessas variáveis através de um planejamento fatorial fracionário, e a seguir com as variáveis selecionadas, determinaram-se as condições ótimas através de um delineamento composto central rotacional e da metodologia de superfície de resposta. Finalmente, foram avaliados diferentes tratamentos para aumentar a vida útil da lipase imobilizada. Os resultados estão apresentados no manuscrito a seguir, publicado no periódico *Applied Biochemistry and Biotechnology*, v. 152, p. 394-404.

Improved enzyme stability in lipase-catalyzed synthesis of fatty acid ethyl ester from soybean oil

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Improved enzyme stability in lipase-catalyzed synthesis of fatty acid ethyl ester from soybean oil

Abstract

In this work we describe the optimization of the ethanolysis of soybean oil by the enzyme Lipozyme™ TL-IM in the lipase-catalyzed biodiesel synthesis and the improvement of the enzyme stability over repeated batches. The studied process variables were: reaction temperature, substrate molar ratio, enzyme content, and volume of added water. Fractional factorial design (FFD) was used to analyze the variables in order to select those with higher influence on the reaction and then perform a central composite design (CCD) to find the optimal reaction conditions. The optimal conditions found were: temperature, 26 °C; substrate molar ratio, 7.5:1 (ethanol: oil); enzyme content, 25 % in relation to oil weight; and added water, 4 % in relation to oil weight. Under these conditions, the yield conversion obtained was 69 % in 12 hours. The enzyme stability assessment in repeated batches was carried out by washing the immobilized enzyme with different solvents (n-hexane, water, ethanol and propanol) after each batch. In the treatment with n-hexane, around 80 % of the enzyme activity still remains after seven cycles of synthesis suggesting its economical application on biodiesel production.

Keywords: biodiesel; lipases; response surface methodology; enzyme stability; organic solvents.

Introduction

Biodiesel is a mixture of mono-alkyl esters obtained from vegetable oils extracted from plants such as soybean, jatropha, rapeseed, palm, sunflower, corn, peanut, canola and cottonseed, among others, and is considered as a carbon neutral fuel emission due to the fact that the carbon generated by its burnt in the exhaust of motors is originally fixed from the atmosphere (*1*). The reaction can be either catalyzed by inorganic compounds such as acids, alkalis and salts, or by biological catalysts, the lipases enzymes. (*2-4*).

The use of lipases, which allow for mild reaction conditions and easy recovery of glycerol, largely prevent the drawbacks encountered with chemical catalysis, such as, high energy consumption, difficulty in glycerol recovery, and a high amount of alkaline waste water from the catalysts (*5*). Several reports describe lipase-catalyzed alcoholysis reactions in solvents and solvent-free media, especially the reaction parameters affecting the rates of lipase activities in alcoholysis reactions (*4, 6-11*).

Several aspects will have impact on the enzymatic synthesis of biodiesel, such as: enzyme content, molar ratio between reagents oil and alcohol, type of alcohol, use of organic solvent in the reaction, amount of added water in the reaction mixture, reaction temperature, and reuse of the enzyme in repeated operations. The optimization of the reaction parameters involved in lipase-catalyzed biodiesel synthesis is commonly made by varying one factor at a time and keeping the others constant, but this method is costly and inefficient, as it fails to explain relationships between the variables and the response when there is interaction between the variables. (*4, 8, 12*).

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results and it is a faster and less expensive method for gathering research data than the classical method (*13*). The use of RSM was reported by some authors for biodiesel enzymatic production (*9-11, 14*).

Concerning stability over time, enzymes will lose their activity due to a series of factors that must be addressed during enzymatic catalysis. Some of these are the enzyme leakage from supports in which they are attached when immobilized, inhibition by the substrate, thermal inactivation, or the loss of their spatial conformation (change in the active site). Therefore, in order to reduce biocatalysis cost and make it economically competitive, for an industrial use, compared to chemical catalysis, it is important to extend enzyme activity as long as possible, or keep it by the largest number of batch reactions (cycles) possible.

The aim of this work was to optimize the lipase-catalyzed ethanolysis of soybean oil through response surface methodology in a solvent-free system and to improve the stability of the immobilized enzyme in repeated batches. The studied reaction parameters were temperature, substrate molar ratio, enzyme content and added water, in order to evaluate their effects on yield conversion. The stability of the immobilized lipase during repeated batch runs was investigated by washing the immobilized lipases with some solvents, water, ethanol, propanol and n-hexane.

Material and Methods

Chemicals

A commercial immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL-IM) was kindly donated by Novozymes™ Latin America (Araucária, Paraná, Brazil) and used in all experiments. Refined soybean oil was purchased in a local market and used without any previous treatment. Ethanol and other chemicals were of analytical grade.

Synthesis and analysis

Different molar ratios of ethanol were added to 2.75 mmol of soybean oil into 50 mL Erlenmeyer flasks, followed by the addition of different amounts of water and enzyme. The mixtures of soybean oil, ethanol, lipase, and water were stirred in an orbital shaker (200 rpm)

at different reaction temperatures for 12 h according to the experimental design. After this time 5 mL of distilled water were added followed by centrifugation (2,500 g, 15 min, 4 °C). The lower phase containing glycerol was analyzed by HPLC. In order to verify that the glycerol could be related to the liberation of esters, free fatty acids in the soybean oil and in the product reaction were periodically monitored by titration with NaOH (*15*). This was necessary in order to show that the hydrolysis reaction was not favored instead of transesterifacation.

Glycerol concentration was determined by HPLC with a refractive index (RI) detector (Perkin Elmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300 x 7.8 mm), at 80 ºC, using ultrapure water as eluent, flow of 0.6 mL.min^{-1} and sample volume of 20 µL. The percentage yield conversion was calculated as follows:

$$Conversion \quad yield = \left[\frac{mmol\ glycerol}{mmol\ initial\ soybean\ oil} \right] * 100\ \% \qquad (1)$$

Experimental Designs

To determine the optimal conditions for ethanolysis reaction of soybean oil catalysed by Lipozyme TL-IM, we carried out a fractional factorial design 2^{4-1}, with the independent variables temperature, substrate molar ratio, enzyme content and added water, varying in two levels and four replications of the central point. Table 1 presents the variables with the respective levels used in the fractional factorial design (FFD).

Table 1: Process variables and their levels used in FFD 2^{4-1}

Variables	Coded Levels		
	-1	0	1
Temperature (^{o}C)	20	40	60
Substrate Molar Ratio (ethanol:Soybean oil)	3:1	7.5:1	12:1
Enzyme Content (% by oil wt.)	5	15	25
Added Water (% by oil wt.)	0	5	10

After selecting the variables with higher influence on transesterification, a central composite design (CCD) was employed to obtain the optimum conditions for biodiesel synthesis. The variables, along with their coded and uncoded values, are given in Table 2. In the CCD, each of the three selected variables were varied at five levels. The design was made up of eight factorial points, six axial points (two axial points on the axis of design variable) and four replications of the central point. In each case, the percentage yield conversion was determined. Second-order polynomial equation for the variables was as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (2)$$

where Y is the response variable, β_0 the constant, β_i the coefficient for the linear effect, β_{ii} the coefficient for the quadratic effect, β_{ij} the coefficient for the interaction effect, X_i and X_j the coded level of variable x_i and x_j. The above quadratic equation was used to plot surfaces for the variables.

Table 2: Selected variables and their levels used in CCD

Variables	Coded Levels				
	-1.68	-1	0	1	1.68
Temperature (^oC)	20	26	35	44	50
Enzyme Content (% by oil wt.)	5	10	17,5	25	30
Added Water (% by oil wt.)	0	4	10	16	20

* Substrate Molar Ratio was fixed at 7.5:1 (ethanol:soybean oil)

Statistical analysis

The experimental designs and results analysis were carried out using Statistica 7.0 (Statsoft, USA). The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). The significance of the regression coefficients and the associated probabilities, p(t), were determined by Student's t-test; the second order model equation

significance was determined by Fisher's F-test. The variance explained by model is given by the multiple determination coefficients, R^2. For each variable, the quadratic models were represented as contour plots (2D).

Enzyme Stability

After the transesterification reaction, the immobilized enzyme was separated from the reaction medium by simple filtration and submitted to different treatments before reused in a new reaction. The treatments were performed by washing with different solvents. The solvents were n-hexane, propanol, ethanol and water. The enzyme was washed with these solvents and then dried for 24 h at 40 °C. As a control, a parallel experiment was carried out without solvent washing.

Results and Discussion

Fractional Factorial Design

Experimental data and the matrix for the FFD for the optimal conditions for ethanolysis reaction of soybean oil catalysed by Lipozyme TL-IM are presented in Table 3. FFD consisted by eight factorial points and four replications at the central point. The highest yield of conversion (48.83 %) was obtained in treatment 2 (20 °C, 12:1 ethanol:soybean oil, enzyme content 25% and without added water). It was clearly observed the negative effect the temperature exerts over the enzyme activity, with the higher temperature (60 °C, treatments 5-8), producing low yields of conversion. In a FFD 2^{4-1}, the main effects can be calculated and used to indicate which variables must be included in the following design as well as to define the new levels of the variables.

Table 3: Experimental design and results of FFD 2^{4-1}

Treatment	Temperature	Substrate Molar Ratio	Enzyme Content	Added Water	Yield Conversion (%)
1	20	3:1	5	0	30.90
2	20	12:1	25	0	48.83
3	20	12:1	5	20	13.57
4	20	3:1	25	20	26.89
5	60	12:1	5	0	4.47
6	60	3:1	25	0	7.47
7	60	3:1	5	20	4.55
8	60	12:1	25	20	4.73
9 (C)	40	7.5:1	15	10	6.49
10 (C)	40	7.5:1	15	10	8.63
11 (C)	40	7.5:1	15	10	6.39
12 (C)	40	7.5:1	15	10	8.32

Table 4 shows the estimated main effects and their p-values. Temperature, enzyme content and added water presented significant effects ($p<0.1$) and were selected to be optimized in the CCD, but only the enzyme content positively affected the transesterification reaction, which means that an increase in this variable leads to an increase in yields conversion.

Table 4: Statistical analysis of FFD 2^{4-1}

Variables	Effect	p-value
Temperature*	-13.63	0.0253
Substrate Molar Ratio	-2.54	0.6130
Enzyme Content*	9.40	0.0916
Added Water*	-10.25	0.0707

* Statistically significant at 90% of confidence level

Central Composite Design

Central composite design is used to find the optimal conditions that maximize the yield conversion of the lipase-catalyzed transesterification reaction for biodiesel synthesis. As in the initial screening of the variables, the substrate molar ratio showed no significant effect, this variable has been fixed in its central point (7.5:1 - ethanol:soybean oil) for all experiments in CCD. The option for this combination, which is above the stoichiometric molar ratio of 3:1, is justified by the fact that an excess of alcohol ensures higher reaction rates and minimizes diffusion limitations (*16*). Moreover, the presence of larger amounts of substrate generally increases the probability of substrate-enzyme collision leading to an increased yield conversion (*13*). Table 5 shows the 18 treatments of the three selected variables, and the percentage yield conversion for each experiment.

Table 5: Experimental design and results of CCD

Treatment	Temperature	Enzyme Content	Added Water	Yield Conversion (%)
1	26	10	4	32.82
2	44	10	4	6.83
3	26	10	16	25.13
4	44	10	16	3.44
5	26	25	4	72.93
6	44	25	4	8.99
7	26	25	16	51.05
8	44	25	16	5.99
9	35	5	10	12.17
10	35	30	10	24.99
11	35	17.5	0	59.46
12	35	17.5	20	22.11
13	20	17.5	10	24.81
14	50	17.5	10	3.03
15 (C)	35	17.5	10	19.74
16 (C)	35	17.5	10	22.73
17 (C)	35	17.5	10	20.12
18 (C)	35	17.5	10	19.19

The higher yield conversions were obtained in treatments 5, 11 and 7 respectively, with conversions of 50 % or more. Some authors (*10,11*), who showed higher yields of conversion, have carried the transesterification out in the presence of organic solvents. Although in some cases the presence of organic solvents in the reaction can favor the conversion, it represents a further step in the biodiesel purification process thus impacting the costs of its production.

Model fitting and ANOVA

The experimental data have been adjusted to the proposed model by the second-order polynomial equation (2), and the adequacy of the model was performed by analysis of variance and the parameters R and R^2. The second-order polynomial model is presented in equation (3).

$$Y = 1.660 + 1.676X_1 - 0.026X_1^2 + 5.521X_2 - 0.007X_2^2 - 6.627X_3 + 0.210X_3^2 - 0.114X_1X_2 + 0.053X_1X_3 - 0.038X_2X_3 \quad (3)$$

where Y is the percentage yield conversion, and X_1, X_2, and X_3, are the uncoded values of temperature, enzyme content and added water, respectively.

Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA). The computed *F*-value (5.96) was highly significant (p=0.009). The goodness of a model can be checked by the determination coefficient (R^2) and correlation coefficient (*R*). The determination coefficient (R^2 = 0.87) implies that the sample variation of 87 % for biodiesel production is attributed to the independent variables, and can be explained by the model. The closer the value of *R* (correlation coefficient) is to 1, the better the correlation between the experimental and predicted values. Here, the value of *R* (0.93) suggests a satisfactory representation of the process model and a good correlation between the experimental results and the theoretical values predicted by the model equation.

Effect of parameters

Linear, quadratic and interaction effects for the variables temperature, enzyme content and added water are presented in Table 6. The variable that presented the highest effects was temperature, producing a negative effect on enzymatic transesterification reaction. This had already been seen in the FFD. Therefore, in the CCD, the range of this variable has been changed to a maximum of 50 °C, restricting the previous condition that was 60 °C. However, this change had not the desired effect, and we observed some thermal denaturation of the enzyme, causing a decrease in the yields of conversion.

Table 6: Statistical analysis of CCD

Variable	Effect	Standard error	p-value
Mean	20.373	0.783	0.0001
Linear			
X_1^*	-28.472	0.853	<0.0001
X_2^*	13.590	0.853	0.0005
X_3^*	-14.490	0.853	0.0004
Quadratic			
$X_1X_1^*$	-4.182	0.894	0.0184
X_2X_2	-0.827	0.894	0.4234
$X_3X_3^*$	15.161	0.894	0.0004
Interactions			
$X_1X_2^*$	-15.330	1.110	0.0008
$X_1X_3^*$	5.795	1.110	0.0136
X_2X_3	-3.450	1.110	0.0530

* Statistically significant at 95% of confidence level

The enzyme content shows a positive effect in the transesterification reaction, indicating an increase in the yield conversion with the increase of the amount of enzyme. Because the enzyme has a very high cost, it is important to determine the appropriate amount to obtain high yield conversions, but there is a limit above which the increase in

enzyme content will not affect product formation and reaction rate remains constant. Among the limiting factors, low substrate concentration, presence of activators or inhibitors, and mass transfer effects are the most important (*13*).

Although water does not participate in the alcoholysis reactions, the control of the water content is important for several reasons: water acts as a “lubricant”, maintaining the enzyme in the active conformation; water participates in many mechanisms that cause enzyme inactivation; water promotes the aggregation of particles of enzyme; at high water content may occur limitations of diffusion of substrate; water can promote the hydrolysis of the substrate thus decreasing the yield of products (*8*). In our work, the amount of added water presented negative effect on the transesterification reaction, which means that the increase in the water content did not favored the synthesis of biodiesel, decreasing the reaction efficiency. The optimum content of water will be, therefore, a combination of minimizing the reaction of hydrolysis and maximize the enzyme activity for the transesterification reaction (*16*).

The relationship between reaction variables and response can be better understood by examining the series of contour plots depicted in Figures 1a, 1b, and 1c, which were generated from the predicted model.

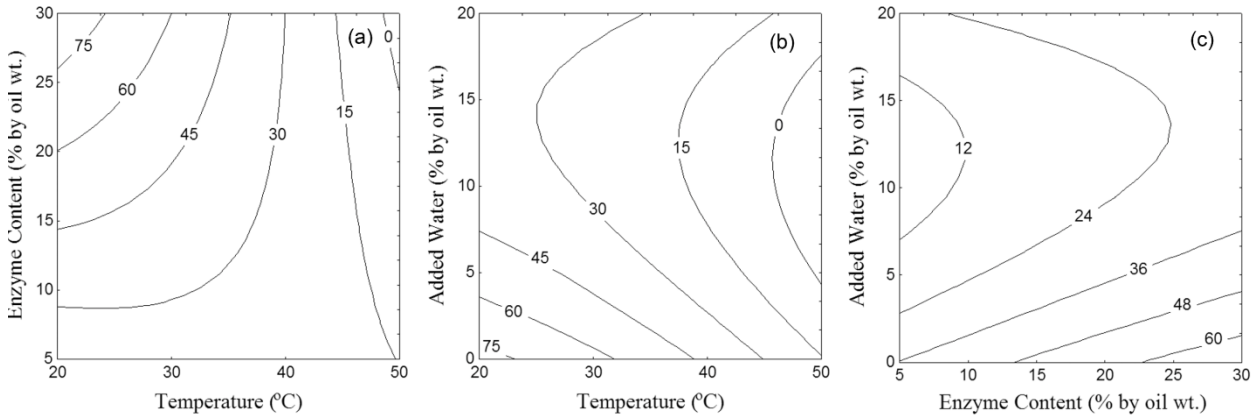

Figure 1. Contour plots of yield conversions of ethanolysis of soybean oil by Lipozyme TL-IM in a solvent-free system. (a) Temperature vs Enzyme Content; (b) Temperature vs Added Water; (c) Enzyme Content vs Added Water. The numbers inside the contour plots indicate yields of conversion (%) at given reaction conditions. In each figure, the missing variable was fixed at the central point.

Figure 1a shows that increasing enzyme content and lowering temperature will have a positive effect in the yield of reaction. High temperatures showed to have no influence on the yield conversion, even when combined with the increase of enzyme concentration. Figure 1b shows that the combined increase of temperature and added water caused a decrease in yields of conversion. Finally, results depicted in Figure 1c shows that the increase in the concentration of water has antagonistic effects in the yields of conversion when compared to the concentration of enzyme.

Optimal Conditions and Model Validation

Thus, the optimal conditions for biodiesel synthesis catalyzed by Lipozyme TL-IM were found to be as: temperature 26 °C; enzyme content 25 %; added water 4%; and a substrate molar ratio, which had been previously defined, 7.5:1 ethanol:soybean oil. Under these conditions, the theoretical value for the yield of reaction predicted by the model is 66.01 %. For the validation of the proposed model, an experiment was conducted under optimized conditions. The test was conducted with four repetitions and the average yield with the standard deviation obtained was 69.04 ± 1.34%, showing good correlation between the experimental results and the statistical predicted by the model. Figure 2 presents the time course of lipase-catalyzed biodiesel synthesis. Samples were taken along the time to accompany the ethyl ester synthesis. It was observed that after 12h, the yield of conversion remains constant.

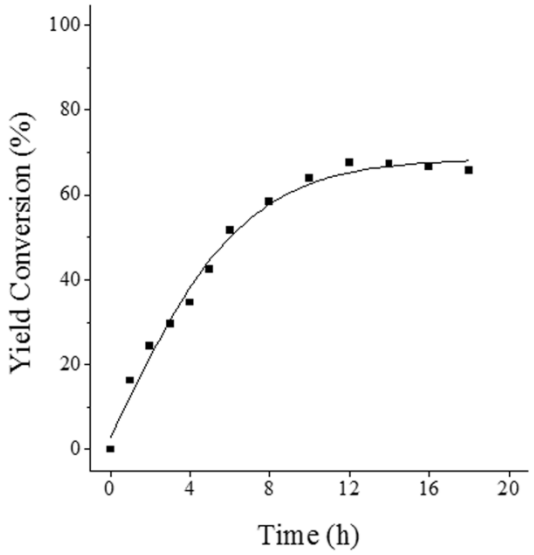

Figure 2. Time course for lipase-catalyzed biodiesel synthesis at the optimum conditions. Conditions were: temperature 26 °C; enzyme content 25 %; added water 4%; and ethanol:soybean oil molar ratio 7.5:1. Experimental points represent the mean of three experiments.

Enzyme Stability

Immobilized enzyme presents the advantage that it can be reused for several times, but its activity decreases along repeated batches due to many factors such as desorption. Therefore, we tried to improve the stability of Lipozyme TL-IM by washing the immobilized system with different solvents after every batch of synthesis. The experiments were done using the optimal conditions previously obtained. Figure 3 shows the results of the relative yield conversions of treatments with ethanol, propanol, n-hexane, water, and the control, as percentage of the first batch reaction. For all polar solvents (ethanol, propanol and water), the enzyme activity remaining after seven batches was approximately of 25 %. In the treatment with n-hexane, the only non-polar solvent tested, around 80 % of the enzyme activity was still maintained in relation to the first batch. During the repeated uses of the lipase, it was also observed that a substrate/product layer gradually formed on the surface of the enzymatic support, which could cause the loss of activity by limiting substrate and

product diffusion (*3*). As the main components of the mixture are non-polar (oil/biodiesel), the use of a non-polar solvent to wash the immobilized lipase helps to remove the substrate/product layer formed on the enzyme surface.

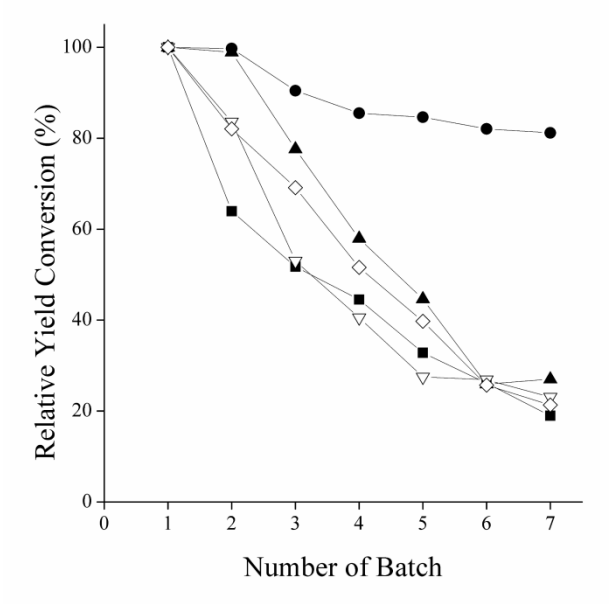

Figure 3. Stability of Lipozyme TL-IM over repeated batches submitted to different treatments. (■) Control; (●) Hexane; (▲) Ethanol; (▽) Propanol; (◇) Water.

Conclusions

The process of biodiesel synthesis can be either achieved by chemical or enzymatic catalysis. While the chemical process has the advantage of being cheaper, it produces highly polluting streams and several operations are required in order to avoid environmental contamination and reuse of its by-products. In contrast, enzymatic bioconversion is cleaner but more expensive at present. Therefore, research must be forward in order to make this technology economically feasible. The optimization of ethanolysis of soybean oil by Lipozyme TL-IM in a solvent-free system through the response surface methodology was successfully obtained in this work and it is an important step towards process improvement. Under the optimized conditions tested by us, a high yield of conversion of 69 % was achieved. Moreover, the comparison between the predicted values by the second-order

polynomial model of and the values obtained experimentally showed good agreement, showing that the empirical model derived from the RMS can be appropriately used to describe the relationships between the reaction parameters and the response and can, therefore, be used in the enzymatic biodiesel synthesis. Repeated batches of enzyme washes with n-hexane showed the high stability of the system, with activities of 80 % still remaining after seven cycles. Further research is granted to scale-up this model system and to check for its economical feasibility.

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CAPÍTULO IV - ENZYMATIC SYNTHESIS OF BIODIESEL FROM TRANSESTERIFICATION REACTION OF VEGETABLE OILS AND SHORT CHAIN ALCOHOLS

Neste trabalho foram avaliados diferentes óleos vegetais e álcoois de cadeia curta na reação de transesterificação catalisada por três diferentes lipases imobilizadas, assim como diferentes tratamentos para aumentar a estabilidade da lipase imobilizada no uso repetido na reação de transesterificação. Os resultados estão apresentados no manuscrito a seguir, publicado no *Journal of the American Oil Chemists' Society*, v. 85, p. 825-830.

Enzymatic synthesis of biodiesel from transesterification reaction of vegetable oils and short chain alcohols

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Enzymatic synthesis of biodiesel from transesterification reaction of vegetable oils and short chain alcohols

Abstract

Biodiesel synthesis by alcoholysis of three vegetable oils (soybean, sunflower and rice bran) catalyzed by three commercial lipases (Novozym 435, Lipozyme TL-IM and Lipozyme RM-IM), and the optimization of the enzymes stability over repeated batches is described. The effects of the molar ratio of alcohol to oil and the reaction temperature with methanol, ethanol, propanol and butanol were also studied. All three enzymes displayed similar reaction kinetics with all three oils and no significant differences were observed. However, each lipase displayed the highest alcoholysis activity with a different alcohol. Novozym 435 presented higher activity in methanolysis, at a 5:1 methanol:oil molar ratio; Lipozyme TL-IM presented higher activity in ethanolysis, at a 7:1 ethanol:oil molar ratio; and Lipozyme RM-IM presented higher activity in butanolysis, at a 9:1 butanol:oil molar ratio. The optimal temperature was in the range of 30 to 35 °C for all lipases. The assessment of enzyme stability over repeated batches was carried out by washing the immobilized enzymes with different solvents (n-hexane, water, ethanol, or propanol) after each batch. When washing with n-hexane, approximately 90 % of the enzyme activity remained after seven synthesis cycles.

Keywords: biodiesel; lipases; alcoholysis; vegetable oil; enzyme stability; organic solvents.

Introduction

Biodiesel is composed of a mixture of fatty acid alkyl esters. It is a natural substitute for petroleum-derived diesel fuel and has similar or better specifications concerning density, viscosity, cetane number, flash point, among others. Because biodiesel is formed from renewable resources such as plant oils, it is considered CO_2-neutral, biodegradable and will help conserve fossil fuels. Compared to traditional diesel fuels, its combustion leads to a substantial reduction in polluting emissions [1, 2].

Industrially, biodiesel can be produced by the transesterification of vegetable oils and short chain alcohols, usually methanol, with alkaline or acid catalysts. The reaction products are constituted of a mixture of the desired esters, mono- and di- glycerides, glycerol, water and the catalysts. Compared to the process mediated by enzymes, this process is more energy-consuming. Due to the presence of soap by-products separation and purification of the chemically produced biodiesel requires somewhat more complex steps than enzymatically produced biodiesel. Therefore, the use of biocatalysts could be an interesting alternative because it is more environmentally attractive because biodiesel synthesized enzymatically can be used directly without purification [3-5]. Lipase-catalyzed transesterification of vegetable oils has been investigated by many researchers in the last years [4-12].

For cost reasons, methanol is the alcohol most frequently used for triglyceride transesterification. Nevertheless, other alcohols are also used. In Brazil, one the biggest world plant oil producers, biodiesel is obtained by ethanolysis of triglycerides, since ethanol is a cheap and abundant commodity produced from the fermentation of sucrose from sugarcane. Alternatively, either propanol or butanol can also be used in this process, especially because these two alcohols promote a better miscibility between the alcohol and the oil phases [7].

The use of a triglyceride feedstock for biodiesel production depends on regional availability and economics and many vegetable oils can be used, such as soybean [3, 13-

15], sunflower [16, 17], and rapeseed [3]. The main differences among these oils are their fatty acid compositions, which strongly affects some important properties of the biodiesel (cetane number, heat of combustion, melting point and viscosity) [1]. Oxidation of biodiesel is a common problem, depending on the source of vegetable oil. For instance, rice bran, sunflower, and soybean oils contain high contents of linoleic acid with low resistance to oxidation as a result of the presence of two double bonds [18].

In enzymatic catalysis, enzymes will lose their activity due to a series of factors that must be addressed. These factors include leakage of enzyme from supports to which they are attached when immobilized; inhibition by the substrate; thermal inactivation; and the loss of their spatial conformation leading to changes in the active site. Therefore, in order to reduce the cost of biocatalysts and make lipase-catalyzed biodiesel manufacture economically competitive for industrial scale, it is important to extend enzyme activity as long as possible. This can be done by developing methods for catalyst re-use in as many reaction cycles as possible.

The purpose of this work was to study the alcohol-enzyme specificity, the enzyme stability, and the optimal alcohol:substrate molar ratio on the enzymatic synthesis of biodiesel. We evaluated the reactions of three vegetable oils (soybean, sunflower and rice bran) and different alcohols (methanol, ethanol, propanol and butanol) in alcoholysis catalyzed by three immobilized lipases. We also investigated the effects of the alcohol concentration and temperature on the lipase activity. The stability of the immobilized lipases during repeated batches was investigated by washing the immobilized lipases with the solvents water, ethanol, propanol, and n-hexane.

Materials and Methods

Chemicals

Refined soybean, sunflower, and rice bran oils were purchased in a local market and used without any previous treatment. The acrylic resin immobilized lipase from *Thermomyces lanuginosus* (Lipozyme™ TL-IM), the anion-exchange resin immobilized lipase from *Rhizomucor miehei* (Lipozyme™ RM-IM), and the macroporous resin immobilized lipase from *Candida antarctica* (Novozym™ 435) were kindly donated by Novozymes™ Latin America (Araucária, Paraná, Brazil) and used in all experiments. Methanol, ethanol, 1-propanol and 1-butanol and other chemicals were of analytical grade. All experiments were performed in duplicates.

Synthesis reaction

The reaction conditions were determined in a previous study [15]. To evaluate the different lipases, oils and alcohols, 2.5 g of each oil were mixed with each alcohol (7.5:1 alcohol:oil molar ratio), 15 % (based on oil weight) of each immobilized lipase and 4 % (based on oil weight) of water. The reactions were carried out in 50 mL Erlenmeyer flasks in an orbital shaker (200 rpm) at 30 °C for 6 h. After this time, 5 mL of distilled water were added to tube reactions and centrifuged at 2,500 g, 15 min, 4 °C. The lower phase containing glycerol was analyzed by HPLC. In the experiments to check the effects of alcohol concentration, the alcohol:oil molar ratio was varied from a stoichiometric ratio (3:1 molar ratio) to a large excess of alcohol (12:1 molar ratio). Temperatures were varied in the range of 20 °C to 50 °C, with 5 °C increments. In order to verify whether the glycerol could be related to the liberation of esters, free fatty acids in the vegetable oils and in the product reactions were periodically monitored by titration with NaOH [19]. This was necessary in order to quantify the degree of unwanted hydrolysis.

Enzyme Stability

After the transesterification reaction, the immobilized enzymes were separated from the reaction medium by filtration and submitted to different treatments before being reused. The treatments were performed by washing with different solvents. The solvents were n-hexane, propanol, ethanol, and water. The enzymes were washed with these solvents and after that dried for 24 h at 40 °C. As a control, a parallel experiment was carried out without solvent washing.

HPLC analysis

Glycerol concentration was determined by HPLC with a refractive index (RI) detector (Perkin Elmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300 x 7.8 mm), at 80 ºC, using ultrapure water as eluting solvent, flow of 0.6 mL.min^{-1} and sample volume of 20 µL. The percentage yield conversion was calculated as follows:

$$Conversion \quad yield = \left[\frac{mmol\ glycerol}{mmol\ initial\ oil} \right] * 100\,\% \qquad (1)$$

Results and Discussion

Control of hydrolysis

In order to determine whether undesired hydrolysis, instead of transesterification, was liberating glycerol during the formation of biodiesel, free fatty acid formation in the medium reaction was measured by titration in control hydrolysis (no alcohol added) and transesterification. This was tested for all three lipases and in Fig. 1 results are shown for the transesterification and control hydrolysis of soybean oil catalyzed by Novozym 435. This behavior was observed for all system reactions tested in this research. As can be seen, free fatty acids were formed during hydrolysis (control reaction) but never during

transesterification, indicating that it is possible to measure transesterification by the quantification of glycerol.

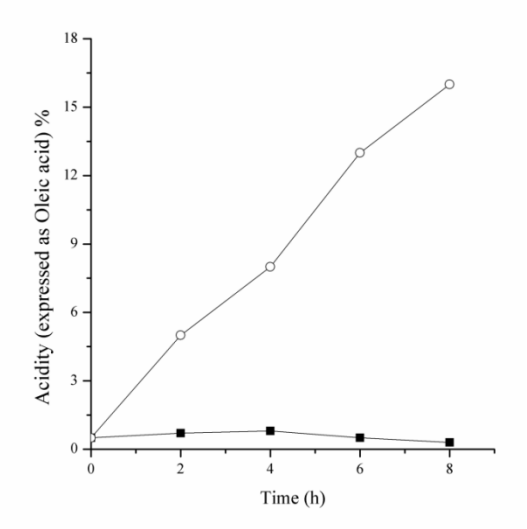

Figure 1. Free fatty acid formation during (■) transesterification and (○) hydrolysis of soybean oil catalyzed by Novozym 435. All reactions were carried out at T = 30 °C. Transesterification conditions: alcohol:oil molar ratio = 7.5:1; enzyme = 15%, water = 4%. Hydrolysis conditions: enzyme = 15%, water = 4%.

Screening of the alcohols and oils

Methanol is the most widely used alcohol in chemically catalyzed biodiesel production. Methanol is easily available and recoverable as an absolute alcohol. The high temperatures used in the chemical process improve the miscibility between methanol and oil. On the contrary, when a biocatalyst process is used, the relatively low temperature (25-35 ^{o}C) of the process does not allow for a good mixing system. Therefore, we tested four short chain alcohols (methanol, ethanol, propanol and butanol) in the transesterification reaction catalyzed by three different immobilized lipases in order to compare their performances. We also compared the alcoholysis of various vegetable oils at a fixed temperature and substrate:enzyme ratio, and the results are summarized in Fig. 2.

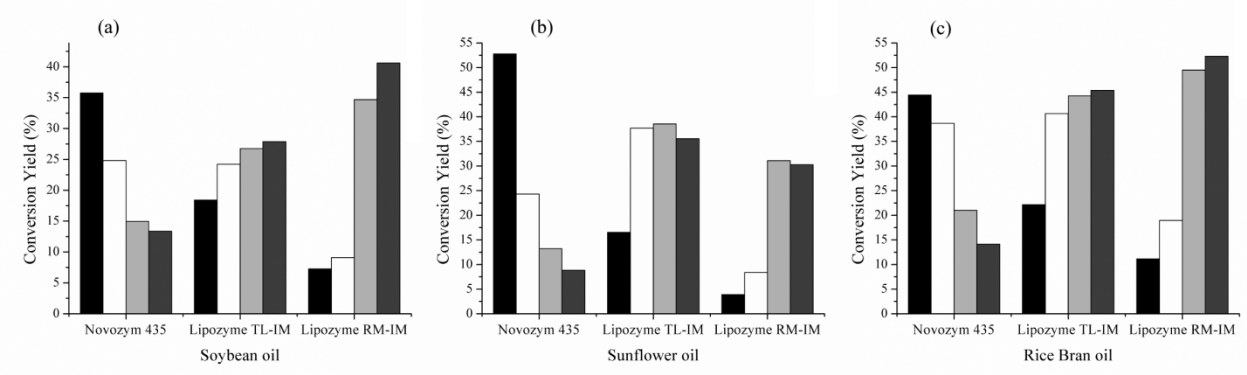

Figure 2. Alcoholysis of vegetable oils catalyzed by the different lipases. (a) Soybean oil; (b) Sunflower oil; (c) Rice bran oil. Black: methanol; White: ethanol; Light gray: propanol; Dark gray: butanol. All reactions were carried out at 30 °C, alcohol:oil molar ratio = 7.5:1, enzyme = 15%, water = 4% for 6 h.

Although the vegetable oils tested in this study have different fatty acid compositions [20], no significant differences were observed in the kinetics of their alcoholysis. The highest conversion yield was obtained in the transesterification with rice bran oil as compared with the soybean and sunflower oils. For enzymatic biodiesel production, almost all sources of triglycerides can be considered as enzyme substrates. The differences showed in the results may be due to the low viscosity of the rice bran oil, which facilitated the miscibility of the substrates in our solvent-free system. Each enzyme showed a different kinetic pattern depending on the alcohol used. Novozym 435 displays high activity in methanolysis, and the conversion yield was lower for other alcohols proportional to the increase in carbon chain length of the alcohol. Novozym 435 has been shown to be more active in the presence of low molecular weight alcohols, thereby facilitating its ability to catalyze methanolysis or ethanolysis reactions [21]. For Lipozyme TL-IM and Lipozyme RM-IM, the highest conversion yields were obtained with the higher molecular weight alcohols. Lipozyme TL-IM displayed no significant differences in reactions with ethanol, propanol, or butanol, and the conversion yield obtained with these alcohols were almost twice that obtained in methanolysis for all vegetable oils. Lipozyme RM-IM presented the highest conversion yield in butanolysis, possibly indicating that this enzyme is easily deactivated by substrates with low molecular

weight alcohols (methanol and ethanol). Soybean oil was chosen for subsequent experiments. There were no significant differences between the vegetable oils in the transesterification reaction, and soybean oil is cheap and widely produced. One alcohol was selected for experiments with each enzyme: methanol for the experiments with Novozym 435, ethanol for Lipozyme TL-IM, and butanol for Lipozyme RM-IM.

Effects of the concentrations of alcohols

In order to verify the effects of the concentration of alcohol in the transesterification reaction, we tested the alcohol:oil molar ratio from a stoichiometric ratio (3:1 molar ratio) to a large excess of alcohol (12:1 molar ratio) (Fig. 3). Above certain alcohol concentrations, the transesterification reactions were inhibited. In the reaction catalyzed by Novozym 435, the highest yield conversion was obtained for a substrate molar ratio of 5:1.. In the ethanolysis catalyzed by Lipozyme TL-IM, the highest yield conversions were obtained in the range of 7:1-8:1 in agreement with that previous work [15] on the optimization of ethanolysis of soybean oil catalyzed by a free lipase of *T. lanuginosus*. In the reaction catalyzed by Lipozyme RM-IM, large amounts of butanol (9:1) were necessary to obtain higher yields of conversions.

The use of an excess of alcohol is necessary to ensure high reaction rates, to minimize diffusion limitations, and to keep the glycerol formed during the reaction in solution. This will reduce the glycerol-mediated deactivation of the immobilized lipase which can take place when glycerol liberated in biodiesel synthesis blocks the entrance of catalyst pores [8, 21]. However, high ratios of alcohol to oil increase the polarity of the medium, and this is often associated with the inactivation of the biocatalysts [9]. From these results, the alcohol:substrate molar ratios selected were: methanol, 5:1; ethanol, 7:1; and butanol 9:1, for subsequent experiments with Novozym 435, Lipozyme TL-IM, and Lipozyme RM-IM, respectively.

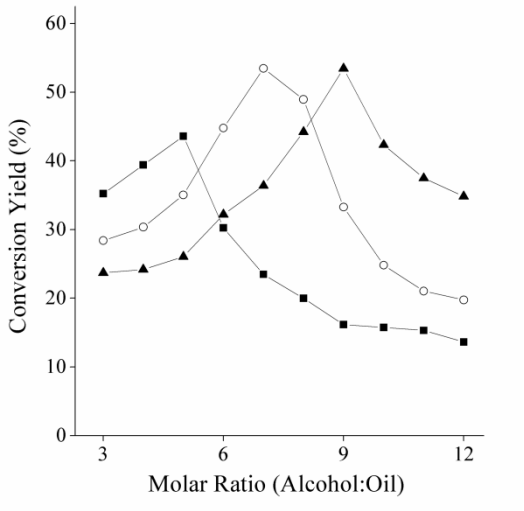

Figure 3. Effect of alcohol concentration in the transesterification of soybean oil. (■) Novozym 435, methanol; (○) Lipozyme TL-IM, ethanol; (▲) Lipozyme RM-IM, butanol. All reactions were carried out at 30 °C, enzyme = 15%, water = 4% for 6 h.

Effects of the temperature of reaction

The effects of temperature on the catalytic activity of the three immobilized lipases in the transesterification of soybean oil were investigated. Temperatures were varied in the range from 20 °C to 50 °C, with 5 °C increments at alcohol concentrations selected in the previous experiment. The highest conversion yields conversions were obtained in the range of 25-35 °C (Fig. 4). In all cases, catalytic activity decreased at temperatures above 40 °C, indicating a possible thermal deactivation of the biocatalysts. Because the inactivation of lipases is significantly greater at higher temperatures, we selected 30 °C for subsequent experiments with all the studied lipases.

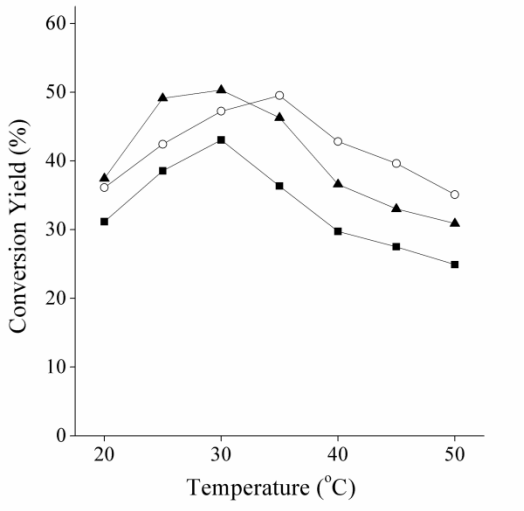

Figure 4. Effect of the temperature in the transesterification of soybean oil. (■) Novozym 435, 5:1 methanol:oil; (○) Lipozyme TL-IM, 7:1 ethanol:oil; (▲) Lipozyme RM-IM, 9:1 butanol:oil. All reactions were carried out with enzyme = 15%, water = 4% for 6 h.

Enzyme Stability

Immobilized enzymes present the advantage that they can be reused several times, but their activity eventually decreases due to many factors, such as desorption, substrate deactivation, and product inhibition. Therefore, we tried to improve the stability of the immobilized lipases after each use. After each transesterification reaction, the three lipases were recovered by filtration and washed with different solvents before being reused under the optimal conditions previously obtained. Figures 5 to 7 show the results of the relative yield conversions of the treatments with ethanol, propanol, n-hexane, water, and the control, expressed as percentage of the yield of the first batch reaction of each lipase preparation. In all cases, washing with n-hexane, the only non-polar solvent tested, caused greater retention of lipase activity than that obtained when washing with the polar solvents and the unwashed control. About 90 % of the activity of Novozym 435 was maintained over seven batch reaction, while in the control the enzyme was almost completely deactivated (Fig. 5).

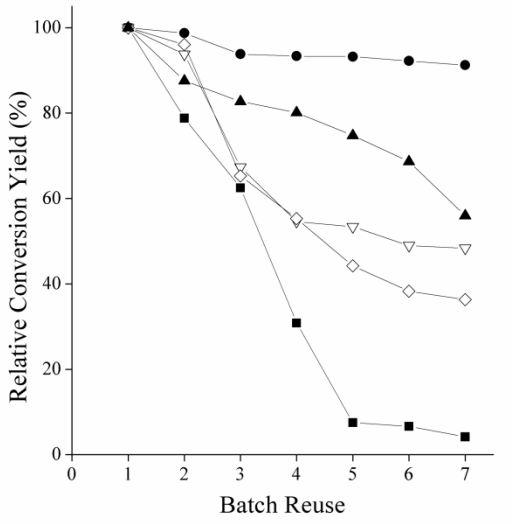

Figure 5. Stability of Novozym 435 over repeated batches submitted to different treatments. (■) Control; (●) Hexane; (▲) Ethanol; (▽) Propanol; (◇) Water. All reactions were carried out at 30 °C, 5:1 methanol:oil, enzyme = 15%, water = 4% for 6 h.

Lipozyme TL-IM and Lipozyme RM-IM retained 80 % and 75% of their initial activity after seven batches in the treatment with n-hexane, respectively (Figs. 6 and 7). After washing with ethanol and propanol, 60 % and 50 %, respectively, of lipase activity was retained in reactions catalyzed by Novozym 435 and Lipozyme RM-IM. In the ethanolysis catalyzed by Lipozyme TL-IM, for all polar washing solvents (ethanol, propanol and water) used, the enzyme activity remaining after seven batches was approximately 25 %.

During the repeated uses of lipases the formation of a separate layer was observed. On analysis, the layer was shown to be a heterogeneous mixture of oil and biodiesel. This substrate/product layer formed on the surface of the enzymatic support could cause loss of activity by limiting substrate and product diffusion [11]. As the main components of the mixture are non-polar (oil/biodiesel), the use of a non-polar solvent to wash the immobilized lipase helped to remove the substrate/product layer formed on the enzyme surface and to preserve the enzyme activity.

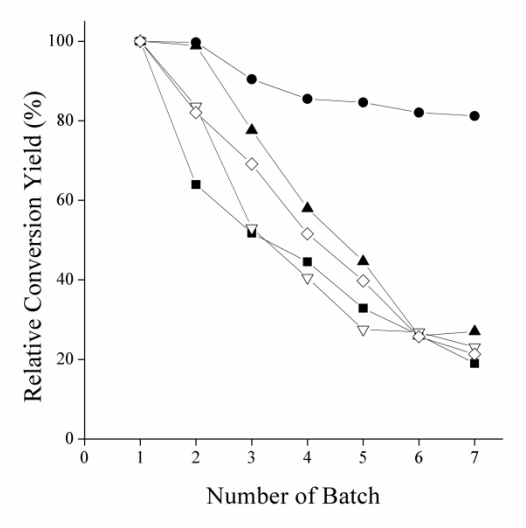

Figure 6. Stability of Lipozyme TL-IM over repeated batches submitted to different treatments. (■) Control; (●) Hexane; (▲) Ethanol; (▽) Propanol; (◇) Water. All reactions were carried out at 30 °C, 7:1 ethanol:oil, enzyme = 15%, water = 4% for 6 h.

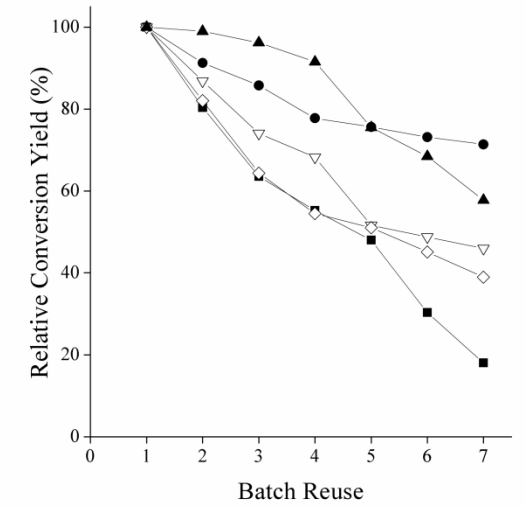

Figure 7. Stability of Lipozyme RM-IM over repeated batches submitted to different treatments. (■) Control; (●) Hexane; (▲) Ethanol; (▽) Propanol; (◇) Water. All reactions were carried out at 30 °C, 9:1 butanol:oil, enzyme = 15%, water = 4% for 6 h.

Acknowledgments

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CAPÍTULO V - IMMOBILIZATION-STABILIZATION OF THE LIPASE FROM *Thermomyces lanuginosus*: CRITICAL ROLE OF CHEMICAL AMINATION

Neste trabalho estudou-se a imobilização e estabilização da lipase de *Thermomyces lanuginosus*. A imobilização foi realizada através de ligação covalente multipontual em suportes glioxil agarose. Juntamente avaliou-se a aminação química da superfície da enzima para obter maior estabilidade quanto a inativação por temperatura ou solventes orgânicos. Os resultados estão apresentados no manuscrito a seguir submetido para publicação no periódico *Process Biochemistry*.

Immobilization-stabilization of the lipase from *Thermomyces lanuginosus*: critical role of chemical amination

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Immobilization-stabilization of the lipase from *Thermomyces lanuginosus*: critical role of chemical amination

Abstract

This paper describes the immobilization and stabilization of the lipase from *Thermomyces lanuginosus* (TLL) on glyoxyl agarose. Enzymes attach to this support only by the reaction between several aldehyde groups of the support and several Lys residues on the external surface of the enzyme molecules at pH 10. However, this standard immobilization procedure is unsuitable for TLL lipase due to the low stability of TLL at pH 10 and its low content on Lys groups that makes that the immobilization process was quite slow. The chemical amination of TLL, after reversible immobilization on hydrophobic supports, has been shown to be a simple and efficient way to improve the multipoint covalent attachment of this enzyme. The modification enriches the enzyme surface in primary amino groups with low pKb, thus allowing the immobilization of the enzyme at lower pH values. The aminated enzyme was rapidly immobilized at pH 9 and 10, with activities recovery of approximately 70%. The immobilization of the chemically modified enzyme improved its stability by 5-fold when compared to the non-modified enzyme during thermal inactivation and by hundreds of times when the enzyme was inactivated in the presence of organic solvents, being both glyoxyl preparations more stable than the enzyme immobilized on bromocyanogen.

Keywords: *Thermomyces lanuginosus* lipase, enzyme immobilization, enzyme stabilization, glyoxyl agarose, chemical amination, multipoint covalent attachment.

Introduction

The use of enzymes is essential in many industrial areas by different reasons, among other things because they catalyze the most complex reactions under the mildest experimental conditions [1]. However, enzymes, to be used as industrial biocatalysts, in many instances need to be improved. For example, enzymes usually require to be immobilized and stabilized in order to facilitate the design of the reactor and to allow for enzyme recovery and reuse [1-4].

The combination of immobilization and stabilization, would be extremely interesting. In this context, the enzyme immobilization via multipoint covalent attachment is one of the most powerful tools for this purpose [5, 6]. However, really many covalent attachments are not easily accomplished, requiring the selection of a suitable immobilization support and proper immobilization conditions [7]. Glyoxyl agarose beads have been shown to produce very high stabilization factors (from 2 to 5 magnitude orders) when used to immobilize several different enzymes [8]. The enzymes only become immobilized on the support when at last two simultaneous enzyme-support attachments are produced. Moreover, enzymes become immobilized on these supports only at pH values over 10, when the ε-amino of Lys residues present in their external surfaces are reactive enough [9]. Moreover, the degree of the enzyme-support multipoint covalent reaction is strongly dependent on the amount of reactive groups in both the support (aldehyde) and the enzyme (amines) [10].

The enrichment of enzyme surfaces with amino groups through different techniques has been shown to successfully improve the process of multipoint covalent immobilization [10, 11]. For example, the chemical amination of the protein surface via reaction of the carboxylic groups, such as the side group of Asp and Glu, with ethylenediamine after activation with carbodiimide, is a well described and easy-to-control reaction [12-16]. More recently, the chemical amination of lipases reversibly immobilized on hydrophobic supports via interfacial activation has been reported to be a way of simplifying this chemical amination previous to its covalent attachment [17].

One further advantage of this chemical amination is the reactivity of the new amino group. The new amino group has a pK of 9.2 [18], much lower than the pK of the ε-amino of Lys (around 10.7). This allows to immobilize an aminated protein on glyoxyl supports at pH below 10 (e.g., pH 9). The possibility of immobilizing a protein at lower pH may be critical when the enzyme intended for immobilization presents a very low stability at pH 10.

In this research, this strategy was used to achieve a stabilized-immobilized preparation of the lipase from *Thermomyces lanuginosus* (previously *Humicola lanuginosa*) (TLL) on glyoxyl agarose beads. TLL is the enzyme responsible for the lipolytic activity of Lipolase®, a commercial lipase preparation supplied by Novozymes. This enzyme has been broadly used in many biotransformations [19-22]. Its structure has been solved at 3.25 Å [23] and more recently for another crystal form at 1.8 Å [24]. Lipases share a common fold of the alpha/beta-hydrolase type, and the structure usually contains a small alpha-helix or loop, referred to as lid, or flat which covers the active site pocket. This conformation is termed the closed conformation. When the lipase is adsorbed to an interface, the lid is displaced so that the active site becomes accessible to substrate. This conformation is termed the open conformation [25-27]. It maintains activity reasonably well at 55–60 ^{o}C [28]. This lipase tends to form bimolecular aggregates, being necessary to use diluted solutions of the enzyme in the presence of detergents to have the monomeric form of it [29].

Materials and Methods

Materials

Lipase from *Thermomyces lanuginosus* (TLL) was obtained from Novozymes (Denmark). Ethanolamine hydrochloride, 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), hexadecyltrimethylammonium bromide (CTAB), and p-nitrophenyl butyrate (p-NPB) were from Sigma. 1,4-Dioxane, and 1,2-ethylenediamine (EDA), were from Fluka. Octyl-sepharose CL-4B and cyanogen bromide activated Sepharose 4B (CNBr) were purchased from GE Healthcare (Uppsala, Sweden). Cross-linked agarose (10 BCL) was kindly donated by Hispanagar S.A. (Burgos, Spain) and its modification to glyoxyl agarose (activated with 200 μmols/g of support) was performed as described elsewhere [9]. 2,4,6-Trinitrobenzensulfonic acid (TNBS) was from Fluka. Other reagents and solvents were of analytical or HPLC grade.

Methods

Lipase enzymatic activity assay

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the released p-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C, using a spectrum with continuous magnetic stirring and a thermostatized cell. To initiate the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of pNPB activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPB/min (U) under the conditions described above. Supernatant of suspensions containing supports were obtained using a pipette-tip-filter, suspensions were assayed using cut-pipette-tips.

Purification of TLL

The enzyme was adsorbed on octyl-sepharose beads under continuous stirring in 10 mM sodium phosphate at pH 7.0, following a previously described procedure [30]. At different times, the activities of suspensions and supernatants were measured by using the pNPB assay. After enzyme adsorption, the lipase preparation was vacuum filtered using a sintered glass funnel and washed with an excess of distilled water. TLL was desorbed from octyl-sepharose by suspending the immobilized enzyme in a ratio of 1/10 (w/v) in 25 mM sodium phosphate at pH 7.0 containing 0.6% (v/v) of CTAB for 1 h at room temperature.

Chemical amination of immobilized TLL

In order to fully modify all exposed carboxylic groups of the protein, the following procedures were used [12-16]. A total of 1 g of immobilized lipase either covalently bound on CNBr agarose beads as described below, or through adsorption on octyl-sepharose beads was added to 10 mL of 1 M EDA at pH 4.75 under continuous stirring. Solid EDC was added to the suspension to a final concentration of 10 mM. After 90 min of gentle stirring at 25 °C, the immobilized-modified preparations were vacuum filtered using a sintered glass funnel and incubated for 4 h in 0.1 M hydroxylamine at pH 7 and 4 °C to recover the EDC-modified tyrosines [31]. The enzyme preparations were filtered and washed with 25 mM sodium phosphate at pH 7.5 and with an excess of distilled water. The aminated TLL (TLL-A) preparations, immobilized on CNBr or octyl-sepharose (used to obtain the soluble aminated enzyme) were stored at 4 °C.

Immobilization of TLL on CNBr-Activated Support

TLL was desorbed from octyl-sepharose as described in the purification section. The immobilization of TLL on CNBr-activated support (10 mg of the purified enzyme per g of support) was performed for 15 min at 4 °C and pH 7 in order to reduce the possibility of multipoint covalent attachments between enzyme and support [9]. During the immobilization

and further blocking of the support, the suspension was submitted to continuous gentle stirring. The enzyme-support reaction was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized TLL preparation was vacuum filtered using a sintered glass funnel and washed with abundant distilled water in order to remove any traces of detergent. This immobilized enzyme system was named CNBr-TLL. These preparations, with just some few enzyme-support bonds [7, 9] use to have a stability very similar to the free enzyme, therefore it is a good reference of the behavior of the soluble enzyme, but avoiding the problems of aggregations or other intermolecular problems.

Immobilization of TLL on Glyoxyl-Agarose Beads

TLL or TLL-A were desorbed from octyl-sepharose as previously described and the pH was adjusted to 9 or 10 with 1 M sodium bicarbonate to a final concentration of 100 mM of this buffer. The immobilized enzyme derivatives were prepared using 1 g of glyoxyl-support and 10 mL of purified TLL or TLL-A. The biocatalysts were prepared to obtain 10 mg of protein per g of support. The mixture was maintained at the desired temperatures during the desired times. Samples of supernatant and suspension were withdrawn and their activities and /or protein concentration determined using the Bradford`s method [32]. Reduced glyoxyl-agarose was used as a control in order to discard unspecific adsorptions. As reaction end-point, to transform the aldehyde in inert hydroxyl groups and the imine bonds in very stable secondary amino bonds, solid sodium borohydride was added to a concentration of 1 mg/mL to the immobilization suspension [33] and the mixture was maintained at 25 °C under gentle stirring. After 30 min, the immobilized and reduced derivatives were washed thoroughly with distilled water.

The glyoxyl biocatalysts prepared were the following: **Gx-TTL** was prepared by immobilization of TLL during 15 h at 4 °C and pH 10; **Gx(9/10)-TLL-A** was prepared by immobilization of TLL-A on glyoxyl-agarose at 25 °C and pH 9 (8 h) and further incubated at

pH 10 and 25 °C overnight; **Gx(10)-TLL-A** was prepared by immobilization of TLL-A during 20 h at 25 °C and pH 10.

Thermal Inactivation of Different TLL Immobilized Preparations

The different TLL preparations were incubated in 25 mM sodium phosphate at pH 7.0 and 70 °C. Samples were withdrawn at different times using a pipette with a cut-tip and under vigorous stirring to have a homogeneous biocatalyst suspension. The activity was measured by the pNPB assay, above described. The experiments were carried out in triplicates and the standard error was under 5%.

Inactivation of Different TLL Immobilized Preparations in the Presence of Organic Cosolvent.

Enzyme derivatives were washed with 60% dioxane/50 mM sodium acetate aqueous solution at pH 5 and 4 °C. Subsequently, the enzyme derivatives were resuspended in the same solution and incubated at 4 °C. Samples were withdrawn at different times, and the activity was checked. Experiments were carried out in triplicates, and standard error was under 5%.

Titration of the Amino Groups Using Picrylsulfonic Acid

Primary amines residues were titrated using the picrylsulfonic acid methodology [34, 35]. To perform these tests it was used the immobilized preparations of TLL and TLL-A adsorbed on octyl-sepharose. One hundred milligrams of each derivative was suspended in 0.4 ml of 100 mM sodium bicarbonate at pH 9. The suspension was incubated at 25°C, and 0.1 mL of picrylsulfonic acid (5% w/w solution) was added; after 60 minutes, the colored derivatives were filtered and washed with a saturated NaCl solution, distilled water, and with 100 mM sodium bicarbonate at pH 9. A total of 50 mg of the colored preparations were then resuspended in 2 mL of 100 mM sodium bicarbonate, pH 9, and their spectra were determined. As control, the enzyme-free support was treated in a similar way.

Results and Discussion

Immobilization of TLL on glyoxyl agarose

TLL is rapidly inactivated under the standard conditions of immobilization on glyoxyl agarose (pH 10 and 25ºC), [36] suggesting a very low stability of the enzyme under these conditions (Figure 1a). Moreover, it can be seen that the enzyme is slowly immobilized on the support , very likely as result of the low content of Lys residues of this protein, (only 7) [24] and its dispersion on the protein surface (Figure 2). At the end of the immobilization process, protein could not be found in the supernatant, suggesting the full immobilzaiiton of TLL. The decrease of the immobilization temperature to 4ºC (Figure 1b) allowed to slow down the rate of inactivation, and to obtain an enzyme preparation expressing around 30% of its initial activity. This was, however, a very slow process, taking 15 h to fully immobilize the enzyme. Curiously, the decrease of enzyme activity is more pronounced for the control solution, which was fully inactivated well before this time. This suggested that the immobilized TLL was already stabilized at pH 10 during the first stages of the immobilization, and that the enzyme–support reaction did not significantly affect the enzyme structure. However, at pH 9, the enzyme is fully stable, although the enzyme is not immobilized as expected [9] on the glyoxyl-support (Figure 1c).

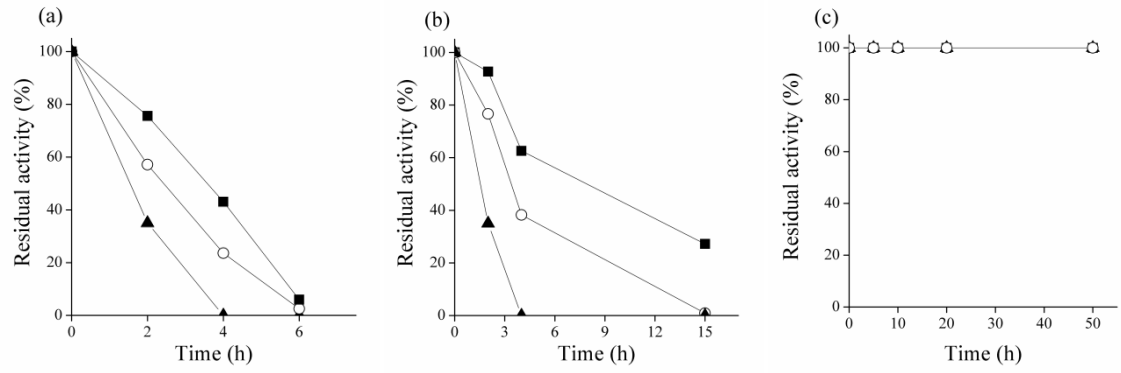

Figure 1. The time course of immobilization of TLL. (a) pH 10, 25 °C; (b) pH 10, 4 °C; (c) pH 9, 25 °C. (▲) Reference suspension; (■) immobilization suspension; (○) supernatant of the immobilization suspension.

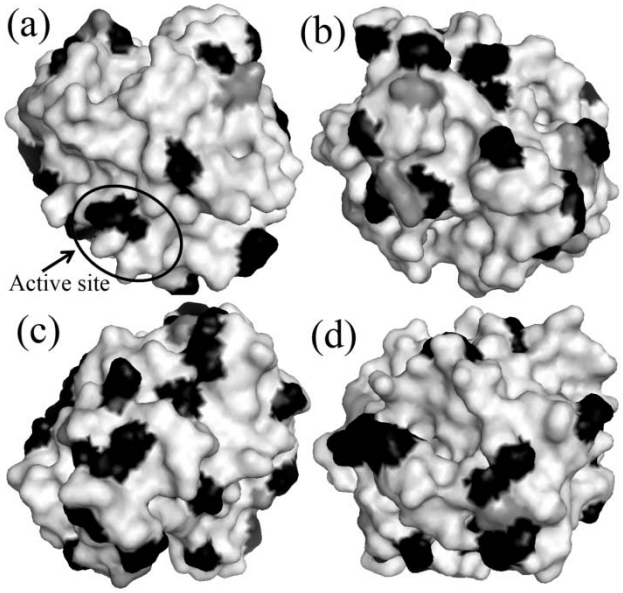

Figure 2. Distribution of Lys, Asp, and Glu residues on four faces of the surface of TLL. Lys are shown in grey; Asp and Glu are shown in black. (a) Front face showing the active site or external face of the lid; (b) 90° rotation of the first face; (c) 180° rotation of the first face; (d) 270° rotation of the first face. The 3D structure was obtained from the Protein Data Bank (PDB) using Pymol vs. 0.99. The pdb code for TLL is 1DTB.

Chemical amination: effect on TLL properties

The full chemical amination [11, 17] of the enzyme was then carried out in order to further improve these results by introducing more amino groups with a higher reactivity than the ε-amino groups of the Lys moieties, at lower pH values [18]. At first, the amination of covalently immobilized preparations was performed in order to check the effect of the amination on covalently immobilized preparations (as a reference for future comparisons). The full chemical amination of the immobilized enzyme increased the colour developed by the reaction with picrylsulfonic [35] by a 5 fold factor of the enzyme immobilized in either octyl-sepharose or CNBr TLL preparations beads (Figure 3). This result approximately fits with the 4 fold excess of carboxylic acids regarding amino groups: the number of external Lys was 7 (plus the terminal amino group) versus the number of external Asp and Glu (19 and 12, respectively) (and terminal carboxylic) (Figure 2) [23].

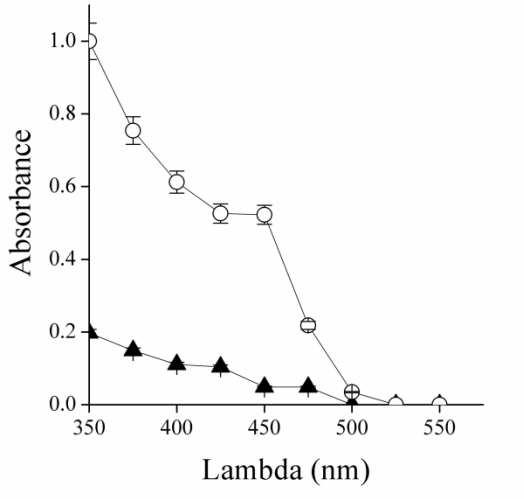

Figure 3. UV spectrum of different TLL immobilized preparations modified with TNBS. (▲): TLL immobilized on octyl agarose and then titrated with TNBS; (○) TLL-A immobilized on octyl agarose and then titrated with TNBS.

The enzyme activities of both TLL immobilized preparations were not apparently affected by this treatment (enzyme activity was 100% after the chemical amination under standard conditions).

However, the pH/activity profiles of modified and non-modified CNBr-TLL were quite different (Figure 4). The non-modified CNBr-TLL presented the highest activity at pH 9, while the modified enzyme presented its highest at the pH 10. The modified enzyme was 4 fold more active than the non-modified enzyme at pH 10. The activities were similar at pH 7, while at pH 5 the modified enzyme was nearly 5 times less active. These modifications in the enzyme activity/pH profile may be due to the changes in the surface physical properties of the lipase, that have been to greatly alter their properties by this kind of chemical modifications [37, 38].

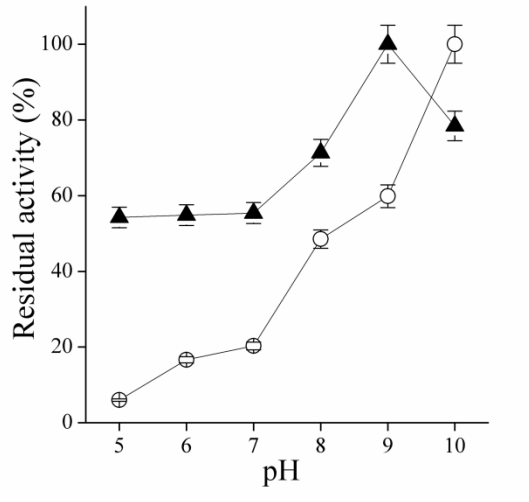

Figure 4. Effect of the chemical amination on the pH/activity profile of CNBr-TLL. Experiments were performed at 25 °C using pNPB as substrate. A solution of 25 mM sodium acetate /25 mM sodium phosphate /25 mM sodium borate was used as buffer. (▲) CNBr-TLL; (○) CNBr-TLL-A.

Figure 5 shows that the chemical amination of the TLL surface did not affect the stability of this immobilized enzyme during thermal inactivation or inactivation in the presence of solvents.

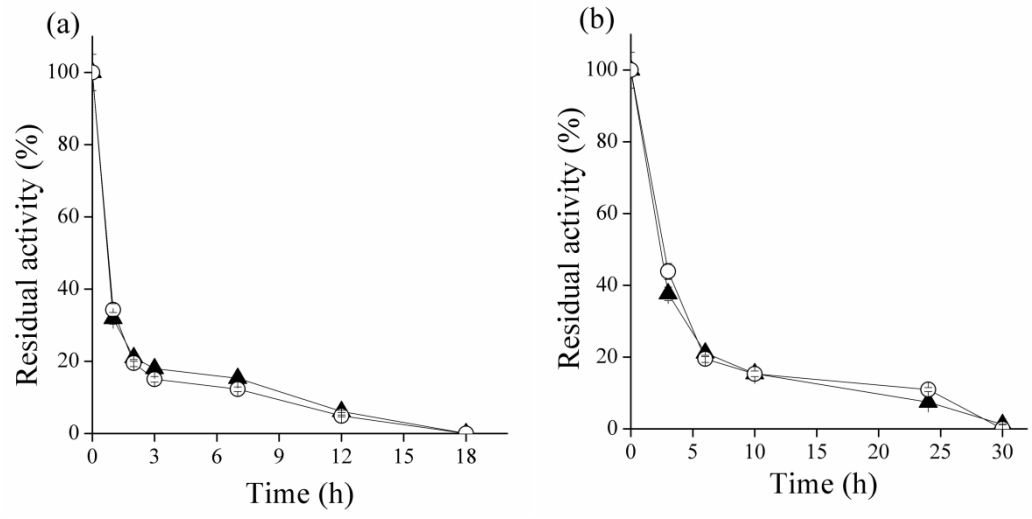

Figure 5. Effect of the chemical amination on the stability of CNBr-TLL. (a) Inactivation courses at 70 °C in 25 mM sodium phosphate pH 7. (b) Inactivation courses in the presence of 60% of dioxane, at pH 5 and 4°C. (▲): CNBr-TLL; (○): CNBr-TLL-A.

This is very important because the strong decrease in the enzyme stability after full amination in the case of Penicillin G Acylase or Glutaryl Acylase was the reason that forced to use partial amination of the enzyme surface, decreasing the potential impact of the strategy to improve the multipoint covalent attachment [11]. Even using the lipase from *Bacillus thermocatenulatus* the stability after the chemical amination was reduced by a 5 fold factor [17]. Thus, the full amination of TLL seems to have negligible effects on the enzyme stability, and TLL-A could be used to be immobilized on glyoxyl agarose.

Immobilization of aminated TLL on glyoxyl agarose

Figure 6 shows the courses of immobilization of TLL-A on glyoxyl at pH 9 and 10. Immobilization was achieved at both pH values in a few minutes, allowing recovering around 65%-70% of the enzyme activity under both conditions, even though the free enzyme remained quite unstable at pH 10 and 25 ºC. Thus, the amination of TLL allowed immobilizing the enzyme under milder conditions (pH 9). Moreover, because of the rapid immobilization and stabilization even during the first steps of the immobilization, the chemical amination permits to recover higher activity even when the immobilization was performed at pH 10. Under these conditions, the free TLL becomes inactivated before it became immobilized because of the slow immobilization rate.

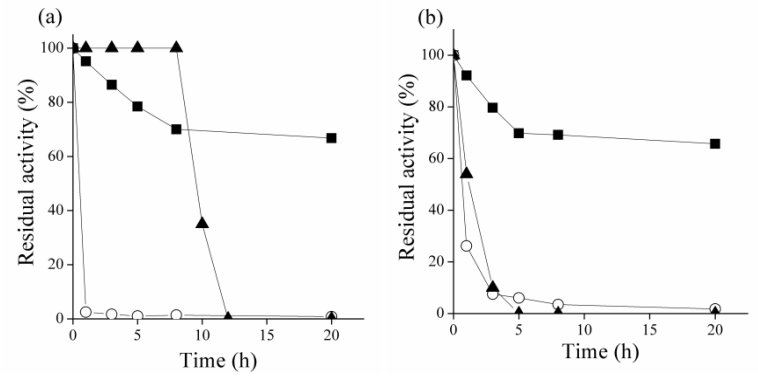

Figure 6. The time course of immobilization of TLL-A. (a) pH 9, 25 °C for 8h and then incubated at pH 10 overnight; (b) pH 10, 25 °C. (▲) Reference suspension; (■) immobilization suspension; (○) supernatant of the immobilization suspension.

Stability of the different TLL immobilized preparations

The immobilization at pH 9 or pH 10 could involve different enzyme surface areas. At pH 10, Lys residues can react with the support, and the enzyme will be immobilized through the region that is the richest in Lys+Asp+Glu residues [9]. However, at pH 9, only the new amino groups will be relevant, [18] and the enzyme will be immobilized through the region having the highest Asp+Glu densities. In this case, immobilization at pH 9 or 10 did not yield differences in the activity recovery. However, the likely implication of different enzyme regions on the enzyme immobilizaiiton could have a different effect on the final stabilization achieved, as in other studied enzymes, thus both enzymes preparations were evaluated [11, 17].

In order to enhance the enzyme support-reaction of the immobilized enzyme at pH 9, it was further incubated at pH 10 for 12 h [36]. This treatment only produced a very small additional reduction of the enzyme activity of around 5%.

The stabilities of Gx-TTL (non-modified enzyme immobilized at pH 10 at 4ºC and reduced), Gx(9/10)-TLL-A (aminated enzyme, immobilized at pH 9, incubated at pH 10 and reduced), and Gx(10)-TLL-A (aminated enzyme, immobilized at pH 10 and reduced) were compared, using CNBr-TLL and CNBr-TLL-A (no stabilized preparations) as references. Figure 7 shows that all the glyoxyl preparations were more thermo-stable than the respective CNBr-TLL, which presented a half-life of 0.5 h under the conditions of inactivation. The non-modified enzyme immobilized at pH 10 and 4 ºC showed the lowest stabilization factor, 8-fold (Figure 7a). The stability of the aminated enzyme immobilized at pH 9 and then incubated at pH 10 was improved to a 20-fold factor compared to the previous TLL derivative. The aminated enzyme directly immobilized at pH 10 was even more stable, reaching a factor of 40-fold increase in the enzyme half life. These results confirm the initial hypothesis that at pH 10 TLL should become immobilized through the richest surface area in reactive groups, area in which the first compulsory multipoint covalent attachments will be the easiest [9]. Thus, the

amination improved the enzyme thermal stability of the immobilized enzyme by a 5-fold factor

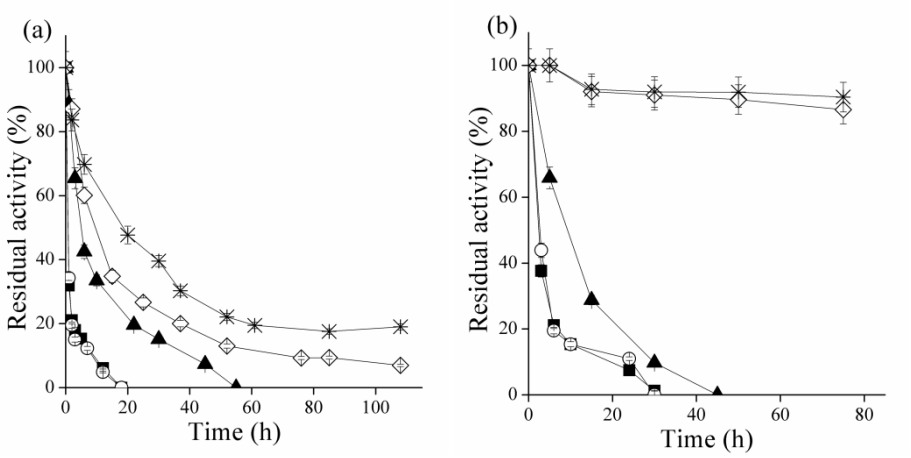

Figure 7. Effect of the chemical amination on the stabilization of TLL by immobilization on glyoxyl agarose. (a) Inactivation courses in 25 mM sodium phosphate at 70 °C and pH 7. (B) Inactivation courses in the presence of 60% of dioxane, at pH 5 and 4°C. (■) CNBr-TLL; (○) CNBr-TLL-A; (▲) Gx-TLL; (◇) Gx(9/10)-TLL-A; (✳) G(10)-TLL-A.

In the presence of organic solvents (Figure 7b), the differences are even clearer. Gx-TTL was only 3 to 4-fold more stable than CNBr-TLL, while Gx(9/10)-TLL-A and Gx(10)-TLL-A become highly stabilized, showing no significant decrease in activity after 80 h of incubation Since there were no differences between the two aminated preparations immobilized on glyoxyl agarose, both were incubated under more drastic conditions (95% dioxane, pH 7, 25ºC). Figure 8 shows that both stabilized preparations remained very stable under these conditions. However, the increase of the dioxane to 95% inactivated both preparations, being slightly more stable Gx(9/10)-TLL-A.

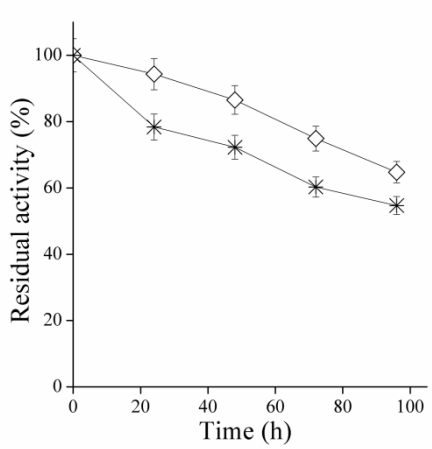

Figure 8. Effect of the chemical amination on the stabilization of TLL by immobilization on glyoxyl agarose. Inactivation courses in the presence of 95% of dioxane, at pH 7 and 25°C. (◇) Gx(9/10)-TLL-A; (✳) G(10)-TLL-A.

Conclusions

The chemical amination of lipases reversible-immobilized on hydrophobic supports is a very simple way to enrich the lipases surface on primary amino groups [11, 17]. The modified enzyme may be easily desorbed from the support after the modification by incubation with detergents [17]. Moreover, the modification enriches the enzyme surface in primary amino groups with a lower pKb [18], that makes the enzyme more reactive with glyoxyl supports at lower pH values, allowing immobilizing the enzymes on these supports at pH of 9 [11, 17]. The higher content of very reactive amino groups permit to also accelerate the immobilization, that depends on the density of reactive groups ion the support and in the enzyme [9].

In the case of TLL, the non-modified enzyme could only be immobilized on glyoxyl agarose at low temperatures since the enzyme become inactivated at pH 10 and 25 ºC and could not be immobilized at pH 9 [9], giving very low activity recovery (30%) and very low stabilization factors (4-8). However, the aminated enzyme immobilized very rapidly at pH 9 and 10, giving good activities recoveries (around 70%) and high stabilization factors (several hundreds in the case of inactivation in the presence of solvents). The new preparation was

more stable in 95% dioxane than the non-modified enzyme immobilized on glyoxyl agarose in 60% dioxane.

The immobilization on the glyoxyl support at pH 9 or pH 10 might permit to alter the enzyme area involved in the immobilization. In the first case the only groups involved in the first immobilization were that generated by the chemical modification, with a lower pKb [18] (in fact the non-modified enzyme cannot be immobilized under that conditions), while at pH 10 also the Lys was implied [9]. This was not very relevant for the enzyme activity, but the stabilization achieved when immobilizing the enzyme at pH 10 (where the maximum possibilities of enzyme-support reaction was achieved) [9] was slightly higher than immobilizing at pH 9 and later incubating at pH 10.

Acknowledgments

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Capítulo VI - REACTIVATION OF COVALENTLY IMMOBILIZED LIPASE FROM *Thermomyces lanuginosus*

Neste trabalho avaliaram-se as possibilidades de reativação do derivado de lipase imobilizado em agarose ativado com brometo de cianogênio parcialmente inativado por temperatura ou solvente orgânico. Estudou-se o tratamento do derivado em agentes caotrópicos antes de submeter o derivado à reativação em meios aquosos, com e sem a presença de detergente. Os resultados estão apresentados no manuscrito a seguir, aceito para publicação na revista *Process Biochemistry*, doi: 10.1016/j.procbio.2009.02.001.

Reactivation of covalently immobilized lipase from *Thermomyces lanuginosus*

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Reactivation of covalently immobilized lipase from *Thermomyces lanuginosus*

Abstract

Lipase from *Thermomyces lanuginosus* (TLL) immobilized on cyanogen bromide agarose (CNBr) may be fully inactivated when incubated in saturated solutions of guanidine. When this inactivated enzyme is re-incubated in aqueous medium, 20% of the activity may be recovered for several cycles. However, if the activity is determined in the presence of a detergent (CTAB, an activator of this enzyme), 100% of the initial activity in the presence of detergent was recovered. The enzyme was also inactivated in the presence of organic solvents and at high temperatures. Inactivations were more rapid when the activity was determined in absence of detergent. In both cases, some activity could be recovered just by incubation under mild conditions, and this increase was higher if the activity measurements were performed in the presence of CTAB. These suggested that the opening of the lipase could be a critical step in the inactivation or reactivation of immobilized TLL. In inactivations in the presence of solvents, 100% of activity could be recovered during several cycles, while in thermal inactivations, the recovered activity decreased in each inactivation-reactivation cycle. The incubation of the thermally inactivated enzyme in guanidine improved the results, but still 100% could not be achieved during several cycles even measured in the presence of CTAB.

Thus, the simple incubation of the partially or fully inactivated enzyme under mild conditions permitted to recover some activity (enhancing the half life of the biocatalysts), even in thermal inactivations.

Keywords: enzyme inactivation, enzyme reactivation, unfolding-refolding, immobilized enzymes, operational stabilization

Introduction

The use of enzymes in the presence of organic solvents may have interest for different purposes: to improve the solubility of substrates or products [1, 2], to shift thermodynamic equilibrium [3-5], to improve the enzyme properties [6, 7] or even to prevent microbial contamination.

One problem of the use of organic solvents and cosolvents in biotransformations is the fact that enzymes may be readily inactivated in the presence of high concentrations of these compounds [8-10]. Immobilized enzymes may be very rapidly inactivated by the presence of high concentrations of inert solvents, even at neutral pH values and low temperatures. At pH 7 and low temperatures, it is unlikely that the primary structure of the enzyme can be modified. Moreover, if the enzyme is immobilized and dispersed on the support surface, aggregations will be impossible. Thus, under these chemically mild conditions, the only cause of inactivation of immobilized monomeric enzymes should be the distortion of their tridimensional structure that can drive to an incorrect and inactive conformation of the enzyme [11]. In this way, if this incorrect conformation may be reversed, the recovery of the enzyme activity could be achieved.

Lipases are one of the most interesting enzymes for use in biocatalysis [12-14]. These enzymes are monomeric proteins that exhibit a complex catalytic mechanism, called interfacial activation [15-18]. Usually, in homogenous medium, lipases mainly have their active center secluded from the reaction medium by an oligopeptide chain called flat or lid [19]. This lid has a very hydrophobic internal side that interacts with hydrophobic aminoacids near to the active center. In the presence of a hydrophobic interface (e.g., an oil drop [20, 21], a hydrophobic support [22, 23], a hydrophobic protein [24, 25]), the lid moves exposing the active center of the lipase to the medium, and this open form becomes adsorbed to the hydrophobic surface, shifting the conformational equilibrium towards the open form.

In this way, strategies for reactivation of immobilized lipases should regenerate not only the active center of the lipase, but also the mechanism of opening and closing of the

lipases. As a result, the recovery of active forms of lipases after their inactivation may be a bit more complex than the reactivation of the standard protein [26, 27].

In this context, it has been described that detergents may help the lipases to give an open form [28, 29]. It is suggested that these reagents may stabilize the open form of the lipase by reducing the interaction of the large hydrophobic pocket present in the open form of the lipase with the medium. In fact, the use of detergents has been proposed as a way to have hyperactivated form of lipases, just by adding the detergents to the reaction medium [30], or by preparing the immobilized lipase in the presence of detergent [31, 32]. Thus, it may be interesting to analyze the recovery of the enzyme activity in the presence or absence of detergents. Moreover, there are some reports stating that some detergents could help to the correct refolding of some proteins [33, 34].

Lipase from *Thermomyces lanuginosus* (TLL) is the enzyme responsible for the lipolytic activity of Lipolase®, a commercial lipase preparation supplied by Novozymes. This enzyme has been broadly used in many biotransformations [35-38]. Its structure has been solved at 1.8 Å [39], presenting a large flat that, in the closed form, isolates the active centre from the reaction medium. This lipase presents a high tendency to form bimolecular aggregates, making it necessary to use diluted solutions and the presence of detergents to have a monomeric form of the enzyme [24].

In this paper, lipase from *Thermomyces lanuginosus* (TLL) immobilized on cyanogen bromide agarose (CNBr) was inactivated by heat and organic cosolvents, and later the recovery of the activity was analyzed by incubation under milder conditions. The inactivation and the activity recovery were analyzed in both, aqueous medium or aqueous-detergent medium. The effect of the previous full unfolding of the lipase by incubation in saturated guanidine hydrochloride to destroy any incorrect structure but stable lipase structure [26, 27] was also studied.

Materials and Methods

Materials

Lipase from *Thermomyces lanuginosus* (TLL) was obtained from Novozymes (Denmark). Ethanolamine hydrochloride, hexadecyltrimethylammonium bromide (CTAB), and p-nitrophenyl butyrate (p-NPB) were from Sigma. 1,4-Dioxane, guanidine hydrochloride and urea were from Fluka. Octyl-sepharose CL-4B and cyanogen bromide activated Sepharose 4B (CNBr) were purchased from GE Healthcare (Uppsala, Sweden). Other reagents and solvents used were of analytical or HPLC grade.

Methods

The experiments were carried out at least by triplicate and the standard error was always under 5%.

Standard Enzymatic Activity Assay.

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the released p-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C, using a thermostatized spectrum with continuous magnetic stirring. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of pNPB activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPB/min (IU) under the conditions described above.

In some instances, 0.01% of CTAB was added to the substrate solution.

Purification of TLL

The TLL was purified prior to use. The used strategy was the interfacial adsorption on hydrophobic supports. The enzyme was adsorbed on octyl-sepharose beads under continuous stirring in 10 mM sodium phosphate at pH 7.0, following a previously described

procedure [22, 23]. Periodically, the activity of suspensions and supernatants was measured by using the pNPB assay. After enzyme adsorption, the lipase preparation was vacuum filtered using a sintered glass funnel and abundantly washed with distilled water. TLL was desorbed from octyl-sepharose by suspending the immobilized enzyme in a relation 1/10 (w/v) in 25 mM sodium phosphate at pH 7.0 containing 0.6% (v/v) of CTAB during 1 h at room temperature. The SDS-PAGE of this preparation revealed just one protein band.

Immobilization of TLL on CNBr-Activated Support

TLL was desorbed from octyl-sepharose as described in the purification section. The immobilization of TLL on CNBr-activated support was performed for 15 min at 4°C and pH 7 to reduce the possibilities of getting a multipoint covalent attachment between the enzyme and the support [40]. During the immobilization and further blocking of the support, the suspension was submitted to continuous gentle stirring. The enzyme preparations were carried out by using 10 mg of the purified enzyme per g of support. The enzyme-support reaction was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized TLL was vacuum filtered using a sintered glass funnel and washed with abundant water, to eliminate the detergent. This immobilized enzyme was called CNBr-TLL

Incubation of CNBr-TLL on caotropic agent solutions

CNBr-TLL was incubated in 50 mM phosphate buffer containing 8M of urea or guanidine hydrochloride at pH 7.0, 25 °C. Samples were withdrawn periodically to check the remaining activity of the immobilized enzyme.

Thermal inactivation

CNBr-TLL was incubated in 50 mM sodium phosphate at pH 7.0 and 60 °C. Samples were withdrawn periodically using a pipet with a cut-tip and under vigorous stirring to have a

homogeneous biocatalyst suspension. The activity was measured using the pNPB assay described above in the presence and absence of CTAB in cuvette.

Inactivation by organic solvent

CNBr-TLL was washed with 80% dioxane/50 mM Tris-HCl aqueous solution at pH 7 and 4 °C. Subsequently, the enzyme derivatives were resuspended in such solution and incubated at 4 °C. Samples were withdrawn periodically, and the activity was checked following the above assay.

Reactivation experiments

Fully or partially inactivated CNBr-TLL preparations (sometimes after incubation in saturated guanidine solution) were resuspended in aqueous buffer (50 mM sodium phosphate at pH 7) and the activity was determined along the time. When a constant value of residual activity was achieved, this was considered the maximum recovered activity. In some cases, several consecutive cycles of inactivation/reactivation of immobilized CNBr-TLL were performed.

Results and Discussion

Immobilization of TLL on CNBr agarose beads

The immobilization of TLL on CNBr agarose beads was performed under very mild conditions (pH 7, 4 ºC), in order to have an immobilized but non-modified enzyme. These mild conditions permitted to recover 100% of the enzyme activity. Figure 1 shows the thermal inactivation of CNBr-TLL compared to the soluble enzyme. The inactivation courses were very similar, suggesting that the immobilized enzyme presented the same rigidity than the soluble enzyme. Thus, this immobilized preparation, with the enzyme immobilized in a nearly inert surface, will be used to study the inactivation and reactivation of TLL in absence of any artefact caused by protein-protein interactions. The use of 0.01% CTAB during the assay permitted to increase the enzyme activity by a 80 fold factor, suggesting that TLL could be strongly hyperactivated by this reagent, perhaps by stabilizing the open form of the lipase [41]. TLL was also one of the lipases that exhibited a higher hyperactivation upon adsorption on octyl agarose [22]. In fact, the large lid of TLL [39] could strongly unfavour the open structure of this lipase. Thus, TLL seems to be a lipase that presents some difficulties to give the open form in homogenous aqueous solution.

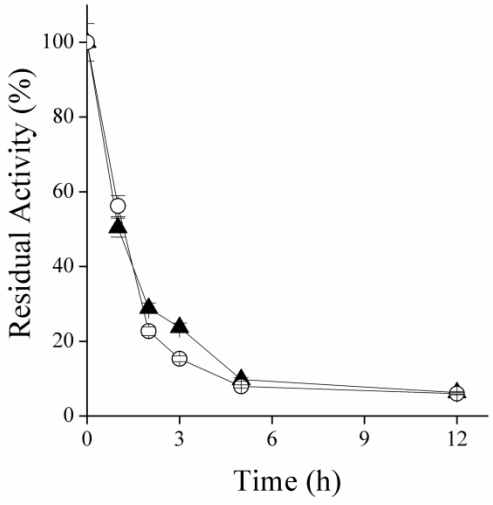

Figure 1. Inactivation courses of soluble and immobilized TLL in 50 mM sodium phosphate at 60 °C and pH 7. (▲) soluble-TLL; (○) CNBr-TLL.

Inactivation /reactivation of CNBr-TLL by incubation in the presence of caotropic agents and aqueous buffer.

The incubation of CNBr-TLL in the presence of saturated solutions of guanidine or urea could permit the unfolding of the enzyme (Figure 2) [26, 27, 33, 34]. However, urea seems to be unable to fully destroy the enzyme activity, while guanidine was able to fully inactivate it.

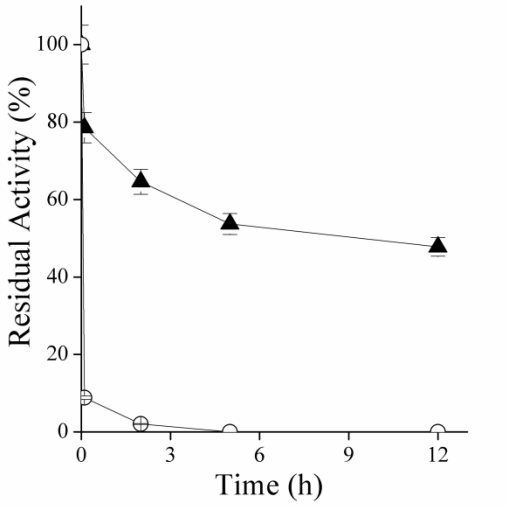

Figure 2. Inactivation course of CNBr-TLL by different caotropic agents. (▲) Urea; (○) Guanidine. Other specifications as described in Methods.

Thus, guanidine was chosen to unfold the immobilized preparation. The incubation of the enzyme inactivated by guanidine in pure aqueous buffer permitted to recover only around 20% of the enzyme activity after some minutes of incubation, while measuring in the presence of CTAB it was possible to recover 100% of the initial activity of the enzyme (measured also in the presence of CTAB). These values could be repeated during several unfolding-refolding cycles (Figure 3).

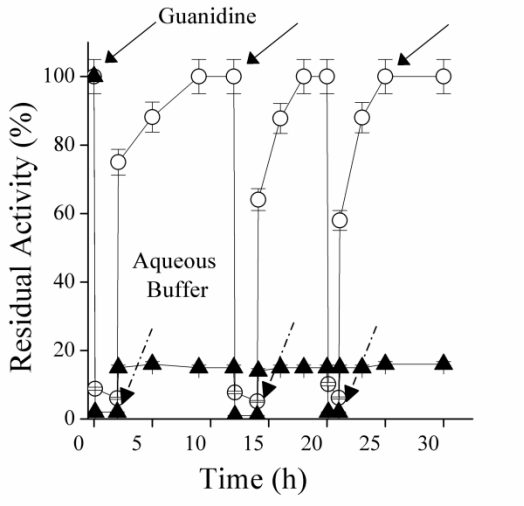

Figure 3. Inactivation/reactivation cycles of CNBr-TLL by successive incubation in guanidine and sodium phosphate 50 mM pH 7. (▲) measured in the absence of CTAB; (○) measured in the presence of CTAB. The arrows show the moment when the enzyme preparations were incubated in guanidine or aqueous buffer. Other conditions are described in Methods.

The difference of activity recovery in the presence and absence of CTAB suggested that the recovering of the capacity of the lipase to give the open form could be the most difficult event in the reactivation process.

When CTAB was present during the whole reactivation process, the recovered activity was similar to that found when the reactivation was performed just in buffer, suggesting that the detergent effect was positive only when the correct enzyme structure was almost formed, having no relevance in the first reactivation steps.

Inactivation of CNBr-TLL in the presence of organic solvents

Figure 4a shows the inactivation course of CNBr-TLL in 50% dioxane. When the enzyme activity was assayed in aqueous buffer, the activity decreased by around 50% in 30 minutes. Afterwards the enzyme activity decreased very slowly. However, when the activity was assayed in the presence of CTAB, the enzyme activity remained unaltered for 24 h. This result suggested that the first inactivation cause of this lipase (and perhaps of other lipases) could be an increasing difficulty of the opening mechanism of the lipase. The presence of

small concentrations of detergent can “help” to open the lipase, although it could be also to help in the correct folding of the lipase [32].

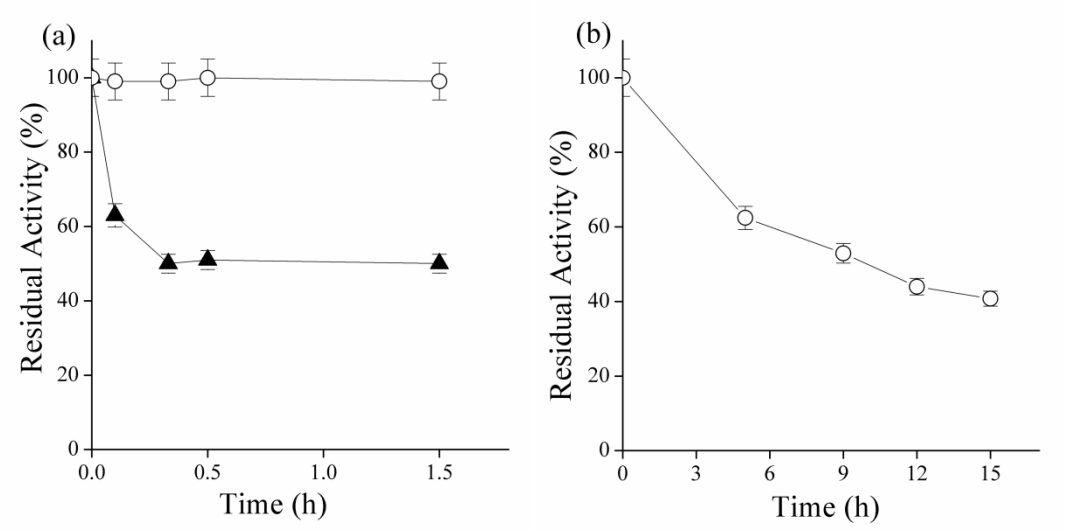

Figure 4. Inactivation courses of CNBr-TLL in the presence of (a) 50% of dioxane or (b) 80% of dioxane, at pH 7 and 4 °C. Other specifications are described in methods. (▲) Activity was measured in the absence of CTAB; (○) Activity was measured in the presence of CTAB.

When the CNBr-TLL was inactivated in 80% dioxane (Figure 4b), the decrease in activity was clear even when measured in the presence of CTAB, although the stability was still very high, with a half life of around 10 h.

To check if the unsuitable structure of the enzyme was spontaneously reversible, the partially inactivated preparation was incubated in buffer as described in methods. Figure 5 shows that the enzyme could recover 100% of the activity after 5 minutes of incubation at pH 7 in absence of solvents, when measured in the presence of CTAB. This suggested that the inactivation of the enzyme was reversible and mainly due to the production of reversible incorrect structures induced by the presence of organic solvent. These structures could be (at least partially) reversed to the active form just by incubating the immobilized enzyme in aqueous buffer.

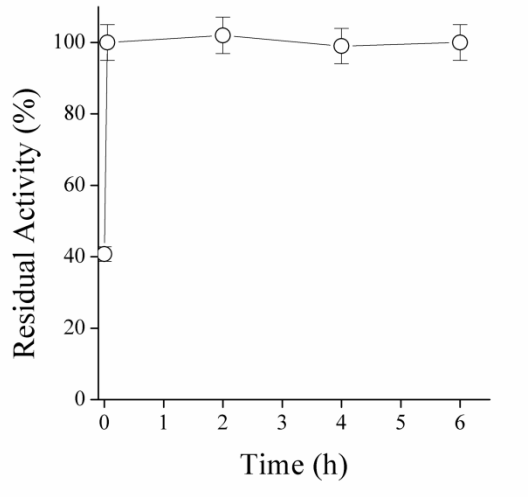

Figure 5. Reactivation course of CNBr-TLL inactivated in the presence of 80% of dioxane at pH 7 and 4 °C by incubation in 50 mM sodium phosphate at pH 7 and 25 °C. The activity was measured in the presence of CTAB.

Thermal inactivation of CNBr-TLL

Figure 6a shows the thermal inactivation of the enzyme. Again, the inactivation was slower when the activity was measured in the presence of CTAB than when the activity was measured in pure buffer. In this case, the re-incubation of the enzyme at low temperature was not enough to fully recover the enzyme activity even measuring in the presence of CTAB (Figure 6b), although a significant percentage of the enzyme activity could be recovered. This could be due to a chemical modification of the protein due to the high temperatures or to the production of an inactivated but stable new enzyme structures.

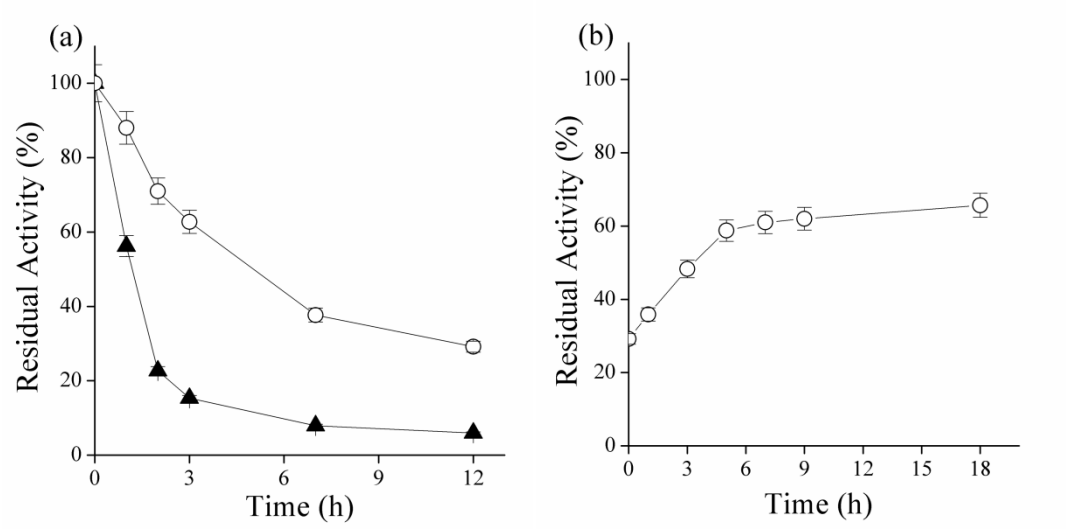

Figure 6. Inactivation courses of CNBr-TLL in 50 mM sodium phosphate at 60 °C and pH 7: (a) (▲) measured in the absence of CTAB; (○) measured in the presence of CTAB; (b) reactivation by incubation in sodium phosphate 50 mM and pH 7, measuring in the presence of CTAB.

Reactivation of CNBr-TLL inactivated by the presence of organic solvents or high temperature.

Figure 7 shows several cycles of inactivation-reactivation of CNBr-TLL by organic solvents and temperature. Figure 7a shows the reactivation of CNBr-TLL inactivated by incubation in the presence of 80% dioxane at pH 7 and 4 ºC, just by incubation in aqueous buffer or by a previous unfolding by incubation in a saturated solution of guanidine hydrochloride. In both cases, measuring in the presence of CTAB, 100% of the activity could be achieved during several cycles.

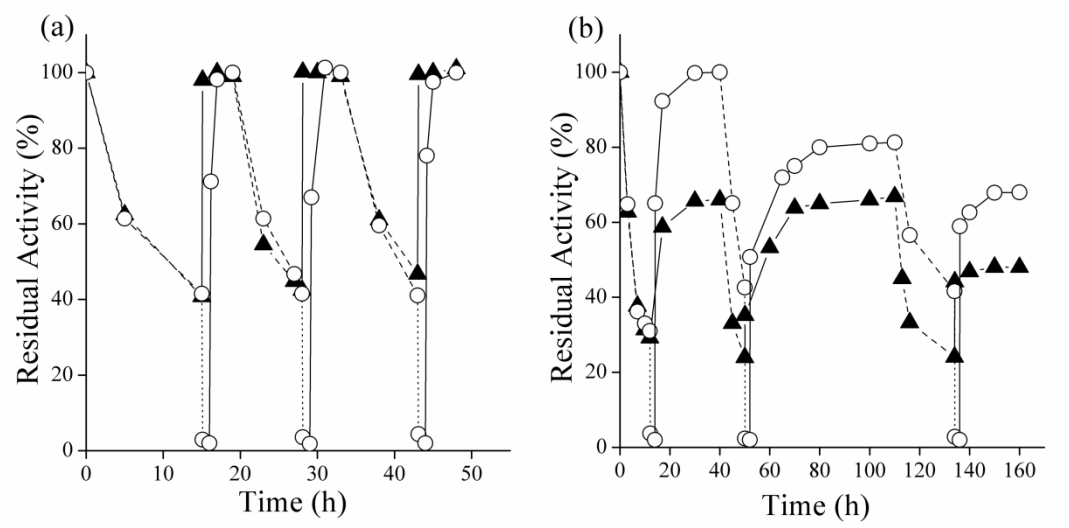

Figure 7. Inactivation/Reactivation cycles of CNBr-TLL inactivated by (a) 80% of dioxane, at pH 7 and 4 °C; (b) 50 mM sodium phosphate at 60 °C and pH 7. All the measures were performed in the presence of CTAB. (▲) incubation in aqueous buffer solution; (○) incubation in guanidine and incubation in aqueous buffer solution. Dashed line: incubation in inactivating condition; Solid line: incubation in aqueous buffer at 25 °C; Dotted line: incubation in saturated guanidine solution.

Figure 7b shows that when the enzyme was inactivated by incubation at high temperatures, reactivation was much more complex. In any case, always some reactivation was observed. In the first cycle, by incubating the enzyme at low temperature during 5 hours, the enzyme activity went from 30 to 60%, while the previous incubation of the inactivated enzyme in guanidine permitted to recover 100% of the initial activity. However, in the second cycle the recovered activity decreased and in the third cycle there was a further decrease in the recovered activity in both cases (50% by incubation in aqueous buffer at low temperature, 75% by incubation in guanidine and incubation in buffer). Considering that the incubation of the enzyme in saturated sodium guanidine seemed to be able to fully unfold the enzyme structure, the results suggested that the inactivation at high temperature could produce some chemical modification of the enzyme, making more difficult the achievement of the correct enzyme structure.

Conclusions

The use of immobilized and dispersed TLL has permitted to recover some enzyme activity by using relatively simple strategies, thus enlarging the operational stability of the biocatalyst. However, in the case of TLL, it seems that the recovering of the capacity of the enzyme to give a stable “open structure” is the critical step. The inactivation rate of the enzyme measured in the presence of an agent that may help to stabilize this open structure (as a detergent) [30, 31] was slower than in the absence of this reagent, in both, thermal and solvent induced inactivations.

On the other hand, the simple incubation of inactivated enzyme in aqueous buffer permitted to recover a certain percentage of enzyme activity, improving the operational half life of the enzyme derivatives. However, if the activity was determined in the presence of detergent, enzymes inactivated by dioxane or by incubation with saturated guanidine, recovered 100% of the initial activity during several cycles. This suggested that dioxane only produces an inactive structure, which could be easily reversed by incubation in aqueous medium. When the inactivation was due to enzyme exposition to high temperatures, the activity recovering was more difficult. The incubation at low temperatures permitted to recover (multiplying by a 2 fold factor the activity in the first cycle), and the previous incubation in saturated guanidine improved the results. However, in the successive cycles the recovered activity decreased even after incubation in guanidine solutions, suggesting that some chemical modification of the enzyme structure could take place.

Acknowledgments

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Capítulo VII - POSITIVE EFFECTS OF THE MULTIPOINT COVALENT IMMOBILIZATION IN THE REACTIVATION OF PARTIALLY INACTIVATED DERIVATIVES OF LIPASE FROM *Thermomyces lanuginosus*

Neste trabalho, após estabelecer as metodologias de reativação no Capítulo VI foram estudados os efeitos da imobilização por ligação covalente multipontual na reativação de derivados parcialmente inativados da lipase de *Thermomyces lanuginosus*. Avaliaram-se as possibilidades de reativação de derivados imobilizados em glioxil agarose (multipontual) e brometo de cianogênio (unipontual) parcialmente inativados por temperatura ou solvente orgânico, assim como o efeito da aminação química no processo de reativação. Os resultados estão apresentados no manuscrito a seguir aceito para publicação no periódico *Enzyme and Microbial Technology*, doi: 10.1016/j.enzmictec.2009.02.009.

Positive effects of the multipoint covalent immobilization in the reactivation of partially inactivated derivatives of lipase from *Thermomyces lanuginosus*

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Abstract

Different immobilized preparations of lipase from *Thermomyces lanuginosus* (TLL) have been inactivated by exposure to high temperatures, guanidine or 95% of dioxane. The studied preparations were: non-stabilized cyanogen bromide (CNBr-TLL), aminated CNBr-TLL (CNBr-TLL-A), and two stabilized preparations of aminated TLL by immobilization on glyoxyl support, Gx(9/10)-TLL-A (TLL-A immobilized at pH 9 and later incubated at pH 10) or Gx(10)-TLL-A (directly immobilized at pH 10). The reactivation of the partially inactivated immobilized enzymes under mild conditions by incubation in aqueous buffer, allowed recovering some activity, which was improved when it was pre-incubated in guanidine. Amination produced a fairly negative effect on the reactivation of the enzyme, but the multipoint covalent attachment of this aminated enzyme reversed the effect (e.g., recovered activity increased from 20% for CNBr-TLL to 80% for Gx(9/10)-TLL-A). The negative effect of the amination was clearer when the inactivation was caused by exposure to high temperatures, although the multipoint attachment of aminated enzyme was able to improve the recovered activity. The determination of enzyme activity in the presence of hexadecyltrimethylammonium bromide slowed the inactivation rates of all preparations and improved the recovery of activity after incubation under mild conditions, suggesting that the opening mechanism of the lipase could be a critical step in the TLL inactivation/reactivation. The use of multipoint attached TLL preparations did not only improve enzyme stability, but it also increased activity recovery when the preparation was incubated under mild conditions.

Keywords: enzyme reactivation, multipoint covalent attachment, glyoxyl, aminated enzymes, stabilized enzymes, operational stability

Introduction

Lipases are one of the most widely used enzymes in biocatalysis [1-3]. These enzymes are monomeric proteins that usually, in homogenous medium, mainly have their active center secluded from the reaction medium by an oligopeptide chain called flat or lid [4]. In the presence of a hydrophobic interface (e.g., an oil drop [5, 6], a hydrophobic support [7, 8], a hydrophobic protein [9, 10]), the lid moves, exposing the active center of the lipase to the medium, and this open form becomes adsorbed to the hydrophobic surface, shifting the conformational equilibrium towards the open form.

In many instances, the use of enzymes (and lipases in particular) may be improved by the addition of organic solvents or co-solvents to the reaction medium, or by the utilization of moderately high temperatures. This may increase the solubility of substrates or products [11, 12], or shift thermodynamic equilibrium [13-15], improve enzyme properties [16, 17], or even prevent microbial contamination. However, enzymes may be readily inactivated in the presence of high concentrations of organic co-solvents or high temperatures by different mechanisms [18-20].

The use of lipases immobilized over inert supports (e.g., glyoxyl-agarose [21]) may prevent some of these inactivation causes. Immobilized enzymes may not aggregate or suffer any other kind of intermolecular interaction. If the lipase molecules are bonded to the support via multipoint covalent attachment, these immobilized preparations are more resistant to any kind of distortion, and therefore to the action of organic solvents or heat [22]. However, even highly stabilized lipase preparations may become inactivated after incubation in the presence of organic co-solvents or to high temperatures for long periods of time, reducing the operational lifespan of the biocatalyst. The situation will be different depending on the inactivation cause. At pH 7 and low temperatures, for instance, it is unlikely that the primary structure of the enzyme would be chemically modified. Thus, the only cause of inactivation of immobilized monomeric enzymes should be the distortion of their three-dimensional structure that can drive to an incorrect and inactive conformation of the enzyme

[23]. In this way, if this incorrect conformation may be reversed towards the active form, the recovery of the enzyme activity could be achieved.

When the enzyme inactivation is produced by its exposition to high temperatures, beside to the production of incorrect enzyme conformations, it is possible that some chemical modifications of the enzyme may also occur [20, 24-27]. This chemical modification may produce some difficulties to recover enzyme activity.

Concerning immobilized lipases, strategies for reactivation should regenerate not only the catalytic center of the lipase, but also the mechanism of opening and closing of the molecule. In this context, it has been suggested that the use of detergents may help to stabilize the open forms of lipases [28, 29]. This may be a good tool to help in the lipase reactivation if this conformational change is a key point in the lipase reactivation. Immobilization of lipases in the presence of detergents [30, 31] or the subsequent addition of detergents to the reaction medium [32], have been proposed as ways of improving the lipase activity. Moreover, there are some reports stating that some detergents could help in some instances to the correct refolding of some proteins [33, 34]. Therefore, it may be interesting to analyze the recovery of the enzyme activity in the presence or absence of detergents.

In the reactivation of the inactivated enzymes, the enzyme-support multipoint covalent attachment may play a double role. On the one hand, it may render more difficult the movements of the polypeptide chains, which might interfere in both the inactivation and reactivation. On the other hand, this multiple enzyme-support bonds may behave as reference points, easing the recovery of enzyme activity.

Lipase from *Thermomyces lanuginosus* (TLL) is the enzyme responsible for the lipolytic activity of Lipolase®, a commercial lipase preparation supplied by Novozymes. This enzyme has been broadly used in many biotransformations [35-38]. Its structure has been solved at 1.8 Å [39], presenting a large flat that, in the closed form, isolates the active centre from the reaction medium. In solution, this lipase tends to form bimolecular aggregates by interaction between the open form of both lipase molecules, making the use of diluted

solutions and the presence of detergents necessary in order to have an individual lipase molecule [9]. After immobilization under these conditions, the enzyme will be in a monomeric form and dispersed on the support surface.

This enzyme has been recently stabilized by coupling the chemical amination of the enzyme surface to the multipoint covalent attachment of the modified enzyme to glyoxyl-agarose beads [40]. The amination of the enzyme was necessary in order to immobilize the TLL to the glyoxyl support giving high values of enzyme stabilization, and did not affect either the enzyme stability or its activity. The glyoxyl TLL preparations presented very good activity recovery and a fairly high stabilization against inactivation at high temperatures or in the presence of organic solvents. The achieved stability suggested a very intense multipoint covalent attachment [40].

The drastic change in the physical properties of the enzyme surface due to the substitution of carboxylic groups by the amination reaction could have some effects on the ability of the enzyme to reactivate, despite both carboxylic and amino groups being polar. This could present some effects on the final reactivation of these immobilized preparations.

In this paper, immobilized preparations of aminated lipase from *Thermomyces lanuginosus* immobilized following the multipoint strategy on glyoxyl-agarose were inactivated by either heat or organic co-solvents, followed by analysis of the recovery of activity after incubation under milder condition in either aqueous or aqueous-detergent media and compared to results obtained in a mildly immobilized TLL preparation. The effect of the previous incubation in saturated guanidine hydrochloride of the lipase to destroy any incorrect but stable lipase structure [41, 42] was also studied.

Materials and Methods

Materials

Lipase from *Thermomyces lanuginosus* (TLL) was obtained from Novozymes (Denmark). Ethanolamine hydrochloride, 1-ethyl-3-(dimethylaminopropyl) carbodiimide, hexadecyltrimethylammonium bromide (CTAB), and *p*-nitrophenyl butyrate (p-NPB) were from Sigma. 1,4-Dioxane, guanidine hydrochloride, and 1,2-ethylenediamine, were from Fluka. Octyl-sepharose CL-4B and cyanogen bromide activated Sepharose 4B (CNBr) were purchased from GE Healthcare (Uppsala, Sweden). Cross-linked agarose (10 BCL) was kindly donated by Hispanagar S.A. (Burgos, Spain) and its modification to glyoxyl agarose (activated with 200 μmol/g of support) was performed as described elsewhere [21, 43]. Other reagents and solvents used were of analytical or HPLC grade.

Methods

The experiments were carried out at least by triplicate and the standard error was always under 5%.

Enzymatic activity assay.

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C, using a thermostated spectrum with continuous magnetic stirring. To initialize the reaction, 0.05 mL of lipase solution or suspension were added to 2.5 mL of substrate solution. One international unit of pNPB activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPB/min (IU) under the conditions described above. In some instances, 0.01% of CTAB was added to the substrate solution.

Purification of TLL

TLL was purified prior to use by interfacial adsorption on hydrophobic supports. The enzyme was adsorbed on octyl-sepharose beads under continuous stirring in 10 mM sodium phosphate at pH 7.0, according to a previously described procedure [7, 8].The activity of suspensions and supernatants was periodically measured by using the pNPB assay. After enzyme adsorption, the lipase preparation was vacuum filtered using a sintered glass funnel and abundantly washed with distilled water. TLL was desorbed from octyl-sepharose by suspending the immobilized enzyme in a relation 1/10 (w/v) in 25 mM sodium phosphate at pH 7.0 containing 0.6% (v/v) of CTAB during 1 h at room temperature.

Immobilization of TLL on CNBr-Activated Support

Immobilization was performed as previously described [40]. TLL was desorbed from octyl-sepharose as described in the purification section. The immobilization of TLL on CNBr-activated support was performed for 15 min at 4 °C and pH 7 to reduce the possibilities of getting a multipoint covalent attachment between the enzyme and the support [43]. During the immobilization and further blocking of the support, the suspension was submitted to continuous gentle stirring. The enzyme-support reaction was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized TLL was vacuum filtered using a sintered glass funnel and washed with abundant water to eliminate the detergent. This immobilized enzyme was termed CNBr-TLL.

Chemical amination of immobilized TLL

Chemical amination was performed as previously described [44]. A total of 1 g of immobilized lipase on octyl or CNBr-Sepharose beads was added to 10 mL of 1 M 1,2-ethylenediamine at pH 4.75 under continuous stirring. Solid 1-ethyl-3-(dimethylaminopropyl) carbodiimide was added to the suspension to a final concentration of 10 mM. These reaction conditions have been reported as capable of fully modifying all exposed carboxylic groups of

the protein [45-49]. After 90 min of gentle stirring at 25 °C, the immobilized-modified preparations were vacuum filtered using a sintered glass funnel and incubated for 4 h in 0.1 M hydroxylamine at pH 7 and 4 °C to recover the 1-ethyl-3-(dimethylaminopropyl) carbodiimide -modified tyrosines [50]. The enzyme preparations were filtered and washed with 25 mM sodium phosphate at pH 7.5 and with an excess of distilled water. The aminated TLL immobilized on octyl-sepharose, later used to obtain the soluble aminated enzyme, was stored at 4 °C. The aminated CNBr-TLL was called CNBr-TLL-A.

Immobilization of TLL on Glyoxyl-Agarose Beads

Immobilization was performed as previously described [40]. TLL-A was desorbed from octyl-sepharose and the pH was adjusted with 1 M sodium bicarbonate at pH 9 or 10 to obtain a final concentration of 100 mM. The immobilized enzyme derivatives were prepared using 1 g of glyoxyl-support and 10 mL of purified TLL-A. The mixture was maintained at the indicated temperatures during the desired times (see below). Reduced glyoxyl-agarose samples were used as controls to discard unspecific adsorptions. As reaction end-point, solid sodium borohydride was added to a concentration of 1 mg/mL [21] and the mixture was maintained at 25 °C under very gentle stirring. After 30 min, the immobilized and reduced derivatives were washed thoroughly with distilled water.

The glyoxyl biocatalysts were the following: **Gx(9/10)-TLL-A**, prepared by immobilization of TLL-A on glyoxyl-agarose at 25 °C and pH 9 (8 h) and further incubated at pH 10 and 25 °C for 16 h; **Gx(10)-TLL-A**, prepared by immobilization of TLL-A during 24 h at 25 °C and pH 10.

Incubation of TLL preparations on caotropic agent solutions

The different TLL preparations were incubated in 50 mM phosphate buffer containing 8M of guanidine hydrochloride at pH 7.0 and 25 °C for different times. Samples were periodically withdrawn and the remaining activity of the immobilized enzyme was determined.

When activity did not decrease for further 30 minutes of incubation, the immobilized enzyme was washed with buffer and incubated as described below.

Thermal inactivation of different TLL immobilized preparations

TLL preparations were incubated in 50 mM sodium phosphate at pH 7.0 and 70 °C. Samples were periodically withdrawn using a pipette with a cut-tip and under vigorous stirring to have a homogeneous biocatalyst suspension. The activity was measured using the pNPB assay described above in the presence or absence of CTAB. Initial activity was determined in the absence or the presence of CTAB.

Inactivation of different TLL immobilized preparations in the presence of organic cosolvent.

TLL preparations were washed with 95% dioxane/ 5% 50 mM Tris-HCl aqueous solution at pH 7 and 4 °C. Subsequently, the enzyme derivatives were resuspended in the same solution and incubated at 25 °C. The activity was measured using the pNPB assay described above in the presence or absence of CTAB. Initial activity was determined in the absence or the presence of CTAB.

Reactivation experiments

Fully or partially inactivated TLL preparations (sometimes after incubation in saturated guanidine solution) were resuspended in 50 mM sodium phosphate at pH 7 and their activities were determined over time. When a constant value of residual activity was achieved, this was considered the maximum recovered activity. In some cases, several consecutive cycles of inactivation/reactivation of immobilized TLL were performed. The activity was measured using the pNPB assay described above in the presence or the absence of CTAB. Initial activity was determined in the absence or the presence of CTAB.

Results and Discussion

Effect of the immobilization strategy on the recovered activity after incubation on saturated guanidine solution

Figure 1 shows the effect of incubating the different immobilized preparations of TLL described in Methods in saturated solutions of guanidine, and later in aqueous buffer. The effect of the amination on the possibilities of reactivating immobilized TLL was studied by comparing CNBr-TLL and CNBr-TLL-A, which are immobilized but not stabilized derivatives (linked to the support by just some few bonds) [43]. In aqueous medium, both preparations recovered only a small percentage of activity, around 20% using CNBr-TLL, and only 10% using CNBr-TLL-A. When the activity was assayed in the presence of CTAB, CNBr-TLL recovered full activity (compared to the initial activity in the presence of similar concentrations of detergent), while CNBr-TLL-A recovered less than 60% of its initial activity in the presence of CTAB. When the reactivation was performed in the presence of CTAB and the activity determination was carried out in the absence of this detergent, activity recoveries were even slightly smaller than using pure aqueous buffer solution, suggesting that CTAB did not play a important role in the initial refolding of the enzyme, just in the last steps.

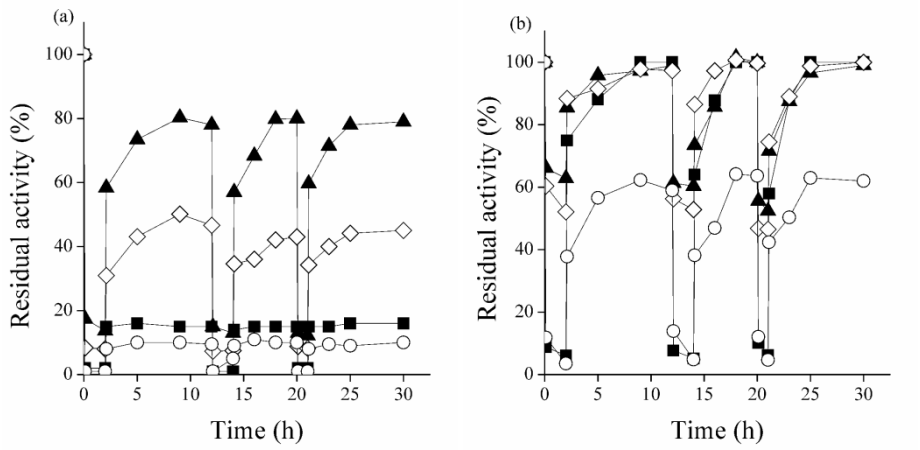

Figure 1. Effect on TLL immobilized preparations activity of their incubation in guanidine followed by the incubation in sodium phosphate 50 mM pH 7. Figure shows 3 cycles. (a) measured in the absence of CTAB; (b) measured in the presence of CTAB. 100% was taken in each case as the activity measured in the absence or presence of CTAB, respectively. (■) CNBr-TLL; (○) CNBr-TLL-A; (▲) Gx(9/10)-TLL-A (◇) Gx(10)-TLL-A.

The much higher activity recovery when using detergent in the activity measurement suggests that the correct opening of the lipase may be a key step in the recovering of enzyme activity. Detergent may have a role in stabilizing the open form of the lipase, making this conformational change easier [31]. Notwithstanding, the amination of the carboxylic groups in the enzyme surface presented a negative, but not drastic, effect on the capability of the enzyme to recover an active form after incubation in guanidine. These inactivation/reactivation steps could be repeated for several cycles with very similar values, suggesting that the preparations are reaching similar structures in each cycle.

The recovered activity in absence of detergent was greatly improved when using both multipointly immobilized preparations, although both derivatives were prepared using aminated TLL, and the amination apparently presented a negative effect on the enzyme reactivation. Considering the fairly negative effect of the amination on the possibilities of TLL reactivation, the reasons for this improved activity recovery should be attributed to the multipoint covalent attachment of the enzyme to the support. The groups attached to the support must keep their relative positions during any conformational change, and it also seems to remain in the right relative positions even after incubation with saturated solutions of caotropic reagents. These reference points may help to regain the active form of the enzyme when incubated under mild conditions. Curiously, although the stabilities of both glyoxyl preparations were similar [40], Gx(9/10)-TLL-A seemed to be better considering the recovered activities. Gx(10)-TLL-A recovered around 50% of the activity, while Gx(9/10)-TLL-A recovered 80%. The difference in activities recoveries using both preparations suggests that the orientation of both preparations could not be identical, and that the groups involved in the multipoint attachment in one of the orientations could be more relevant to get a correct reactivation than in the other orientation.

In the presence of detergent, both glyoxyl preparations recovered 100% of their initial activity (also measured in the presence of detergent), as the CNBr-TLL preparation. Again, this higher activity recovery could be explained hypothesizing that the correct opening of the

lipase may be one of the hardest points in TLL reactivation, even in these rigidified preparations.

Effect of the immobilization strategy on the enzyme stability in the presence of organic solvents and their further reactivation

The four immobilized TLL preparations were inactivated in the presence of 95% dioxane (Figure 2). As previously described [40], the multipoint covalent attached preparations were much more stable than the CNBr-TLL and CNBr-TLL-A preparations. On the other hand, both CNBr preparations and both glyoxyl preparations presented almost identical stabilities.

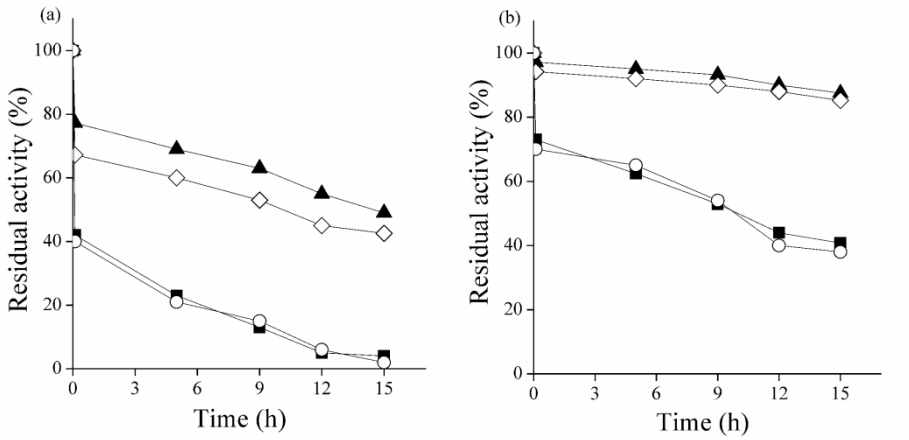

Figure 2. Inactivation courses of TLL preparations in the presence of 95% of dioxane at pH 7 and 25 °C. (a) measured in the absence of CTAB; (b) measured in the presence of CTAB. 100% was taken in each case as the activity measured in the absence or presence of CTAB, respectively (■) CNBr-TLL; (○) CNBr-TLL-A; (▲) Gx(9/10)-TLL-A (◇) Gx(10)-TLL-A.

However, inactivation courses were slower when assaying the enzyme activity in the presence of detergent, suggesting that the correct opening of the lipase was a key point in the inactivation of the enzyme, and perhaps one of the first steps in the enzyme inactivation process was an obstacle in yielding the open form of the lipase.

To check the reversibility of the inactivation, the different immobilized enzyme preparations were incubated in aqueous buffer, and the evolution of the activity was recorded

over time (Figure 3). Both CNBr preparations recovered only a small percentage of their initial activities after re-incubation in aqueous buffer in only 1 hour (around 5-10%) and remained constant for 15 h, while both glyoxyl preparations recovered 65-75% of their initial activities. In this case, the reactivation was slower and the maximum activity recovery was after 10 hours of incubation, as could be expected by the higher rigidity of the preparation. Thus, it seems that the inactivation of the immobilized TLL in 95% dioxane was mainly due to the establishment of an incorrect conformation, which can partially be reversed to a more active form when incubated in aqueous medium. The amination of TLL has a slightly negative effect for the enzyme reactivation. However, this negative effect was compensated by the multipoint covalent attachment of the aminated enzyme with the glyoxyl.

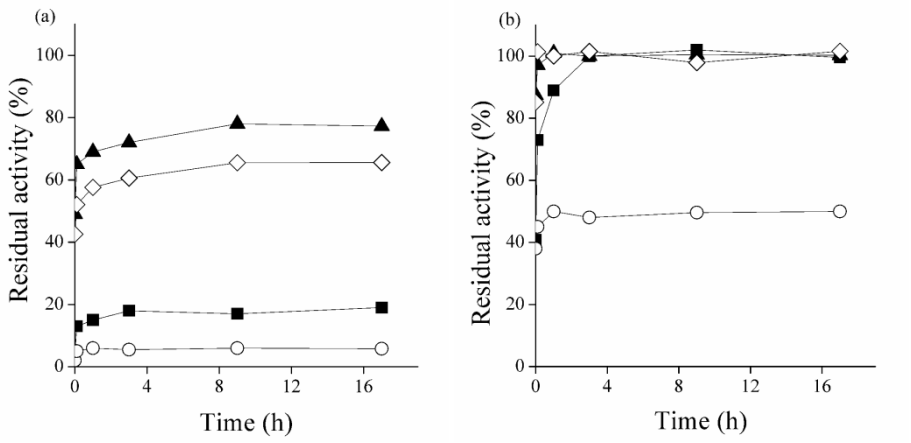

Figure 3. Effect of the incubation in 50 mM sodium phosphate at pH 7 and 25 °C on the activity of TLL preparations inactivated in the presence of 95% of dioxane at pH 7 and 25 °C. 100% is considered the activity before enzyme inactivation by the organic solvent. (a) measured in the absence of CTAB; (b) measured in the presence of CTAB. 100% was taken in each case as the activity measured in the absence or presence of CTAB, respectively (■) CNBr-TLL; (○) CNBr-TLL-A; (▲) Gx(9/10)-TLL-A (◇) Gx(10)-TLL-A.

When measured in the presence of detergent, both glyoxyl preparations and CNBr-TLL recovered 100% of their initial activities (measured also in the presence of detergent). However, CNBr-TLL-A only recovered around 50% of its initial activity. Again, the presence of detergent during the measurement, a condition that helps the opening mechanism of the lipase, posed a positive effect for recovering enzyme activity. This allowed reaching to the

initial activities for all enzyme preparations, with the exception of CNBr-TLL-A (non stabilized and chemically modified enzyme). The correct opening of the lipase, mediated by the detergent [31], seems to be a critical step in the TLL recovering of the activity after inactivation caused by organic solvents.

The results using CNBr-TLL-A suggest that the amination makes it more difficult to recover the initial conformation of the lipase, perhaps due to the change of ionic bridges in the surface by repulsion forces. However, even in this more negative case, the recovered activity could reach 50% by just incubating it in aqueous buffer. The “reference system” obtained by the multipoint covalent attachment is able to diminish the negative effects of the amination, and even to recover higher activity than using non stabilized preparations of unmodified TLL.

Effect of the immobilization strategy on the enzyme stability at high temperature and their further reactivation

Figure 4 shows the inactivation of the four preparations of TLL. Again, the amination did not affect enzyme stability, while the multipoint covalent attachment presented a clear enhancement of the enzyme stability [40]. Moreover, a positive effect of the presence of detergent during the activity measurement could also be detected; suggesting that during thermal inactivations the loss of the opening capability of the lipase could also be a key point in enzyme inactivation. However, enzyme inactivation rates determined in absence or presence of detergents did not vary as significantly as when the inactivation was caused by organic solvents.

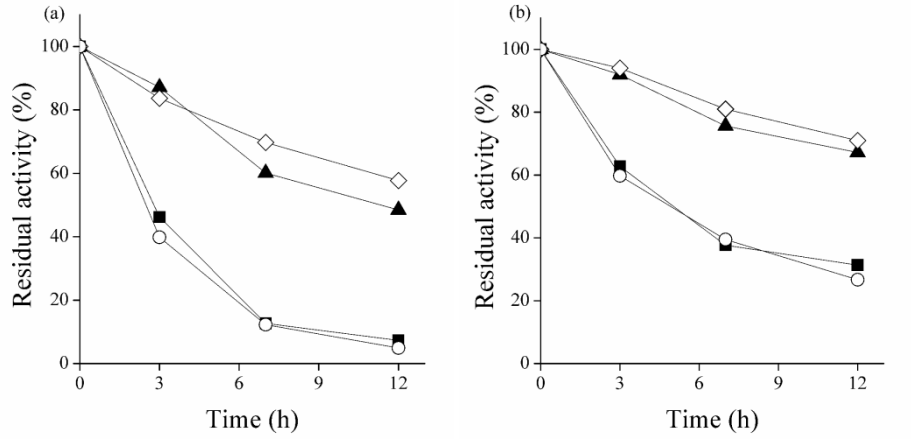

Figure 4. Inactivation courses of TLL immobilized preparations in 50 mM sodium phosphate at 70 °C and pH 7 (a) measured in the absence of CTAB; (b) measured in the presence of CTAB. (100% was taken in each case as the activity measured in the absence or presence of CTAB, respectively ■) CNBr-TLL; (○) CNBr-TLL-A; (▲) Gx(9/10)-TLL-A (◇) Gx(10)-TLL-A.

When the partially thermal-inactivated enzymes were incubated at 25 °C (Figure 5), the activity of the immobilized preparations using the aminated enzyme remained almost unaltered even after long incubation periods, suggesting that the spontaneous reactivation was not possible. CNBr-TLL recovered a small percentage of the activity. Similarly, when measured in the presence of detergent, all the preparations using TLL-A remained with their activities unchanged. However, CNBr-TLL was able to recover a significant amount of enzyme activity when measured in the presence of detergent (from 30% to 60%). The results suggest that the amination of TLL did not produce negative effects upon enzyme activity, stability, and reactivation capabilities when the inactivation is caused by solvents, where the enzyme was not expected to change chemically. However, when the inactivation cause was the exposition to high temperatures, this presented a negative effect on the possibilities of enzyme reactivation, perhaps by the chemical sensitivity of the amino groups to these moderately aggressive conditions. For example, the new amino groups could be oxidized to nitro groups [24-27], and this could produce a more difficult reactivation of the enzyme.

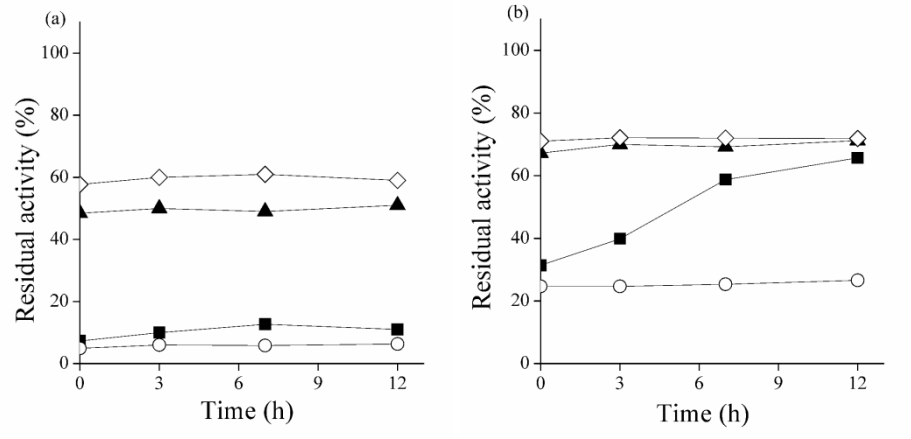

Figure 5. Effect of the incubation in 50 mM sodium phosphate at pH 7 and 25 °C on the activity of TLL preparations inactivated by incubation in 50 mM sodium phosphate at 70 °C and pH 7. (a) measured in the absence of CTAB; (b) measured in the presence of CTAB. 100% is considered the activity before enzyme inactivation by incubation at 70 °C, measured in the absence or the presence of CTAB (■) CNBr-TLL; (○) CNBr-TLL-A; (▲) Gx(9/10)-TLL-A (◇) Gx(10)-TLL-A.

Reactivation of partially inactivated enzymes

Finally, the possibilities of improving enzyme reactivation by incubating the partially inactivated enzyme preparations on saturated guanidine solutions before incubating under mild conditions was studied. Figure 6 shows that when the glyoxyl preparations inactivated in 95% dioxane were incubated in saturated guanidine before the incubation in aqueous buffer, the recovered activity was slightly higher than when directly incubated in aqueous medium (mainly when using Gx(9/10)-TLL-A). However, the recovered activity after each inactivation cycle was slightly lower (in both cases, direct incubation in aqueous buffer or passing by guanidine), suggesting that the incubation in saturated guanidine was not enough to fully unfold the enzyme structure. That way, some incorrect structure remained after incubation in guanidine which produced this progressive decrease in the recovered activity. The CNBr preparations, either aminated or not, recovered a very small amount of their initial activity, as expected according to the results of recovered activity after incubation in guanidine. However, in the case of CNBr-TLL, the recovered activity was almost identical in each cycle

when incubated with guanidine, allowing the recovery of similar activities after several solvent inactivation-guanidine incubation-reactivation cycles.

After incubation in saturated guanidine and when measured in the presence of detergent (results not shown), all preparations reversed to 100% of their initial activities, except for CNBr-TLL-A that recovered 60% in the first cycle and in each cycle decreased by an additional 10%.

These results suggest that the amination of TLL could have a negative effect on the enzyme capabilities of reactivation, and multipoint covalent attachment was enough to revert this negative effect and allowed to recover a much higher activity during several cycles, either by direct incubation in aqueous buffer or by previous incubation in saturated guanidine solutions. The presence of detergent during the measurements allowed recovering 100% of the enzyme activity in all 3 cycles, suggesting that the main problem for enzyme reactivation is the recovery of the opening mechanism of the lipase.

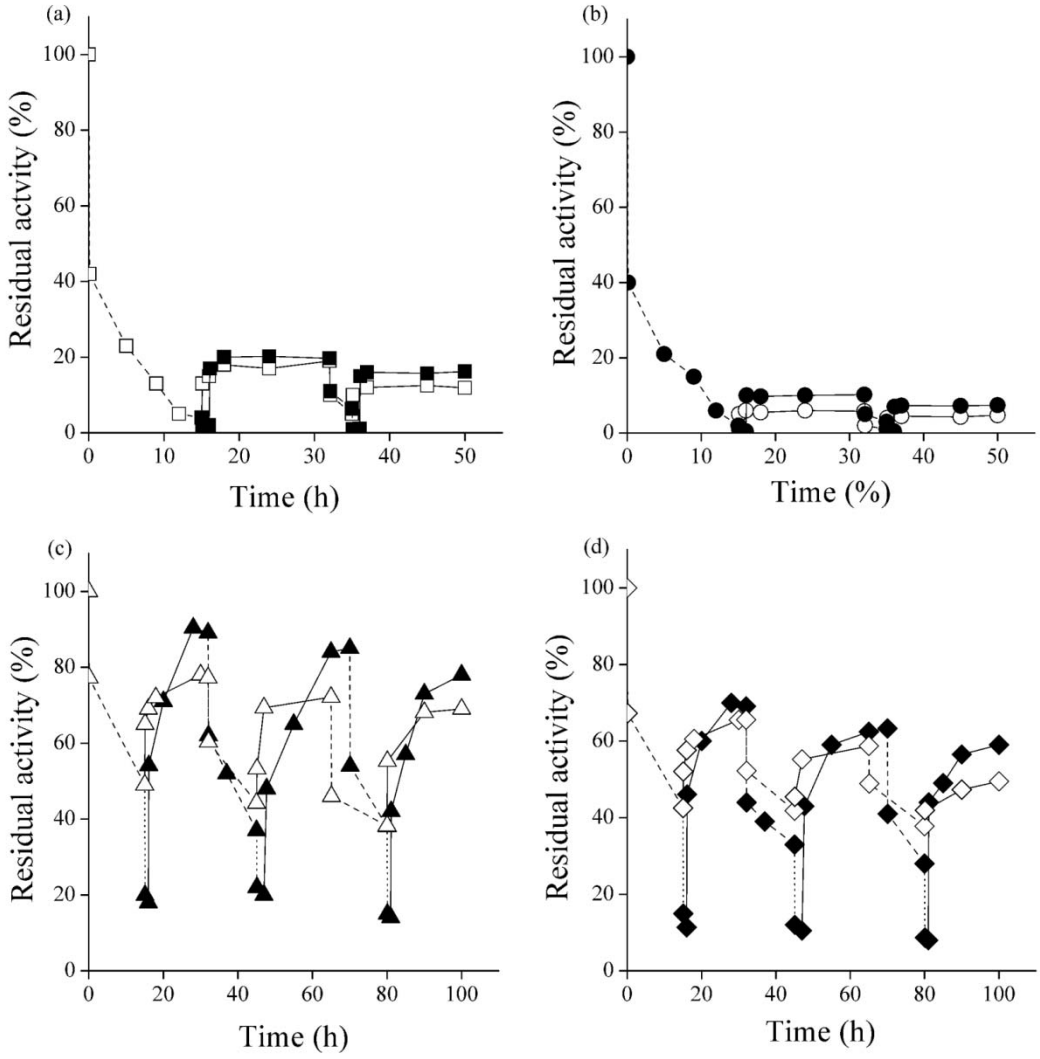

Figure 6. Evolution of the activity of different TLL preparations during incubation in the presence of 95% of dioxane, at pH 7 and 25 °C, followed two different treatments: 1- direct incubation in aqueous medium at 25 °C or 2- an incubation of the partially inactivated biocatalysts in saturated sodium guanidine solutions followed by an incubation in aqueous buffer. (a) CNBr-TLL; (b) CNBr-TLL-A; (c) Gx(9/10)-TLL-A; (d) Gx(10)-TLL-A. All the measures were in the absence of CTAB. Open symbols: incubation in aqueous buffer solution; Filled symbols: incubation in guanidine and incubation in aqueous buffer solution. Dashed line: incubation in inactivation agent; Solid line: incubation in aqueous buffer; Dotted line: incubation in guanidine.

In thermal inactivation (Figure 7), incubation in guanidine slightly improved the recovery of activities of BrCN-TLL. However, the incubation in guanidine of immobilized preparations from TTL-A (both glyoxyl and CNBr) inactivated by heat, produced a decrease in the recovered activities. Using the glyoxyl preparations, the direct incubation at 25 °C

allowed to recover a significant amount of activity, but the recovered activity significantly decreased if the partially inactivated preparation was incubated in guanidine (e.g., from 65 to 50% for Gx(10)-TLL-A). The recovered activity in each cycle clearly decreased; in the third cycle, the recovered activity after incubation at 25 °C was 50%, and after the guanidine step it was 30%.

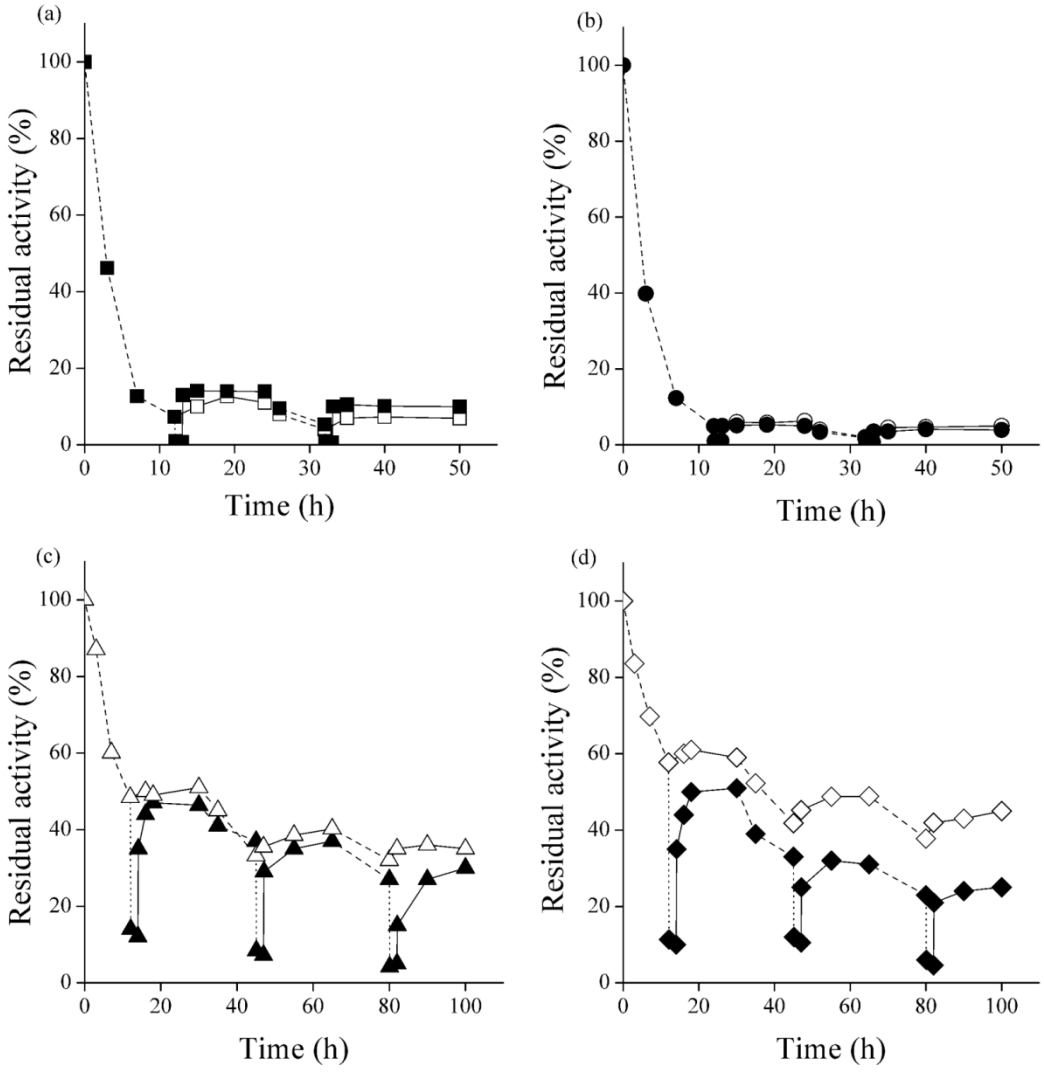

Figure 7. Evolution of the activity of different TLL preparations during incubation in 50 mM sodium phosphate at 70 °C and pH 7, followed two different treatments: 1- direct incubation in aqueous medium at 25 °C or 2- an incubation of the partially inactivated biocatalysts in saturated sodium guanidine solutions followed by an incubation in aqueous buffer. (a) CNBr-TLL; (b) CNBr-TLL-A; (c) Gx(9/10)-TLL-A; (d) Gx(10)-TLL-A. All the measures were in the absence of CTAB. Open symbols: incubation in aqueous buffer solution; Filled symbols: incubation in guanidine and incubation in aqueous buffer solution. Dashed line: incubation in inactivation agent; Solid line: incubation in aqueous buffer; Dotted line: incubation in guanidine.

Therefore, in the case of thermal inactivation, the amination seems to be more negative (for the enzyme reactivation) than when the inactivation was caused by dioxane or guanidine, perhaps due to the sensitivity of these groups to chemical transformations. These modified amino groups could turn the recovery of a more complex active form after incubation in guanidine, promoting a decrease in the enzyme activity recovery.

Conclusions

From a practical point of view, the results show that partially inactivated TLL immobilized preparations, by either heat or solvents, may be partially reactivated by a simple incubation under mild conditions (aqueous buffer and 25 °C for 1 to 10 h), extending the useful lifespan of the biocatalysts. The reactivation is easier to achieve if the inactivation cause is due to incubation in organic solvents, and a previous incubation in saturated guanidine may increase the recovered activity in this case.

Apparently, the key point in the inactivation and reactivation of TLL is the recovery of the mechanism of the opening of the lid. The activity determination in the presence of detergent allows improving the enzyme activity recovery and slowing down the enzyme inactivation. This detergent has been reported to produce a strong hyperactivation of the TLL [32], very likely by stabilizing the open form of the lipase [31], although TLL has been reported to be destabilized by this detergent [29].

The amination of TLL was strictly necessary to get a high stabilization of TLL by multipoint covalent attachment. However, amination of the enzyme seems to produce a negative effect on enzyme reactivation under chemically inert conditions, but this negative effect may be completely reversed after multipoint covalent attachment, allowing for several cycles to recover very high yields of activity. Nevertheless, at high temperatures, the amination seems to be far more negative, with lower activities recoveries and also a negative

effect of the incubation of the inactivated enzyme in guanidine. The reactivity of these primary amino groups may be the cause of this negative effect.

The multipoint attached TLL preparations, although based in aminated enzymes, allowed recovery of high activities after incubation under mild conditions, higher than those for non-aminated enzymes but attached to the support only by one or some few bonds. The two different stabilized preparations recovered different percentages of activity after inactivation, being Gx(9/10)-TLL-A better in reactivations after dioxane or guanidine inactivation. These results suggest a new parameter to be studied when designing an industrial catalyst. Not only enzyme activities and stabilities must be considered, but also the capabilities of reactivation should be included in the optimization of biocatalysts.

Acknowledgments

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CAPÍTULO VIII - TWO STEP ETHANOLYSIS: SIMPLE AND EFFICIENT WAY TO IMPROVE ENZYMATIC BIODIESEL SYNTHESIS CATALYZED BY IMMOBILIZED-STABILIZED LIPASE FROM *Thermomyces lanuginosus*

Neste capítulo serão apresentados os resultados da aplicação dos derivados obtidos nos estudos anteriores na reação de síntese de biodiesel. Inicialmente o derivado foi testado nas condições estabelecidas no Capítulo III, e posteriormente a reação de transesterificação foi aperfeiçoada através da adição de etanol em etapas. Os resultados estão apresentados na forma de um manuscrito submetido para publicação na revista *Biomass and Bioenergy*.

Two step ethanolysis: simple and efficient way to improve enzymatic biodiesel synthesis catalyzed by immobilized-stabilized lipase from *Thermomyces lanuginosus*

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Two step ethanolysis: simple and efficient way to improve enzymatic biodiesel synthesis catalyzed by immobilized-stabilized lipase from *Thermomyces lanuginosus*

Abstract

In this work we describe the immobilization and stabilization of lipase from *Thermomyces lanuginosus* (TLL) on a support containing glyoxyl groups (Gx-TLL). The immobilization was fast, with high activity recoveries, and the Gx-TLL preparation presented higher stability to thermal inactivation than a commercial TLL preparation, Lipozyme TL-IM. Gx-TLL was tested for the enzymatic transesterification reaction of ethanol and soybean oil. The reaction was carried out as a 7.5:1 ethanol:soybean oil molar ratio, 15% of immobilized enzyme and 4% of water at 30 °C. In the presence of n-hexane, the transesterification achieved 100% of yield conversion, while in solvent-free system the yield of conversion was 75%, and at stoichiometric molar ratio 70%. The ethanolysis carried out by three stepwise addition of ethanol produced 80% of conversion, but when two step ethanolysis was employed, 100% of yield conversion was reached in 10 h of reaction, both for solvent and solvent-free systems.

Keywords: Biodiesel, *Thermomyces lanuginosus* lipase, glyoxyl supports, ethanolysis, soybean oil

Introduction

Foreseen crisis of supply and amounting environmental problems associated with the petroleum industry, are pushing the efforts in search for renewable and environmentally friendly biofuels. In this sense, biodiesel becomes an alternative to diesel, since it is made entirely from vegetable oil or animal fats, thus being renewable and biodegradable [1-3].

Biodiesel is produced by transforming triglycerides into fatty acid alkyl esters, in the presence of an alcohol, such as methanol or ethanol, and an acid or alkali catalyst, with glycerol as a byproduct [4]. Alternatively, the enzymatic reaction with lipases can be used as biocatalysts in the biodiesel synthesis [5]. Unlike the conventional chemical routes for synthesis of biodiesel, biocatalysis routes allow to carry out the transesterification of a wide variety of oil feedstocks in the presence of acidic impurities, such as free fatty acids frequently present in oil samples. Moreover, separation and purification of enzymatically produced biodiesel is simplified due to the absence of soap and other by-products. The main disadvantage of this biotechnological process is related to the cost of the biocatalysts, which so far is more expensive than the chemical reaction. Therefore, to turn the biocatalytic route economically competitive is very important the development of stable and active biocatalysts in order to improve conversions in the shortest possible time and to allow the reuse of enzyme for as much batches as possible.

Multipoint covalent attachment is a well described method for enzyme immobilization-stabilization [6] where the enzyme preparations generally present a much higher stability than that of the soluble enzyme. Supports containing glyoxyl groups are the most used to apply this technique to enzyme immobilization [7, 8]. The enzymes only become immobilized on the support when several simultaneous enzyme-support attachments are produced between the reactive aldehyde groups of the support [9] and the primary amines groups of the enzyme [10].

Lipase from *Thermomyces lanuginosus* (TLL) is the enzyme responsible for the lipolytic activity of Lipolase® and Lipozyme TL-IM, commercial lipase preparations supplied

by Novozymes. This enzyme has been recently stabilized by coupling the chemical amination of the enzyme surface to the multipoint covalent attachment of the modified enzyme to glyoxyl-agarose beads [11]. The amination of the enzyme was strictly necessary in order to immobilize the TLL to the glyoxyl support with good activity recovery, and neither the enzyme stability nor activity was affected. The glyoxyl-TLL preparations presented very good activity recovery and a fairly high stabilization against inactivation at high temperatures or in the presence of organic solvents. The achieved stability suggested a very intense multipoint covalent attachment.

Some reports show the ability of TLL to synthesize biodiesel from vegetable oils, either in a free or immobilized form [12-18]. However, TLL is well known as one of the lipases with a strict *sn*-1,3 regiospecificity, thus, to obtain a full transesterification, acyl migration from *sn*-2 position to *sn*-1,3 position should occur during the reaction. Some works deal with the acyl migration in reactions catalyzed by TLL and its cause [12, 17, 19, 20].

In this work, we studied the enzymatic synthesis of biodiesel catalyzed by the lipase from *Thermomyces lanuginosus*. The reaction was carried out with soybean oil and ethanol, and two derivatives of TLL were used: one preparation was multipoint covalently immobilized on a glyoxyl support (Gx-TLL), and the commercial Lipozyme TL-IM. To improve the content of ethyl esters, the following strategies were tested: single step ethanolysis under the optimal conditions determined in a previous study [15] and using the stoichiometric molar ratio between alcohol and oil (3:1); and the two or three stage stepwise methodology developed by Watanable et al. [21], where the alcohol was added one molar equivalent at time in three steps, and 2 molar equivalent initially, followed by 1 molar equivalent at desired time in two steps.

Material and Methods

Materials

Lipase from *Thermomyces lanuginosus* (TLL) free and immobilized (Lipozyme TL-IM) were kindly donated by Novozymes (Denmark). 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), *p*-nitrophenyl butyrate (p-NPB), 1,2-ethylenediamine (EDA), and hexadecyltrimethylammonium bromide (CTAB) were from Sigma. Octyl-Sepharose CL-4B was purchased from GE Healthcare (Uppsala, Sweden). Lewatit VP OC1600 was purchased from Bayer (Leverkusen, Germany) and its modification to glyoxyl (activated with 200 μmols/g of support) was performed as described elsewhere for glyoxyl-agaorse [7]. Refined soybean oil was purchased in a local market. Ethanol and other reagents and solvents used were of analytical or HPLC grade.

Methods

Enzymatic activity assay.

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM pNPB in 25 mM sodium phosphate at pH 7 and 25 °C, using a thermostatized spectrum with continuous magnetic stirring. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One unit of pNPB activity was defined as the amount of enzyme necessary to hydrolyze 1 *μ*mol of pNPB/min (U) under the conditions above described.

Purification of TLL

Prior to use, TLL was purified by interfacial adsorption on hydrophobic supports. The enzyme was adsorbed on octyl-sepharose beads under continuous stirring in 10 mM sodium phosphate at pH 7.0, according to a previously described procedure [22, 23]. Periodically, the activity of suspensions and supernatants was measured by the pNPB assay. After

enzyme adsorption, the lipase preparation was vacuum filtered using a sintered glass funnel and abundantly washed with distilled water. TLL was desorbed from octyl-sepharose by suspending the immobilized enzyme in a relation 1/10 (w/v) in 25 mM sodium phosphate at pH 7.0 containing 0.6% (v/v) of CTAB during 1 h at room temperature.

Chemical amination of immobilized TLL

Chemical amination was performed as previously described [24]. A total of 1 g of immobilized lipase on octyl-sepharose beads was added to 10 mL of 1 M EDA at pH 4.75 under continuous stirring. Solid EDC was added to the suspension to a final concentration of 10 mM. These reaction conditions have been reported as capable of fully modifying all exposed carboxylic groups of the protein [25-29]. After 90 min of gentle stirring at 25 °C, the immobilized-modified preparations were vacuum filtered using a sintered glass funnel and incubated for 4 h in 0.1 M hydroxylamine at pH 7 and 4 °C to recover the EDC-modified tyrosines [30]. The enzyme preparations were filtered and washed with 25 mM sodium phosphate at pH 7.5 and with an excess of distilled water. The aminated TLL immobilized on octyl-sepharose, later used to obtain the soluble aminated enzyme, was stored at 4 °C.

Immobilization of TLL on Glyoxyl-Lewatit Beads

Lewatit was modified to obtain glyoxyl groups [7] on its surface which allows the immobilization by covalent attachment. The immobilization procedure followed the previous protocol determined by immobilization on glyoxyl-agarose [11]. TLL-A was desorbed from octyl-sepharose and the pH was adjusted with 1 M sodium bicarbonate at pH 9 to obtain a final concentration of 100 mM using as immobilization buffer. The immobilized enzyme derivatives were prepared using 1 g of glyoxyl-support and 10 mL of purified TLL-A and 25% of glycerol to avoid hydrophobic adsorption with the support and the immobilization takes place only by covalent attachment. The mixture was maintained at pH 9 and 25 °C by 3 h

and further incubated at pH 10 and 25 °C for 4 h. Reduced glyoxyl-agarose was used as controls to discard unspecific adsorptions. As reaction end-point, solid sodium borohydride was added to a concentration of 1 mg/mL [8] and the mixture was maintained at 25 °C under very gentle stirring. After 30 min, the immobilized and reduced derivatives were washed thoroughly with distilled water. The glyoxyl derivative was termed Gx-TLL.

Single step alcoholysis reaction

The reaction conditions were determined in a previous study [15]. To evaluate the different lipase preparations, 2 g of soybean oil were mixed with ethanol (7.5:1 alcohol:oil molar ratio), 15% (based on oil weight) of immobilized lipase and 4% (based on oil weight) of water. The other tested condition was the alcoholysis under the stoichiometric alcohol:oil molar ratio (3:1 molar ratio). The reactions were carried out in 50 mL Erlenmeyer flasks in an orbital shaker (200 rpm) at 30 °C for 10 h, in the presence or absence of 2 mL of n-hexane as solvent. Samples were periodically withdrawn from the flasks and analyzed.

Stepwise alcoholysis reaction

The modified stepwise ethanolysis of soybean oil proposed by Watanabe et al. [21] was conducted as follows. For three stage ethanolysis, 2 g of soybean oil were mixed with 1:1 alcohol:oil molar ratio, 15% (based on oil weight) of immobilized lipase and 4% (based on oil weight) of water. 0.11 g of ethanol (1/3 the molar equivalent of the initial soybean oil) was added at 0, 3 and 6 h reaction. For two stage ethanolysis, 2 g of soybean oil were mixed with 2:1 alcohol:oil molar ratio, 15% (based on oil weight) of immobilized lipase and 4% (based on oil weight) of water. 0.22 g of ethanol (2/3 the molar equivalent of the initial soybean oil) was added at the start of reaction and 0.11 g of ethanol was added after 6 h. The reactions were carried out in 50 mL Erlenmeyer flasks in an orbital shaker (200 rpm) at 30 °C for 10 h, in the presence or absence of 2 mL of n-hexane as solvent. Samples were periodically withdrawn from the flasks and analyzed.

HPLC analysis

To samples, 5 mL of distilled water were added and centrifuged at 2,500 *g*, 15 min, and 4 °C. The lower phase containing glycerol and remaining ethanol was analyzed by HPLC. In order to verify whether the glycerol could be related to the liberation of esters, free fatty acids in the vegetable oils and in the product reactions were periodically monitored by titration with NaOH [31]. This was necessary in order to quantify the degree of unwanted hydrolysis.

Glycerol and ethanol concentrations were determined by HPLC with a refractive index (RI) detector (Perkin Elmer Series 200, USA) and a Phenomenex RHM monosaccharide column (300 x 7.8 mm), at 80 ºC, using ultrapure water as eluting solvent, flow of 0.6 mL.min^{-1} and sample volume of 20 µL. The percentage yield conversion was calculated as follows:

$$Conversion = \left[\frac{mmol\ remaining\ ethanol}{mmol\ initial\ ethanol} \right] * 100\ \% \quad (1)$$

Results and Discussion

Immobilization of TLL on Glyoxyl-Lewatit

Lewatit VP OC 1600 is a macroporous resin that consists of poly(methyl methacrylate-*co*-divinylbenzene) and has average values of particle size, surface area, and pore diameter of 315–1,000 μm, 130 $m^2.g^{-1}$, and 150 Å, respectively [32]. It has been used by Novozymes to immobilize the lipase B from *Candida antarctica* by hydrophobic adsorption [33]. In this work, Lewatit was modified to allow the covalent immobilization of the enzyme. Fig. 1 shows the time course of TLL immobilization on glyoxyl-Lewatit initially at pH 9 for 3 h, followed by incubation at pH 10 for 4 h. Full immobilization was achieved in 2 h, and the additional time of immobilization was necessary to obtain a multipoint interaction between enzyme and support [9].

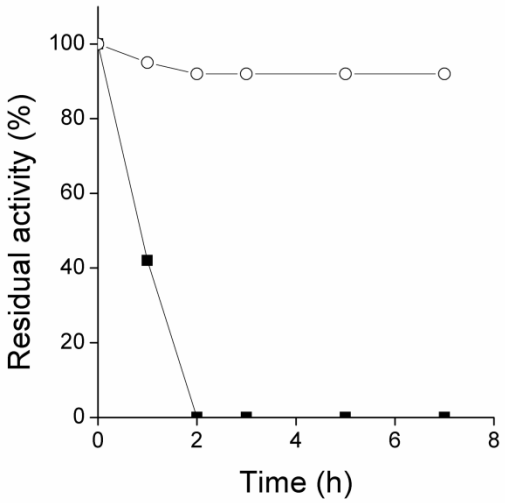

Figure 1. Time course of TLL immobilization on glyoxyl-Lewatit. The immobilization was carried out at 25 °C and pH 9 for the first 3 h and then at pH 10 until the end of reaction. (○) immobilization suspension; (■) supernatant of the immobilization suspension.

In order to verify the stability of the prepared derivative, it was carried out the thermal inactivation at 60 °C and compared with the commercial preparation Lipozyme TL-IM and a mild covalent immobilization on cyanogen bromide activated agarose. Fig. 2 shows that Gx-TLL was much more stable than the other two preparations, probably because the lipase was

multipointly attached to the support. Thus, this very active and stable preparation was tested on the enzymatic transesterification reaction.

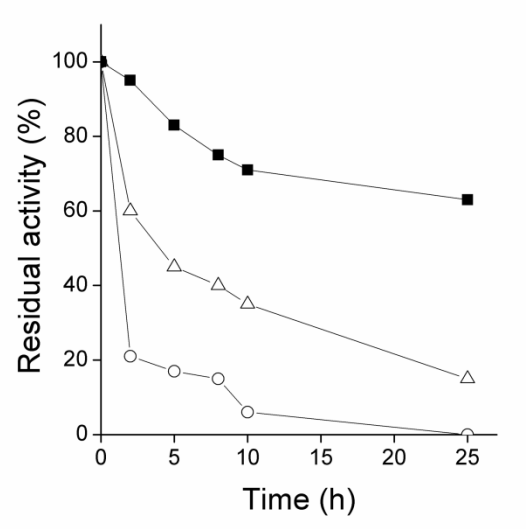

Figure 2. Inactivation courses of TLL preparations in 20 mM sodium phosphate at 60 °C and pH 7. (■) Gx-TLL; (○) CNBr-TLL; (△) Lipozyme TL-IM.

Single step alcoholysis

The ethanolysis of soybean oil catalyzed by Lipozyme TL-IM (Novozymes) from *Thermomyces lanuginosus* has been previously optimized [15], producing a yield conversion of 66% after 12 h of reaction. In this research, Gx-TLL was evaluated in the transesterification reaction under the optimized conditions described in Methods, and the results are presented in Fig. 3. As stated in the Introduction, TLL presents a 1,3-regio specificity, which allows a theoretical conversion of 66% and, in order to obtain higher conversions, it is necessary an acyl migration. Thus, the reaction was carried out in a solvent-free system, as well as in the presence of n-hexane, because it was reported that solvents could be help the acyl migration [19, 20].

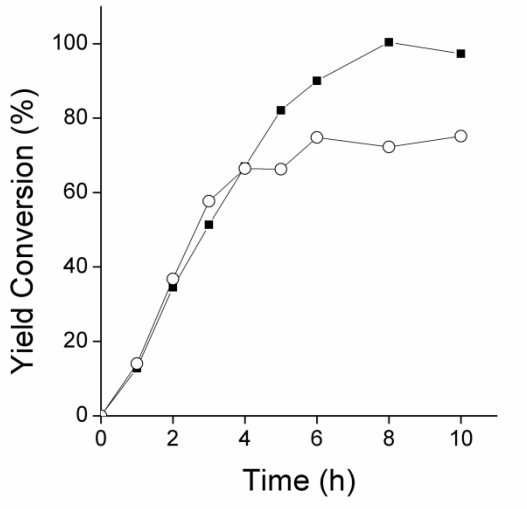

Figure 3. Time course for biodiesel synthesis catalyzed by Gx-TLL. Conditions: 30 °C, 7.5:1 ethanol:soybean oil molar ratio, 15% of enzyme and 4% of added water. (■) Reaction with n-hexane in the reaction medium; (○) Reaction in solvent-free system.

In the solvent-free system, around 75% of yield conversion was obtained in 6 h, higher than for Lipozyme TL-IM [15], and slightly higher than the predicted theoretical conversion, indicating that some acyl migration occurred. However, when the reaction was performed in a solvent medium, full conversion was reached in 8 h. This complete reaction could be explained by the effect of the non-polar solvents, as n-hexane, exert on the rates of acyl migration [19]. However, the use of organic solvent in the reaction medium to improve the enzymatic biodiesel synthesis catalyzed by TLL, will add a step in the biodiesel purification, because this solvent needs to be removed along with the remaining ethanol at the end of reaction. In this way, we tested the transesterification under the stoichiometric molar ratio, since when the reaction was complete, all the ethanol should be consumed, leaving only the solvent to be removed. These results are shown in Fig. 4. Under these conditions, 70% of yield conversion was obtained, either in the presence or absence of n-hexane. It was verified that the excess of ethanol was necessary, not only to ensure high reaction rates, but also to minimize diffusion limitations, and to keep soluble the glycerol formed during the reaction [13, 34], as observed before to increase the rates of acyl migration. However, the presence of solvent was not enough in this case to obtain a higher

yield conversion and, therefore, we tested the stepwise alcoholysis proposed by Watanable et al. [21].

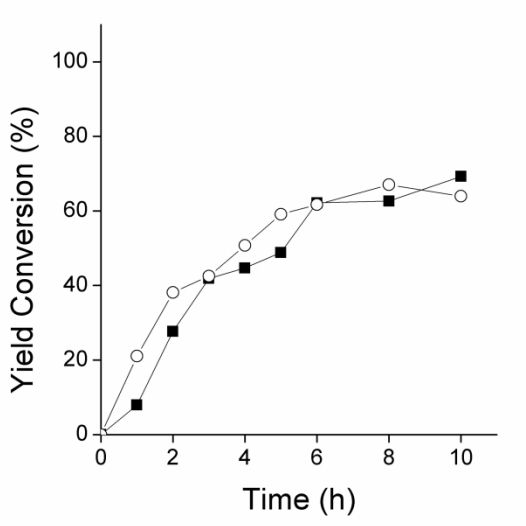

Figure 4. Time course for biodiesel synthesis catalyzed by Gx-TLL. Conditions: 30 °C, 3:1 ethanol:soybean oil molar ratio, 15% of enzyme and 4% of added water. (■) Reaction with n-hexane in the reaction medium; (○) Reaction in solvent-free system.

Three stepwise alcoholysis

Although an excess of alcohol would promote the miscibility of oil, alcohol, and enzyme, higher alcohol:oil ratios increase the polarity of the medium reaction produced by alcohol and water, and this is often associated with the inactivation of the biocatalyst [35]. To avoid the lipase inactivation, therefore, Watanable et al. [21] proposed the stepwise addition of ethanol, which consists in the sequential addition of less than stoichiometric amounts of ethanol to the acylglycerol. This strategy seems to be particularly appropriate for applications involving TLL, since this lipase undergoes major deactivation by low molecular weight alcohols and the stepwise strategy reduces the exposure of this lipase to the nucleophile [13]. Fig. 5 depicts the effect of a three step addition of ethanol in solvent and solvent-free systems, with 15% of Gx-TLL. The alcohol was added at 0, 3, and 6 h of reaction. For both systems, around 85% of yield conversion was obtained, and the conversion was improved from the one step ethanolysis under the stoichiometric molar ratio.

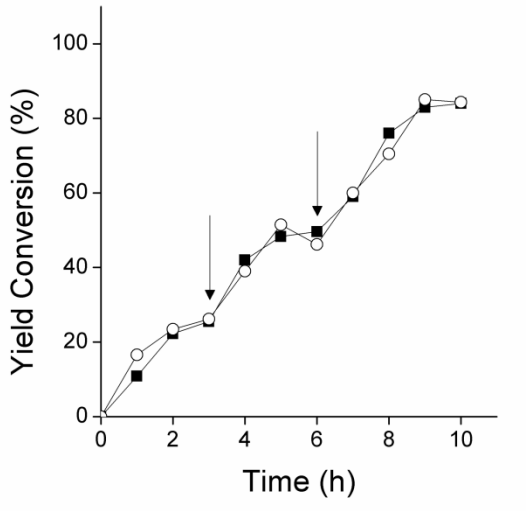

Figure 5. Time course for biodiesel synthesis catalyzed by Gx-TLL. Conditions: 30 °C, three step ethanol addition to soybean oil (1 molar equivalent at 0, 3 and 6 h), 15% of enzyme and 4% of added water. (■) Reaction with n-hexane in the reaction medium; (○) Reaction in solvent-free system.

These results are in agreement with those reported by Hernandez-Martinez and Otero [13], who used the commercial Lipozyme TL-IM in a three step ethanolysis of soybean oil. The authors obtained 84% of yield conversion after 96 h. In our work, using the preparation immobilized by multipoint covalent attachment, the same yield of conversion was reached after 10 h, showing that this preparation could be more active or the orientation of immobilization could facilitate the access of the substrate to the lipase active site. Soumanou and Bornscheuer [36] also found that the stepwise addition of methanol is more effective than a one-step methanolysis reaction. Moreover, the TLL showed initial alcoholysis rate in a solvent-free system compared to reactions in an organic solvent.

Since TLL is 1,3 regiospecific, these results indicate that migration of acyl groups from the *sn*-2 position to the *sn*-1,3 position occurs in the lower glycerides, not only during a single step reaction but also during stepwise alcoholysis. Thus, we proposed the use of a two step ethanolysis, initially with the addition of 2 molar equivalent of ethanol we proceeded the full alcoholysis of the glycerides in the positions *sn*-1,3, followed by the addition of 1 molar

equivalent of ethanol, to complete the reaction by the alcoholysis of the mono-glycerides remaining by the acyl migration from the *sn*-2 position to the *sn*-1,3 position.

Two stepwise alcoholysis

Fig. 6 presents the results of a two step ethanolysis of soybean oil catalyzed by Gx-TLL in solvent and solvent-free medium. Initially, the reaction proceeded with 2 molar equivalent of ethanol to oil; after 6 h of reaction, 1 molar equivalent was added. As can be seen, full ethanolysis was obtained after 10 h. This can be explained by the proposed reaction mechanism. Starting with only 2 molar equivalent of ethanol to oil, it can be predicted [13, 36] that the enzyme is protected by the inactivation caused by short chain alcohol. As explained by the phenomenon of acyl migration [12, 13, 17, 37], proceeding with the addition of 1 molar equivalent of ethanol to oil could direct the acyl migration from sn-2 to sn-1,3 position, eased by the presence of solvent, low water content, or the support [12, 19, 20], and the full conversion of the transesterification reaction could be reached.

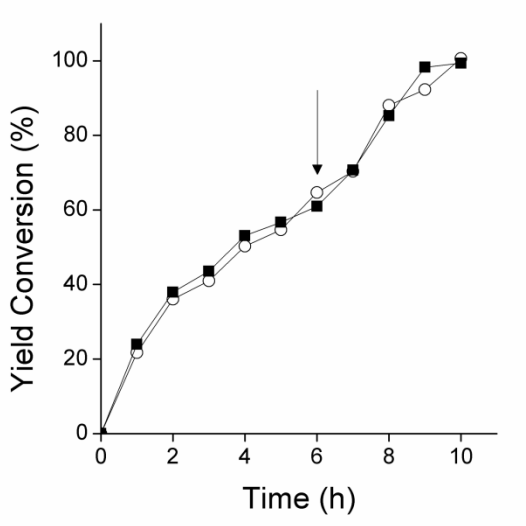

Figure 6. Time course for biodiesel synthesis catalyzed by Gx-TLL. Conditions: 30 °C, two step ethanol addition to soybean oil (2 molar equivalent added initially and 1 molar equivalent at 6 h), 15% of enzyme and 4% of added water. (■) Reaction with n-hexane in the reaction medium; (○) Reaction in solvent-free system.

In order to confirm whether it is a particular feature of Gx-TLL enzyme preparation or it can be applied to other TLL preparations, we tested the commercial preparation Lipozyme TL-IM (Novozymes) in the two stage stepwise ethanolysis of soybean oil, in solvent and solvent-free systems. Fig. 7 shows that, as for Gx-TLL, Lipozyme TL-IM produced the same 100% yield conversion in 10 h. Although the full conversion obtained in the two step ethanolysis was confirmed for Lipozyme TL-IM, this procedure has to be tested for other lipases, because yields could be affected by several factors, among them immobilization support, type of immobilization (adsorption, covalent immobilization, entrapment, etc.), presence of organic solvent in the reaction medium, temperature and reaction time, and the thermodynamic parameters that influence the equilibrium of acyl migration [20].

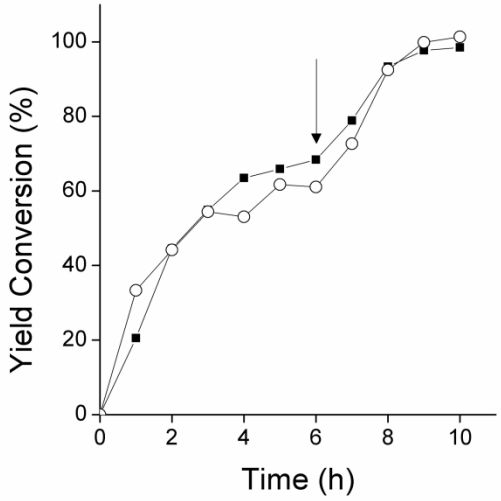

Figure 7. Time course for biodiesel synthesis catalyzed by Lipozyme TL-IM. Conditions: 30 °C, two step ethanol addition to soybean oil (2 molar equivalent added initially and 1 molar equivalent at 6 h), 15% of enzyme and 4% of added water. (■) Reaction with n-hexane in the reaction medium; (○) Reaction in solvent-free system.

Conclusions

Two stage stepwise ethanolysis is a very simple and effective way to improve the biodiesel synthesis catalyzed by the lipase from *Thermomyces lanuginosus*, a strict sn-1,3 regiospecific, in solvent and solvent-free systems. The preparation of TLL based on the multipoint covalent immobilization on glyoxyl supports (Gx-TLL), showed to be very active in

the biodiesel synthesis, reaching 100% of yield conversion in the one step reaction using excess of ethanol (7.5:1 ethanol:soybean oil molar ratio) in the presence of n-hexane in the reaction medium.

Under stoichiometric molar ratio and three stepwise ethanolysis, Gx-TLL was also active, but the full conversion was not reached; even in the presence of n-hexane, no more than 80% of yield conversion was obtained. Using a two stepwise ethanolysis, 100% of yield conversion in 10 h was obtained in the reaction catalyzed by the multipoint immobilized Gx-TLL and by the commercial Lipozyme TL-IM. Therefore, this methodology showed to be an excellent alternative to enzymatic biodiesel synthesis, catalyzed by lipase from *Thermomyces lanuginogus*, in order to help the reduction of the costs, making the enzymatic route competitive with the chemical process.

Acknowledgments

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CONSIDERAÇÕES FINAIS

A presente Tese de Doutorado teve como objetivo estudar a síntese de biodiesel através da reação de transesterificação entre óleos vegetais e álcoois de cadeia curta catalisada por lipase. Dentro do tema proposto, investigou-se os fatores que influenciam na reação de transesterificação, definindo as melhores condições para a reação, avaliou-se diferentes óleos vegetais, álcoois e lipases de diferentes fontes microbianas, estudou-se estratégias para o reuso da lipase imobilizada na reação de transesterificação, e durante uma parte realizada no Laboratório de Ingeniería Enzimática do Instituto de Catálisis y Petroleoquímica do CSIC na Espanha, estudou-se a imobilização e estabilização de lipase por ligação covalente multipontual, assim como estratégias para reativação de biocatalisadores inativados por ação de temperatura e solventes orgânicos. Na parte final, o biocatalisador preparado no período no exterior foi aplicado na reação de síntese de biodiesel e também foram avaliadas metodologias para melhorar o desempenho da lipase imobilizada na reação de transesterificação.

Inicialmente, identificaram-se como variáveis importantes na reação de transesterificação enzimática, a quantidade de enzima, pois é um fator fundamental na parte econômica devido ao custo do biocatalisador, assim como o tempo de reação, a temperatura e a razão molar de substrato entre álcool e óleo, pois representam fatores que podem ser responsáveis pela inativação da enzima e a quantidade de água adicionada no meio reacional, por auxiliar no desempenho da lipase na reação de transesterificação. Nesta etapa utilizou-se a lipase de *Thermomyces lanuginosus* livre como catalisador da reação entre óleo de soja e etanol, em um meio sem solvente orgânico. Esses fatores foram

avaliados mediante um delineamento composto central rotacional e a metodologia de superfície de resposta. As condições ótimas da reação encontradas foram: Temperatura = 31,5 °C; Tempo de reação = 7 h; Razão Molar de Substrato = 7,5:1 etanol:óleo de soja; Conteúdo de enzima = 15%; Água adicionada = 4%. Nestas condições atingiu-se 96% de rendimento de conversão.

No seguinte passo, as condições da reação de transesterificação foram avaliadas para a síntese catalisada pela lipase imobilizada comercial Lipozyme TL-IM. Esta lipase, a mesma utilizada anteriormente, é disponibilizada pela empresa Novozymes imobilizada em silica porosa granulada. Neste estudo, fixou-se o tempo de reação, e as demais variáveis (temperatura, razão molar de substrato, quantidade de enzima e água adicionada) foram avaliadas em um planejamento fatorial fracionário, para uma prévia seleção, no qual a razão molar de substrato não foi estatisticamente significativa. Com esta variável foi fixada, procedeu-se um segundo delineamento composto central rotacional com as três variáveis restantes, onde as condições ótimas definidas foram: temperatura 26 °C, quantidade de enzima 25%, água adicionada 4%, e razão molar de substrato, de 7,5:1 etanol: óleo de soja. Com as condições otimizadas atingiu-se um rendimento de conversão de 69%. Juntamente avaliaram-se estratégias para o reuso da lipase imobilizada. A enzima foi submetida a diferentes tratamentos com solventes (lavagens com água, etanol, propanol e n-hexano) antes de cada ciclo de reação, e a prévia lavagem do derivado com n-hexano manteve a atividade do biocatalizador em torno de 90% da atividade inicial após sete ciclos, enquanto que a enzima reutilizada sem tratamento manteve 20% de sua atividade inicial no mesmo período.

Com estas etapas concluídas avaliou-se diferentes óleos vegetais (óleo de soja, óleo de girassol e óleo de arroz), álcoois (metanol, etanol, propanol e butanol), e lipases microbianas de diferentes fontes (*Thermomyces lanuginosus* – Lipozyme TL-IM, *Candida antartica* – Novozym 435, e *Rhizomucor miehei* – Lipozyme RM-IM). As lipases utilizadas eram imobilizadas, comercialmente disponibilizadas pela empresa Novozymes. Neste

estudo verificou-se que cada lipase apresenta uma especificidade quanto ao álcool utilizado, obtendo diferentes rendimentos de conversão na reação de síntese de biodiesel. A Lipozyme TL-IM apresentou maior afinidade ao etanol, Novozym 435 ao metanol, e Lipozyme RM-IM ao butanol. Além disso, constatou-se que independente do óleo vegetal empregado, as lipases apresentaram comportamento semelhantes, indicando que não há um óleo específico que deva ser utilizada na reação, podendo assim ser determinado de acordo com a disponibilidade da região e principalmente em relação ao fator econômico. Também foi avaliado o reuso da enzima e novamente, lavagem com n-hexano mostrou-se o melhor tratamento, aumentando a vida útil do biocatalisador.

O seguinte passo realizado foi desenvolvido em colaboração com o Laboratório de Ingeniería Enzimática do ICP-CSIC, durante um período sanduíche. Primeiramente estudou-se a imobilização da lipase de *Thermomyces lanuginosus*, em glioxil-agarose. A imobilização neste tipo de suporte procede-se de forma multipontual através da união dos grupos aldeídos do suporte com grupos aminos primários da superfície da enzima. Durante esta etapa observou-se que ao longo da imobilização, que deve ocorrer a pH 10, a enzima sofria inativação por pH alcalino e a imobilização não era satisfatória, pois a enzima possui poucos resíduos de lisinas. No entanto, através da aminação química dos grupos carboxilas de ácidos aspárticos e glutâmicos, aumentava-se em cinco vezes a quantidade de grupos aminos na superfície da enzima, e assim obtinha-se um derivado altamente estável frente a agentes inativadores.

Outra pesquisa desenvolvida no mesmo período foi o estudo de estratégias de reativação de derivados submetidos a inativação por temperatura e solventes orgânicos. Em um primeiro momento, realizaram-se estudos em derivados imobilizados covalentemente, porém de forma suave, imobilização unipontual em agarose ativada com brometo de cianogênio. Assim, foi verificada uma importante propriedade desta lipase de *T. lanuginosus* para reativar-se que é reestruturação do *lid*, e consequentemente o mecanismo de abertura e fechamento, mecanismo este fundamental para a atividade lipolítica. Através do uso de

detergentes, que são capazes de estabilizar a forma aberta (ativa) das lipases, conseguiu-se recuperar 100% da atividade inicial após inativação com dioxano, com simples incubação em meio aquoso ao final da inativação. E, quando o derivado foi inativado a 60 °C, recuperou-se 100% da atividade no primeiro ciclo de inativação, com prévia incubação em guanidina para eliminar qualquer estrutura mal formada e posterior incubação em meio aquoso. Porém neste caso a atividade recuperada foi diminuindo ao final de cada ciclo, provavelmente causa de modificações químicas ocasionadas pela temperatura.

A seguinte hipótese avaliada foi com relação a reativação dos derivados imobilizados multipontualmente. Considerando que um derivado unido por vários pontos a um suporte sólido teria muitos mais pontos de referência no momento de sua reativação, estudou-se a inativação destes derivados e sua posterior capacidade de reativação, comparando com derivados ligados covalentemente a um suporte por apenas um ponto. Constatou-se aqui que derivados imobilizados por ligação covalente multipontual apresentam melhor capacidade de reativação, além do fato que mesmo em ausência de detergente obteve-se maior atividade recuperada, indicando que os vários “pontos de referência” criados pela ligação multipontual, auxiliam também na reestruturação do *lid*. Entretanto neste caso, como a enzima havia sofrido o processo de aminação química para que fosse possível a imobilização em suporte glioxil-agarose, a mesma estava mais suscetível a modificações químicas ocasionadas principalmente por temperatura e assim dificultando a sua total reativação.

Por fim, a última etapa do trabalho realizada foi a aplicação do derivado preparado por imobilização multipontual na reação de síntese de biodiesel. Os primeiros testes foram realizados nas condições otimizadas previamente. Obteve-se aqui, 75% de conversão, quando realizado em meio livre de solvente, e quando n-hexano foi empregado como solvente no meio reacional atingiu-se 100% de conversão em 10 h. Porém, a utilização de solventes orgânicos representaria uma etapa a mais no processo de purificação do

biodiesel. Assim, avaliou-se outras metodologias para a reação de transesterificação catalisadas por lipases, como a etanólise por etapas, que consiste em adicionar o etanol gradualmente na reação. Aqui, encontrou-se que realizando a reação em duas etapas, uma primeira partindo de 2 moles de etanol, e a posterior adição de 1 mol de etanol, ao final do processo obteve-se 100% rendimento de conversão em 10 horas de reação em meio sem solvente orgânico.

Para concluir, pode-se afirmar que o trabalho atingiu plenamente os objetivos propostos. As condições para a reação de síntese enzimática foram otimizadas com sucesso, encontrou-se a especificidade de cada lipase com cada álcool para aplicação no processo, além de uma metodologia para reuso da lipase imobilizada. Mais do que isso, preparou-se um derivado imobilizado da lipase de *Thermomyces lanuginosus*, que ademais de ser altamente estável, apresentou uma alta atividade e eficiência na reação de transesterificação, assim como se constatou ser possível a reativação do derivado quando a causa de inativação não provoca modificações químicas na enzima. Portanto, os resultados obtidos nesta Tese de Doutorado representam contribuições relevantes no campo do desenvolvimento tecnológico para a síntese de biodiesel catalisado por lipase. Contudo, abrem-se perspectivas para trabalhos futuros, pois cada vez busca-se mais a inovação e a melhora tecnológica.

Assim, uma das idéias sugeridas e com fácil aplicação é a ampliação de escala no processo de síntese enzimática de biodiesel, com a utilização de reatores em batelada com maior volume, assim como reatores com leito fixo, onde pode realizar a reação de forma contínua ou batelada alimentada.

Também podem ser realizados estudos de novas metodologias para imobilização da lipase, visando obter derivados que sejam mais estáveis, mais ativos, e que apresentem maior desempenho na reação de síntese de biodiesel. Estas pesquisas podem ser feitas através da utilização de novas orientações para imobilização em glioxil-agarose, ou também com a utilização de outros suportes com grupos funcionais diferentes.

Além disso, estudos de melhoramento do processo de reativação dos biocatalizadores, através de modificações nos derivados imobilizados, que permitam a completa reativação mesmo quando sofram inativações que podem provocar modificações químicas é um ponto fundamental para além de aumentar a vida útil do biocatalizador, auxiliar na diminuição dos custos de produção e aplicação tornando-os competitivos aos catalisadores químicos.

E também, a aplicação de líquidos iônicos como solventes para reação e suporte para imobilização da enzima, tecnologia esta que vem ganhando crescente atenção atualmente, pois trata-se de uma tecnologia limpa, em que não afeta o meio ambiente pois os líquidos iônicos são facilmente recuperáveis e reutilizáveis.

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