UNIVERSIDADE TÉCNICA DE LISBOA

INSTITUTO SUPERIOR DE AGRONOMIA

Produção de Sucedâneos de Gordura de Leite Humano por Catálise Enzimática

Tese apresentada para obtenção do grau de Doutor em Engenharia Alimentar

Orientador: Doutora Maria Suzana Leitão Ferreira Dias Vicente

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Carla Sofia Ramos Tecelão

Lisboa

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Ao Paulo e à Sofia

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RESUMO

Pretendeu produzir-se sucedâneos de gordura de leite humano (SGLH) por interesterificação catalisada por lipases imobilizadas *sn*-1,3 selectivas.

Testaram-se diferentes biocatalisadores na reacção de acidólise entre tripalmitina e ácido oleico (C18:1), em reactor descontínuo, num meio isento de solvente. As preparações comerciais de *Candida antarctica* (Novozym 435), de *Thermomyces lanuginosa* (Lipozyme TL IM) e de *Rhizomucor miehei* (Lipozyme RM IM) conduziram a valores de incorporação molar de C18:1 semelhantes (*c.a.* 27%) após 24h de reacção. Foram alcançados valores idênticos com as lipases não comerciais de *Carica papaya* auto-imobilizada no látex (22%) e de *Rhizopus oryzae*, expressa numa estirpe mutante de *Pichia pastoris*, imobilizada em Accurel MP 1000 (25%) ou em Lewatit VP OC 1600 (30%), nas mesmas condições operacionais. Com a lipase/aciltransferase de *Candida parapsilosis* imobilizada em Accurel MP1000, os níveis de incorporação foram baixos (8,5%), mas quando se substituiu o C18:1 por oleato de etilo, conseguiram-se incorporações de 32 a 45%, dependendo da razão molar. A incorporação de ácidos gordos polinsaturados ómega-3 foi sempre inferior à incorporação de C18:1, com todas as lipases testadas.

A estabilidade operacional em reutilizações sucessivas foi em geral, superior para as lipases comerciais. Todavia, o baixo custo das preparações não comerciais constitui uma mais valia na implementação da síntese de SGLH à escala industrial.

Palavras-chave: Ácido oleico; ácidos gordos polinsaturados omega-3; acidólise; estabilidade operacional; interesterificação; lipase imobilizada; lípidos estruturados; lípidos funcionais; tripalmitina; sucedâneos de gordura de leite humano.

PRODUCTION OF HUMAN MILK FAT SUBSTITUTES BY ENZYMATIC CATALYSIS

ABSTRACT

The aim of this study was the production of human milk fat substitutes (HMFS) catalysed by *sn*-1,3 selective immobilised lipases.

Different biocatalysts were tested for the ability to catalyse the acidolysis reaction between tripalmitin and oleic acid (C18:1), batchwise, in solvent free medium. The commercial preparations of *Candida antarctica* (Novozym 435), *Thermomyces lanuginosa* (Lipozyme TL IM) and *Rhizomucor miehei* (Lipozyme RM IM) lead to similar molar oleic acid incorporations (*c.a.* 27%) after 24h reaction. Similar results were attained with the non commercial lipases of *Carica papaya* self-immobilized on its latex (22%) and of *Rhizopus oryzae*, expressed in a mutant strain of *Pichia pastoris*, immobilised on Accurel MP 1000 (25%) or Lewatit VP OC 1600 (30%), under the same operational conditions. With the lipase/acyltransferase from *Candida parapsilosis* immobilised on Accurel MP1000, low incorporation levels were observed (8.5%), When C18:1 was replaced by ethyl oleate, C18:1 incorporations from 32 to 45% were attained, depending on the molar ratio used.

For all the biocatalysts tested, the incorporation of omega-3 polyunsaturated fatty acids was always lower than the values obtained with C18:1,.

The operational stability in consecutive batches was higher for commercial lipases. However, the low cost of non commercial preparations is an added value for the implementation of HMFS production at industrial scale.

Key-words: oleic acid; omega-3 polyunsaturated fatty acids, acidolysis; operational stability; interesterification; immobilised lipase; structured lipids; functional lipids; tripalmitin; human milk fat substitutes.

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LISTA DE ABREVIATURAS E SÍMBOLOS DOS CAPÍTULOS I, II E VII

AG – Ácido(s) gordo(s)

CpLIP2 – lipase/aciltransferase recombinante de *Candida parapsilosis*

DHA – Ácido docosahexenóico, C22:6

FFA – "Free Fatty Acids" – ácidos gordos livres

GLH – Gordura de leite humano

LCP – Lipase de *Carica papaya*

Omega-3 PUFA – "Omega-3 Polyunsaturated Fatty Acids" - ácidos gordos polinsaturados ómega-3

r-LRO – Lipase recombinante de *Rhizopus oryzae*

SGLH – Sucedâneos de gordura de leite humano

TAG – triacilglicerol(óis)

LISTA DE PUBLICAÇÕES NO ÂMBITO DA PRESENTE TESE

Capítulo de livro – este capítulo faz parte da Revisão da Literatura da tese (em português): Tecelão, C., Valero, F., Sandoval, G., Ferreira-Dias, S, Producción de sucedâneos de grasa de leche humana catalizados por lipasas, *In*: *Obtención Enzimática de Compuestos Bioactivos a partir de Recursos Naturales Iberoamericanos*, (Francisco J. Plou Gasca, Georgina C. Sandoval Fabián, eds.), cap. 4, Editorial CSIC, Madrid, Espanha, pp. 101-116 (no prelo).

Artigos em revistas ISI:

1 – Tecelão, C., Silva, J., Dubreucq, E., Ribeiro, M.H., Ferreira-Dias, S. (2010). Production of human milk fat substitutes enriched in omega-3 polyunsaturated fatty acids using immobilized commercial lipases and *Candida parapsilosis* lipase/acyltransferase, *Journal of Molecular Catalysis B: Enzymatic,* **65**: 122-127 (doi:10.1016/j.molcatb.2010.01.026).

2 – Tecelão, C., Perrier, V., Dubreucq, E., Ferreira-Dias, S. (2011) Production of human milk fat substitutes by interesterification of tripalmitin with ethyl oleate catalyzed by *Candida parapsilosis* lipase/acyltransferase (submetido à *Applied Biochemistry and Biotechnology*).

3 - Tecelão, C., Rivera, I., Sandoval, G., Ferreira-Dias, S. (2011). *Carica papaya* latex: a low-cost biocatalyst for human milk fat substitutes production, *European Journal of Lipid Science and Technology* (aceite) DOI:10.1002/ejlt.201100226.

4 – Tecelão, C., Guillén, M., Valero, F., Ferreira-Dias, S. (2011) Immobilized heterologous *Rhizopus oryzae* lipase: a feasible biocatalyst for the production of human milk fat substitutes, (submetido ao *Biochemical Engineering Journal*).

Capítulo I.

Introdução Geral

1.1. Enquadramento do tema

O aleitamento materno, nos primeiros meses de vida, assume uma importância preponderante no desenvolvimento imunológico, neurológico, psicomotor e emocional do recém-nascido.

A fracção lipídica do leite humano constitui a principal fonte de energia para o lactente e compreende, na sua composição, cerca de 98% de triacilgliceróis (TAG), 1,3 % de fosfolípidos e 0,4% de colesterol. Os ácidos gordos (AG) de cadeia longa prevalecem nas moléculas de TAG, com uma proporção de 50% de saturados e de insaturados, que varia em função da dieta alimentar da mãe. O ácido palmítico (16:0) é o AG saturado mais abundante (18-25%).

No que concerne à distribuição de AG nos TAG, esta caracteriza-se pelo predomínio de AG saturados, essencialmente ácido palmítico, na posição *sn*-2 dos TAG e pela prevalência de AG insaturados nas posições externas. Esta estrutura, única na natureza, condiciona o processo digestivo do recém-nascido. A lipase pancreática *sn*-1,3 selectiva liberta os AG das posições *sn*-1 e *sn*-3 do TAG os quais são, posteriormente, absorvidos a nível do intestino grosso como AG livres, conjuntamente com os *sn*-2-monoacligliceróis formados. A presença de ácido palmítico na posição *sn*-2 proporciona uma absorção mais eficiente deste ácido como *sn*-2 monoacilpalmitato, evitando a complexação do ácido palmítico, na sua forma livre, com o cálcio, que resultaria na formação de sabões de cálcio insolúveis.

As fórmulas de leite infantil surgem com o intuito de suprir as necessidades nutricionais do recém-nascido privado de aleitamento materno nos primeiros meses de vida. Contudo, a grande maioria das fórmulas desenvolvidas com o objectivo de mimetizar o leite materno é obtida a partir de óleos vegetais ou de leite de ruminantes, que contêm o ácido palmítico e os restantes AG saturados predominantemente esterificados nas posições *sn*-1 e *sn*-3 das moléculas de TAG, o que se traduz em problemas de obstipação no recém-nascido e numa deficiente absorção de ácido palmítico e de cálcio.

A criação de fórmulas de leite infantil que reproduzam, tanto quanto possível, a estrutura e composição do leite humano, coloca-se como um desafio à indústria alimentar. Neste contexto, o recurso à catálise enzimática oferece múltiplas vantagens face à utilização de catalisadores químicos. As lipases (triacilglicerol acil-hidrolases, E.C. 3.1.1.3.) são enzimas que catalisam a hidrólise de acilgliceróis na interface óleo/água mas, em meio orgânico com baixa actividade de água, são capazes de promover as reacções de esterificação e interesterificação. As lipases possuem a capacidade de actuar sob condições suaves de temperatura e pressão (temperatura inferior a 70ºC e pressão atmosférica) o que se traduz na redução de efeitos poluentes e de custos económicos associados ao processo. Salienta-se ainda a selectividade das lipases face aos catalisadores químicos, no que respeita quer à posição dos AG no esqueleto de glicerol, quer às características do AG (nomeadamente tamanho da cadeia carbonada e saturação/insaturação). O recurso a lipases com selectividade *sn*-1,3 permite a manutenção do AG na posição interna do acilglicerol, proporcionando a síntese de TAG estruturados como sucedâneos de gordura de leite humano (SGLH).

Os trabalhos de investigação publicados sobre a síntese de SGLH descrevem a utilização de sistemas reaccionais, em presença ou ausência de solvente orgânico, cuja composição obedece, na sua maioria, a um padrão característico. Utilizam (i) uma fonte de ácido palmítico, (ii) lipases imobilizadas com selectividade *sn*-1,3 como biocatalisador e (iii) AG livres (em reacções de acidólise) ou esterificados ao esqueleto de glicerol (em reacções de interesterificação).

Os estudos desenvolvidos e publicados no âmbito desta tese pretendem ser um contributo importante e válido para o avanço na investigação sobre a síntese por catálise enzimática de TAG estruturados como SGLH, numa perspectiva de possível implementação da produção à escala industrial.

1.2. Objectivos e plano geral da dissertação

O objectivo primordial desta tese centra-se na produção de sucedâneos de gordura de leite humano, por reacção de interesterificação, catalisada por lipases imobilizadas *sn*-1,3 selectivas.

Os sistemas reaccionais em estudo compreenderam a utilização de tripalmitina, como fonte de ácido palmítico, e ácido oleico (C18:1) (sistema 1) ou concentrado de ácidos gordos polinsaturados ómega-3, rico em ácido docosahexenóico (DHA) (sistema 2), como fonte de AG livres, em reactor descontínuo de mistura total, num meio livre de solvente.

A primeira fase do trabalho consistiu na utilização das lipases comerciais de *Candida antarctica* (Novozym 435), de *Thermomyces lanuginosa* (Lipozyme TL IM) e de *Rhizomucor miehei* (Lipozyme RM IM) como catalisadores em ambos os sistemas reaccionais. Estudou-se também a utilização da lipase/aciltransferase de *Candida parapsilosis* imobilizada em Accurel® MP 1000, como alternativa às lipases comerciais de preço elevado, pela potencialidade que revelou anteriormente como biocatalisador em reacções de interesterificação para a produção de bases gordas para margarinas em meios orgânicos livres de solvente (Osório et al., 2009a; Osório et al., 2009b). Esta enzima foi descoberta pelo grupo do Prof. Eric Dubreucq de Montpellier Sup Agro (Briand *et al*., 1995; Lecointe *et al*., 1996) e actualmente está protegida por patentes da BASF (Weiss *et al*., 2005; Dubreucq *et al*, 2007). Investigaram-se os seguintes aspectos:

- Efeito da composição do meio reaccional na actividade de interesterificação das diferentes lipases, no que respeita às características dos AG livres: monoinsaturado (sistema 1) *versus* polinsaturados (sistema 2). A actividade de cada biocatalisador foi avaliada em termos de incorporação molar de C18:1 ou de AG polinsaturados no TAG.
- Estabilidade operacional dos biocatalisadores, em reutilizações sucessivas num reactor descontínuo.
- Avaliação da composição em AG na posição *sn*-2 do TAG, uma vez que ocorre sempre a migração de grupos acilo das posições *sn*-1,3 para a posição *sn*-2, mesmo com lipases *sn*-1,3 selectivas.

Os resultados obtidos encontram-se publicados no *Journal of Molecular Catalysis B: Enzymatic* (factor de impacto de 2,330, em 2010) e são apresentados integralmente no capítulo 3 desta dissertação (artigo 1; Tecelão *et al.*, 2010).

Avaliou-se ainda a lipase/aciltransferase de *Candida parapsilosis* como potencial biocatalisador na síntese de SGLH, num sistema diferente do testado anteriormente para esta enzima (capítulo 3). Neste novo sistema reaccional utilizou-se também a tripalmitina, como fonte de ácido palmítico, mas substituiu-se o ácido oleico por oleato de etilo, como dador de grupo acilo. Este estudo considerou a influência da razão molar oleato de etilo/tripalmitina na cinética de incorporação de ácido oleico no TAG. Os resultados obtidos são apresentados no capítulo 4 e foram submetidos a publicação na revista *Applied Biochemistry and Biotechnology* (artigo 2; factor de impacto de 1,879, em 2010).

Uma vez que a implementação industrial de muitos processos catalisados por lipases está limitado pelo preço elevado das preparações comerciais de lipases imobilizadas, testaram-se, para além da lipase/aciltransferase de *C. parapsilosis*, duas outras lipases não comerciais: a lipase de *C. papaya* e a lipase heteróloga de *Rhizopus oryzae*. A lipase de *Carica papaya* auto-imobilizada no látex, é extraída de agro-resíduos de plantações de papaia, e apresenta-se como um biocatalisador de baixo custo em alternativa às preparações comerciais. Este biocatalisador foi preparado pelo grupo da Doutora Georgina Sandoval, do Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ), Guadalajara, México. Testaram-se preparações enzimáticas extraídas do fruto da papaia e do pecíolo nos sistemas reaccionais 1 e 2, descritos no artigo 1 desta tese (Tecelão *et al.*, 2010). Estes ensaios decorreram em condições similares aos apresentados anteriormente. Os estudos incidiram sobre os seguintes aspectos:

- Escolha do sistema reaccional em função da selectividade para o AG, avaliada com base na incorporação molar no TAG.
- Modelação da reacção de acidólise seleccionada, em função da composição do meio reaccional e da temperatura, e optimização das condições reaccionais pela metodologia das superfícies de resposta.
- Validação do modelo empírico obtido.

- Estudos de estabilidade operacional do biocatalisador em reutilizações sucessivas em reactor descontínuo.
- Caracterização dos SGLH obtidos no que respeita à regiodistribuição dos AG no TAG.

Este conjunto de ensaios constitui o capítulo 5 desta tese que corresponde ao artigo 3, aceite para publicação no *European Journal of Lipid Science and Technology* (factor de impacto de 1,487, em 2010).

O capítulo 6 desta tese (artigo 4; submetido ao *Biochemical Engineering Journal*) (factor de impacto de 2,692, em 2010) apresenta os resultados obtidos com a lipase heteróloga de *Rhizopus oryzae*, expressa numa estirpe mutante de *Pichia pastoris*, como biocatalisador no sistema reaccional 1, nas condições operacionais mencionadas anteriormente. Esta lipase heteróloga foi produzida pelo grupo do Prof. Franscisco Valero da Universidade Autónoma de Barcelona, Espanha. Com esta lipase, abordaram-se os seguintes aspectos:

- Selecção do suporte de imobilização e optimização da razão carga de lipase/suporte, nos suportes mais promissores em termos de actividade catalítica.
- Estudos de estabilidade operacional do biocatalisador, nas condições já referidas.

Os ensaios com a lipase/aciltransferase foram desenvolvidos no âmbito da acção bilateral Franco-Portuguesa, programa PESSOA, "*Lipase/acyltransferase-catalyzed Lipid Structuration*", 2009-2010. Os ensaios com as lipases de *C. papaya* e rROL fazem parte dos estudos desenvolvidos no âmbito da "*Rede Ibero-Americana para a Extracção e Transformação Enzimática de Ingredientes Funcionais e Nutracêuticos de Plantas Regionais e Agro-resíduos (ENZNUT)"*, Acção CYTED 108RT0346, e (ii) da acção bilateral integrada Portugal-Espanha E-29/11*, "Produção de Aromatizantes e de Lípidos Estruturados para a Indústria Alimentar, utilizando como Biocatalisador uma Lipase Microbiana Recombinante".*

A finalizar esta tese, há um capítulo de conclusões gerais e perspectivas para trabalhos futuros (capítulo 7).

Capítulo II.

Revisão da Literatura

Tecelão, C., Valero, F., Sandoval, G., Ferreira-Dias, S., Producción de sucedáneos de grasa de leche humana catalizados por lipasas, *In*: Obtención Enzimática de Compuestos Bioactivos a partir de Recursos Naturales Iberoamericanos, (Francisco J. Plou Gasca, Georgina C. Sandoval Fabián, eds.), cap. 4, Editorial CSIC, Madrid, Espanha, pp. 101-116 (no prelo)

PRODUÇÃO DE SUCEDÂNEOS DE GORDURA DE LEITE HUMANO CATALISADA POR LIPASES

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1. INTRODUÇÃO

O leite humano é uma emulsão óleo/água, de elevada riqueza nutricional, que proporciona manifestos benefícios para a criança sob os pontos de vista energético, imunológico, intelectual e motor.

A secreção láctea madura é constituída por cerca de 87% de água e por uma fracção rica em gordura, sais minerais, vitaminas, enzimas e imunoglobulinas, as quais desempenham um papel importante a nível do sistema imunitário da criança. A proteína predominante no leite humano é a lactoalbumina (constituindo 80% do teor proteico) e a lactose é o principal glúcido, presente numa concentração de 7%. O leite humano é rico em aminoácidos como a cisteína e a taurina que desempenham um papel preponderante no desenvolvimento do sistema nervoso do recém-nascido (Silva *et al.*, 2007).

A gordura de leite humano (GLH) constitui uma das principais fontes de nutrientes e energia para o recém-nascido. A composição da sua fracção lipídica compreende cerca de 98% de triacilgliceróis (TAG), 1,3 % de fosfolípidos e 0,4% de colesterol (Jensen, 1996). Os ácidos gordos (AG) são, maioritariamente, de cadeia longa, com uma proporção de 50% de saturados e de insaturados, que varia em função da dieta alimentar da mãe (Yuhas *et al.*, 2006). O ácido palmítico (16:0) é o AG saturado mais abundante (18-25%). Os AG seguintes são também muito importantes na composição do leite materno: ácidos oleico (18:1; 24-39%), linoleico (18:2n-6; 8-18%), linolénico (18:3n-3; 0,4-2%), láurico (12:0; 4-14%), mirístico (14:0; 3-12%), esteárico (18:0; 5-8%) e cáprico (10:0; 1,5-2,5%). Os ácidos gordos polinsaturados de cadeia longa, como o ácido araquidónico (20:4n-6) e docosa-hexenóico (22:6n-3), constituem 0,1 a 0,5% do total de AG no leite maduro (Silva *et al.*, 2009a; Yuhas *et al.*, 2006). A presença destes ácidos influencia o crescimento e desenvolvimento do sistema nervoso central da criança e tem um papel de relevo na massa e mineralização ósseas (Innis, 2007; Mu, 2010).

A composição em AG e a sua distribuição nos TAG obedecem a um padrão característico: cerca de 60 a 70% do ácido palmítico ocupa a posição *sn*-2 dos TAG, 80 a 90% do ácido oleico encontra-se nas posições *sn*-1 e *sn*-3 e 80% do ácido linoleico distribui-se entre as posições *sn*-2 e *sn*-3 (Jensen, 1999). Com efeito, a GLH possui uma estrutura única na Natureza, que se caracteriza por um predomínio de TAG do tipo ISI, isto é, com AG insaturados nas posições *sn*-1 e *sn*-3 e com AG saturados (essencialmente, ácido palmítico) na posição interna, ao contrário do observado nas restantes gorduras naturais.

A composição e a posição dos AG nos TAG afectam dramaticamente a digestibilidade e a posterior absorção de nutrientes. A situação em crianças merece maior atenção dada a imaturidade do seu sistema digestivo (Lien, 1994; Linderborg *et al.*, 2000; Willis *et al.*, 1998).

O processo de metabolismo e transporte de TAG, tanto em crianças como em adultos, ocorre de forma concertada, envolvendo a acção de várias lipases que actuam ao longo do processo digestivo. A lipase pancreática hidrolisa selectivamente os TAG nas posições *sn*-1 e *sn*-3, conduzindo à formação de AG livres e de 2-monoacilgliceróis que

são absorvidos ao nível do intestino grosso (Lien, 1994; Linderborg *et al.*, 2000; Willis *et al.*, 1998).

A absorção dos AG livres é função do tamanho da sua cadeia carbonada. Os AG saturados de cadeia longa (C12:0 a C18:0) não são tão eficazmente absorvidos como os AG de cadeia média (C6:0 a C10:0) e os AG insaturados. Os AG livres saturados de cadeia longa, tal como o palmítico, podem formar complexos de cálcio insolúveis, conhecidos como sabões de cálcio, que contribuem para a deficiente absorção de cálcio e de AG saturados em crianças alimentadas com leite artificial. Esta é também uma das causas de obstipação nos primeiros meses de vida. Salienta-se que, contrariamente ao ácido palmítico livre, o monoacilglicerol *sn*-2-monopalmitina é eficientemente absorvido (Lien, 1994; Linderborg *et al.*, 2000; Willis *et al.*, 1998).

1.1. Influência dos hábitos alimentares na composição da gordura de leite humano

O estado nutricional da mulher, a sua dieta alimentar, a duração e frequência do período de amamentação, bem como factores psicológicos e ambientais que podem afectar a predisposição da mulher para a aleitação, condicionam a composição em ácidos gordos da GLH (Forsyth, 1998; Jensen *et al.*, 1978, Yuhas *et al.*, 2006).

Yuhas e colaboradores (2006) desenvolveram um estudo, sem paralelo, sobre o perfil lipídico do leite maduro de mulheres originárias da Austrália, Canadá, Chile, China, Estados Unidos da América, Filipinas, Japão, México e Reino Unido, saudáveis, não fumadoras, com idades compreendidas entre 14 e 41 anos, em período de amamentação exclusiva de uma criança saudável, com idade compreendida entre 1 e 12 meses.

As principais conclusões apontam para proporções em AG saturados (predominantemente ácido palmítico) relativamente constantes entre os diversos países, enquanto o nível de alguns AG polinsaturados, particularmente o ácido docosa-hexenóico (DHA), é consideravelmente variável. Os níveis elevados de DHA no leite são o reflexo inequívoco de uma dieta alimentar rica em peixe, praticada pela mãe (no Chile, Filipinas e Japão). Estas evidências são confirmadas por vários estudos clínicos que demonstram existir uma relação dose-resposta na composição do leite de mulheres que introduzem suplementos de óleo de peixe ou de DHA na sua dieta alimentar (Helland *et al.*, 1998; Makrides *et al.*, 1996). No estudo de Yuhas *et al.* (2006), os níveis

de ácido linoleico eram superiores no leite de mães cuja dieta era rica em milho (caso do México e Chile), enquanto os maiores teores de ácido oleico se relacionaram com consumos elevados de óleo de colza (Canadá e China). Os níveis mais elevados de ácidos láurico e mirístico, observados em leite de mães filipinas, indicam uma alimentação predominante em glúcidos e baixa em gordura. Com efeito, uma dieta alimentar rica em glúcidos e deficiente em gordura estimula a síntese de AG de cadeia média a partir da glucose, no citoplasma das células da glândula mamária. O incremento destes ácidos pode registar valores entre 10 e 20% (Hayat *et al.*, 1999).

1.2. Importância dos sucedâneos de gordura de leite humano

A Organização Mundial de Saúde (OMS) e a UNICEF recomendam o aleitamento materno em exclusivo nos primeiros seis meses de vida e têm promovido campanhas no sentido de sensibilizar a opinião pública para a importância da amamentação. Todavia, factores de índole cultural, socio-económica e/ou de saúde da mãe podem condicionar a sua predisposição para a aleitação. O desenvolvimento de fórmulas sucedâneas do leite materno, que mimetizem a sua estrutura e composição, coloca-se como um desafio à indústria alimentar.

A grande maioria das formulações de leite infantil é obtida a partir de óleos vegetais ou de leite de ruminantes que contêm o ácido palmítico e os restantes AG saturados predominantemente esterificados nas posições *sn*-1 e *sn*-3 das moléculas de TAG (Forsyth, 1998; Jensen *et al.*, 1978), ao contrário do que sucede na gordura de leite humano, na qual o ácido palmítico ocupa essencialmente a posição interna do TAG. Estas formulações apresentam teores em ácido palmítico muito similares aos encontrados no leite materno. No entanto, a sua esterificação na posição *sn*-2 é consideravelmente baixa. O teor em ácido oleico é, em geral, igual ou superior ao do leite materno, mas encontra-se predominantemente esterificado na posição *sn*-2 (Straarup *et al.*, 2006).

Criar fórmulas de leite infantil que possibilitem ultrapassar os condicionalismos anteriormente expostos justifica o crescente interesse da indústria alimentar por produtos passíveis de serem comercializados.

2. A Biocatálise aplicada à síntese de sucedâneos de gordura de leite humano

Actualmente já existem no mercado sucedâneos de gordura de leite humano (SGLH) produzidos por catálise enzimática. Estas gorduras produzidas em laboratório são exemplos de lípidos estruturados.

Numa perspectiva geral, podem definir-se lípidos estruturados como sendo TAG que sofrem reestruturação, por métodos químicos ou enzimáticos, com o intuito de alterar a composição e/ou distribuição posicional dos AG no esqueleto de glicerol (Ferreira-Dias, 2010; Xu, 2000). A síntese de TAG estruturados como SGLH é levada a cabo por reacções enzimáticas. O recurso à tecnologia enzimática oferece múltiplas vantagens face à síntese química, salientando-se a elevada regio e estereoselectividade dos catalisadores biológicos, as reduzidas ou inexistentes reacções secundárias, que originam subprodutos indesejáveis, e a possibilidade de operar em condições de temperatura e pressão moderadas (pressão atmosférica e temperaturas inferiores a 70ºC).

Importa enfatizar que um produto sintetizado por via enzimática pode ser reconhecido como natural sendo identificado como GRAS (“Generally Recognized as Safe”).

As lipases (triacilglicerol acilhidrolases, E.C. 3.1.1.3.) são enzimas que catalisam a hidrólise de triacilgliceróis na interface óleo/água mas, em meio orgânico ou com baixa actividade de água, podem promover a reacção inversa de união de AG ao esqueleto de glicerol, reacção de esterificação, bem como reacções de interesterificação.

Como exemplos de SGLH são de referir o Betapol™, produzido pela Lipid Nutrition (Holanda) e mais recentemente, o InFat™ fabricado pela Advanced Lipids. Esta empresa resulta da fusão, em 2007, entre a Enzymotec (Israel) e a AAK (Suécia).

Tanto o Betapol™ como o InFat™ são obtidos por acidólise de gorduras vegetais ricas em ácido palmítico em posição *sn*-2 (eg. estearina de palma duplamente fraccionada) ou tripalmitina com misturas de AG rica em ácido oleico. Como catalisadores são utilizadas lipases *sn*-1,3 selectivas, como sejam a lipase de *Rhizomucor miehei* (Lipozyme RM IM) e de *Rhizopus oryzae* (http://www.lipidnutrition.com, Junho 2011; Meiri-Bendek *et al.*, EP 1 681 945 B1).

Um estudo comparativo estabelecido entre a gordura de leite proveniente de mães brasileiras e o SGLH Betapol revelou diferenças substanciais a nível das propriedades

físico-químicas. O teor de acidez foi consideravelmente superior na GLH (2,84g ácido oleico/100g de amostra) contrastando com 0,05g de ácido oleico/100g no Betapol. Relativamente à composição em AG, o Betapol demonstrou possuir maior teor em ácidos gordos saturados (52%) comparativamente à GLH (44%). O Betapol revelou valores mais elevados de ácidos láurico, palmítico e oleico. No que concerne às propriedades físicas das gorduras em estudo, o Betapol apresentou pontos de fusão inferiores aos da GLH em cerca de 3ºC (da Silva *et al.*, 2007).

Os trabalhos de investigação publicados sobre a síntese de SGLH descrevem a utilização de sistemas reaccionais, em presença ou ausência de solvente orgânico, cuja composição obedece, na sua maioria, a um padrão característico. Utilizam (i) uma fonte de ácido palmítico, (ii) lipases imobilizadas com selectividade *sn*-1,3 como biocatalisador e (iii) AG livres (em reacções de acidólise) ou esterificados ao esqueleto de glicerol (em reacções de interesterificação).

A Tabela 1 apresenta exemplos de estudos, às escalas laboratorial e piloto, de produção de SGLH por catálise enzimática. Como se pode observar, estes produtos podem ser obtidos por (i) acidólise entre um TAG rico em ácido palmítico em posição *sn*-2 e AG livres ou (ii) por interesterificação entre TAG, geralmente, de gorduras naturais.

Tabela 1

2.1. Fonte de ácido palmítico

A selecção da fonte de ácido palmítico deve ser criteriosa, de forma a assegurar a localização maioritária deste ácido na posição interna do TAG. Na produção de SGLH pode utilizar-se tripalmitina ou gorduras naturais ricas em ácido palmítico na posição *sn*-2. Assim, a banha, a estearina de palma e a gordura de manteiga constituem substratos adequados à síntese de SGLH.

A banha apresenta uma composição em AG variável, dependente da raça, sexo, idade e regime alimentar do animal de onde é extraída. Apesar da variabilidade, a banha possui uma estrutura em TAG muito semelhante à do leite humano, embora apresente um menor teor em AG polinsaturados de cadeia longa nomeadamente, de AG essenciais como os ácidos linoleico e linolénico. É uma gordura de baixo valor comercial.

Todavia, questões de ordem ética, religiosa e cultural podem constituir um entrave à sua utilização na produção de SGLH. A gordura de manteiga assemelha-se à GLH no que respeita à composição em AG e à sua localização no TAG. Todavia, os SGLH obtidos a partir da gordura de manteiga não possuem os ácidos araquidónico e docosa-hexenóico na sua composição (Mu, 2010). A estearina de palma, obtida por fraccionamento do óleo de palma, possui um conteúdo em ácido palmítico superior ao do leite humano e tem sido utilizada como substrato na síntese de SGLH. Nos estudos laboratoriais utiliza-se frequentemente tripalmitina que tem o inconveniente do seu elevado custo.

2.2. Biocatalisadores

As enzimas frequentemente utilizadas como biocatalisadores em reacções de síntese de SGLH são lipases que provêm, essencialmente, de fontes microbianas e possuem regioselectividade *sn*-1,3.

A selectividade das lipases possibilita a criação de moléculas de TAG estruturadas, isto é, moléculas "construídas à medida" ("Taylor-made fat"), com a consequente modificação das suas propriedades físicas e nutricionais. Esta selectividade compreende (i) a especificidade relativa ao substrato, isto é, a capacidade de a lipase hidrolisar, preferencialmente, um tipo de ésteres de glicerol, (ii) a regioselectividade definida como a capacidade de discriminar entre as posições externas do TAG e a posição interna, (iii) a estereoselectividade que descreve a capacidade exibida por algumas lipases de distinguirem entre as posições *sn*-1 e *sn*-3 do TAG e (iv) a especificidade relativa ao AG que traduz a afinidade da lipase para um AG específico ou, mais frequentemente, para um grupo de AG (Villeneuve, 2003).

A maioria dos estudos publicados recorre à utilização das lipases imobilizadas de *Rhizomucor miehei* (Lipozyme RM IM), de *Thermomyces lanuginosa* (Lipozyme TL IM) e de *Candida antarctica* (Novozym 435), comercializadas pela Novozymes, Dinamarca, a preços elevados (Tabla 1).

Diversos estudos demonstram que a selectividade da lipase é fortemente condicionada pela estrutura do substrato, nomeadamente a dimensão da cadeia carbonada, o grau de

insaturação e a estereoquímica da molécula, bem como por factores físico-químicos na interface óleo/água e na ligação ao centro activo da enzima.

Karabulut *et al.* (2010), em reacções de acidólise entre a tripalmitina e uma mistura equimolar de AG insaturados C18 (ácidos oleico, 18:1; linoleico, 18:2 e linolénico, 18:3), em *n*-hexano, observaram que a incorporação molar dos diferentes ácidos no TAG depende do biocatalisador utilizado. Assim, obtiveram-se incorporações superiores para a Novozym 435, seguida da Lipozyme RM IM e da Lipozyme TLIM. Para todos os biocatalisadores registou-se um incremento da incorporação molar do ácido com o aumento do seu grau de insaturação.

Esta constatação não é sustentada pelo trabalho de outros autores. Assim, Tecelão e colaboradores (2010) estudaram a incorporação de (i) ácido oleico ou de (ii) um concentrado de AG polinsaturados omega-3, rico em DHA, em tripalmitina, num sistema livre de solvente, tendo em vista a síntese de SGLH. Todas as lipases comerciais testadas (Novozym 435, Lipozyme RM IM e Lipozyme TL IM) exibiram uma clara preferência pelo AG monoinsaturado, não se registando diferenças significativas no seu grau de incorporação no TAG. Por outro lado, a Lipozyme TL IM conduziu ao menor valor de incorporação de AG polinsaturados comparativamente aos restantes biocatalisadores testados. Neste mesmo estudo, a lipase aciltransferase de *Candida parapsilosis* imobilizada em Accurel MP 1000 não demonstrou qualquer preferência entre os dois tipos de AG utilizados.

Hamam e Shahidi (2008) defendem que o número de duplas ligações e a sua localização na molécula de AG afectam, dramaticamente, a sua reactividade. Estes autores advogam que a presença de três duplas ligações na molécula de AG lhe confere uma forma característica (em gancho), causando impedimentos estereoquímicos na ligação ao TAG, comparativamente a AG com menor número de duplas ligações.

Nestes estudos, têm sido utilizadas diversas fontes de AG em reacções de acidólise com o objectivo de obter um produto similar à GLH. Na maioria dos estudos publicados, a razão dador de grupo acilo/TAG é consideravelmente elevada (Tabela 1).

Com efeito, é geralmente aceite que um aumento desta razão resulta em níveis superiores de incorporação de AG no TAG, por deslocação do equilíbrio da reacção no sentido da formação do produto. Todavia, elevadas concentrações de AG livres são

indesejáveis na medida em que aumentam o custo do processo associado, quer à recuperação do substrato remanescente, quer do produto.
A utilização de solvente orgânico não é recomendada porque (i) se trata da obtenção de um produto para alimentação infantil, (ii) encarece o processo e (iii) dificulta a recuperação do produto para além de (iv) constituir uma fonte de poluição ambiental.

3. Trabalhos realizados no âmbito da Rede.

Os condicionalismos inerentes à utilização da via enzimática face à via química relacionam-se com o custo do biocatalisador e com a sua instabilidade intrínseca. A estabilidade operacional do biocatalisador, isto é, a capacidade de manter a sua actividade catalítica ao longo do tempo de operação, revela-se como um factor chave em biocatálise. Com efeito, só um biocatalisador com estabilidade operacional elevada é economicamente competitivo. Vários factores podem afectar a actividade enzimática no decurso da reacção nomeadamente, a temperatura, o teor de água, a presença de produtos de oxidação, a configuração do bioreactor e o seu modo de operação (Illanes, 1999; Sandoval *et al.*, 2002; Slotema *et al*., 2003).
A pesquisa de biocatalisadores com estabilidade operacional elevada a mais baixo custo coloca-se como um desafio à comunidade científica, no sentido de implementação dos sistemas enzimáticos à escala industrial.
Neste contexto, os trabalhos desenvolvidos no âmbito da Rede ENZNUT revelaram-se bastante promissores. Com efeito, são de referir os trabalhos desenvolvidos com lipases não comerciais de baixo custo, como alternativa aos biocatalisadores disponíveis no mercado, com o intuito de produzir SGLH. As lipases foram utilizadas num sistema reaccional, livre de solvente, constituído por tripalmitina, como fonte de ácido palmítico, e ácido oleico (razão molar tripalmitina/ácido oleico=1:2). As reacções processaram-se em reactor de parede dupla, a temperatura igual ou superior a 60ºC de forma a garantir a completa fusão da tripalmitina. Testaram-se como biocatalisadores (i) a lipase de *Carica papaya* (LCP), autoimobilizada no látex, extraída de agro-resíduos de plantações de papaia, (ii) a lipase heteróloga de *Rhizopus oryzae* (r-LRO), expressa numa estirpe mutante de *Pichia pastori* e (iii) a lipase 2 de *Yarrowia lipolytica*, imobilizada em diferentes suportes. O extracto enzimático de *Carica papaya* e a lipase 2

de *Yarrowia lipolytica* foram preparados pelo grupo de investigação liderado pela Dra. Georgina Sandoval, CIATEJ, México (Tecelão, 2010). A r-LRO foi produzida e purificada pelo grupo de investigação liderado pelo Prof. Francisco Valero, UAB, Barcelona (Arnau, 2010; Guillén, 2011). Os ensaios de biocatálise foram realizados pelo grupo da Profª Suzana Ferreira-Dias, ISA-UTL, Lisboa (Tabela 2).

Tabela 2

Na reacção de acidólise entre a tripalmitina e o ácido oleico catalisada pela LCP, a incorporação molar deste ácido no TAG rondou os 25%, valor semelhante ao alcançado quando se utilizaram as lipases comerciais Novozym 435, Lipozyme RM IM e Lipozyme TL IM (Tecelão *et al.*, 2010). A utilização da lipase recombinante de *Rhizopus oryzae* imobilizada em Eupergit C conduziu à incorporação de 12,6 mol-% de ácido oleico. A lipase 2 de *Yarrowia lipolytica* revelou, para o mesmo sistema, valores de incorporação substancialmente inferiores (menos de 5,3 mol-%), independentemente do suporte de imobilização testado. Estes resultados parecem indiciar uma desactivação térmica do biocatalisador (Fig. 1). Nesta figura estão também apresentados os rendimentos de incorporação calculados tendo em conta que 100% de incorporação corresponderá à incorporação de AG nas posições *sn*-1 e *sn*-3, ou seja 66,6% de incorporação molar, uma vez que as lipases utilizadas apresentam selectividade *sn*-1,3.

Figura 1

Os resultados promissores obtidos com LCP e r-LRO impulsionaram o desenvolvimento de estudos adicionais, sobre o mesmo sistema reaccional, com o intuito de aumentar o rendimento do processo.

Procedeu-se à modelação e optimização das condições reaccionais para LCP, com o objectivo de pesquisar efeitos significativos das variáveis razão molar e temperatura na incorporação de ácido oleico na tripalmitina. Apenas a razão molar exibiu um efeito positivo e linear na incorporação de ácido oleico indicando que um aumento desta variável se traduz num aumento da incorporação do ácido. Foi possível obter para uma

razão molar 6.8:1 (ácido oleico/tripalmitina) um valor de incorporação de ácido oleico de cerca de 39%-mol (Tecelão *et al.*, submetido).

Os estudos publicados sobre sistemas reaccionais idênticos, em que se utilizam enzimas comercias de elevado custo, revelaram valores de incorporação molar de ácido oleico, em condições optimizadas, muito semelhantes ao obtido com a LCP. Importa salientar que na literatura estes valores foram alcançados com razões dador de grupo acilo/TAG muito elevadas (Sahín *et al.*, 2005b; Sahín *et al.*, 2006).

No âmbito da Red ENZNUT, testaram-se ainda diferentes suportes de imobilização para r-LRO, nomedamente, Accurel MP 1000, Lewatit VP OC 1600 e Sepiolite modificada. Os níveis mais elevados de incorporação de ácido oleico em tripalmitina foram obtidos para a lipase imobilizada em Accurel e Lewatit (cerca de 22 mol-% para ambos os suportes).

Na perspectiva de implementar o sistema reaccional à escala piloto e/ou industrial, é importante seleccionar o biocatalisador tendo em consideração não apenas a sua actividade catalítica, mas também a sua manutenção ao longo do tempo de operação. Neste sentido, avaliou-se a estabilidade operacional de LCP e r-LRO em batches sucessivos de 23h cada. No final de cada batch, a enzima era separada do meio reaccional por filtração e reutilizada em meio fresco. O grau de incorporação molar de ácido oleico em tripalmitina, observado no final de cada batch, foi adoptado como indicador da actividade do biocatalisador nesse batch. O primeiro ensaio foi usado como referência (100% de actividade).

Os resultados obtidos para LCP e r-LRO imobilizada em Accurel e Lewatit são apresentados na fig 2. A LCP foi a que apresentou maior estabilidade operacional, seguida da r-LRO imobilizada em Lewatit. Quando a r-LRO foi imobilizada em Accurel, a actividade diminuiu drasticamente após o 2º batch. Os resultados obtidos com a LCP são extremamente promissores, uma vez que se utiliza uma lipase a custo próximo de zero, proveniente de um agro-resíduo, para a produção de SGLH de elevado valor acrescentado. Os resultados obtidos com r-LRO são muito interessantes uma vez que demonstram que a estabilidade operacional da lipase depende de uma escolha criteriosa do suporte.

Figura 2

Os resultados obtidos no âmbito da Red ENZNUT, para a produção de SGLH, demonstram a viabilidade da substituição das preparações comerciais de lipases imobilizadas por lipases não comerciais, com claros benefícios do ponto de vista da redução dos custos do processo biotecnológico.

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Legenda das figuras

Figura 1: *Incorporação de ácido oleico em tripalmitina e respectivo rendimento molar, após 24h de acidólise, catalisada por diferentes lípases não comerciais (tabela 2), em meio sem solvente, a 60ºC.*

Figura 2: *Ensaios de estabilidade operacional: actividade residual da LCP e da r-LRO imobilizada em Accurel e Lewatit, no final de cada batch de 23h, na reacção de acidólise da tripalmitina com o ácido oleico, à razão molar (TAG:AG) 1:2.*

Tabela 1: Produção de sucedâneos de gordura de leite humano (SGLH) por catálise enzimática.

Reacção	Substratos	Biocatalisador	Modo de operação	Tipo de sistema	Condições Reaccionais optimizadas	Melhores resultados	Referência
Acidólise	PPP +AG de óleo de colza com teor reduzido em ácido erúcico	*Carica papaya* latex Lipozyme RM IM	Batch	Sem solvente	[Biocatalisador] = 9,9 m-% RM= 1:1; T= 60ºC; t= 6h	SGLH com AGPI essenciais	Mukherjee & Kiewitt (1998)
Acidólise	Banha + AG de óleo de soja	Lipozyme RM IM	Batch	Sem solvente	[Biocatalisador] = 13,7 m-% RM= 1:2.4; T= 61ºC; t= 1h	SGLH similar à gordura de leite de mães Chinesas.	Yang *et al.* (2003)
Acidólise	PPP + AG de óleo de avelã + ácido esteárico (C18:0)	Lipozyme RM IM	Batch	*n*-hexano	[Biocatalisador] = 10 m-%; RM= 1:12:1.5; T= 65ºC; t= 24h	SGLH 47% of C18:1	Sahín *et al.* (2005a)
Acidólise	PPP + AG de óleo de avelã + GLA	Lipozyme RM IM Lipozyme TL IM	Batch	*n*-hexano	T= 55ºC; t=24h [Lipozyme RM IM]= 10 % RM=1:14.8 [Lipozyme TL IM]= 6 % RM=1:14	SGLH 10% GLA; 45% C18:1	Sahín *et al.* (2005b)
Acidólise	Banha + AG de óleo de soja	Lipozyme RM IM	Contínuo PBR	Sem solvente	RM= 1:3; T= 65ºC; Tempo de residência= 1.5h	SGLH similar ao Betapol	Nielsen *et al.* (2006)

Tabela 1 (cont.)

Reacção	Substratos	Biocatalisador	Modo de operação	Tipo de sistema	Condições reaccionais optimizadas	Melhores resultados	Referência
Acidólise	PPP + AG de óleo de avelã + AGPI omega-3	Lipozyme RM IM	Batch	*n*-hexano	[Biocatalisador] = 10 m-%; RM= 1:12.4; T= 55ºC; t= 24h	SGLH com AGPI omega-3	Sahín *et al.* (2006)
Acidólise	PPP + ácido oleico (C18:1) PPP + oleato de metilo	LIP1 Lipozyme RM IM	Batch	*n*-hexano	[Lipozyme RM IM]= 10 %; PPP + oleato de metilo RM= 1:3; T= 65ºC; t= 24h	SGLH 49.4% C18:1	Srivastava *et al.* (2006)
Interesterificação	PPP + óleos de coco, de girassol e de soja	Lipozyme RM IM	Batch	*n*-hexano	[Biocatalisador] = 10 m-%; RM= 1:1 (PPP: óleos vegetais); T= 55ºC; t= 12h	SGLH	Maduko *et al.* (2007a)
Interesterificação	PPP + óleos de girassol e de soja	Lipozyme RM IM	Batch	*n*-hexano	[Biocatalisador] = 10 m-%; RM= 1:3 (PPP: óleos vegetais); T= 55ºC; t= 14.4h	SGLH 40% C16:0 na posição *sn*-2	Maduko *et al.*, (2007b)
Interesterificação	Banha + oleo de soja	Lipozyme TL IM	Batch	Sem solvente	[Biocatalisador] = 5 m-%; T= 60ºC; t= 14.4h	SGLH	Silva *et al.* (2009b)

Tabela 1 (cont.)

Reacção	Substrato	Biocatalisador	Modo de operação	Tipo de sistema	Condições reaccionais optimizadas	Melhores resultados	Referência
Acidólise	PPP + ácido oleico PPP + AGPI omega-3	Lipozyme TL IM Lipozyme RM IM Novozym 435 Lipase/aciltransferase de *Candida parapsilosis*	Batch	Sem solvente	[Biocatalyst] = 8,9 m-%; MR= 1:2; T= 60ºC; t= 24h	SGLH com ácido oleico	Tecelão *et al.* (2010)
Acidólise	PPP + AG de óleo de avelã + AG de Neobee (mistura de AG de cadeia média)	Lipozyme RM IM	Batch	*n*-hexano	[Lipozyme RM IM]= 19,78 %; RM= 1:3.35 (AG total); T= 57ºC; t= 24h	SGLH com C8:0, C10:0 e C16:0	Ilyasoglu *et al.* (2011)
Acidólise	Estearina de palma + AG de óleos de colza, girassol e palmiste e AG esteárico e mirístico	Lipozyme RM IM	Batch	Sem solvente	[Lipozyme RM IM] = 10,7 m-%; RM= 1:14.6; T= 57ºC; t= 3.4h	SGLH	Zou *et al.* (2011)
Acidólise	Banha + AG de óleo de camélia	Lipozyme RM IM	Batch	Sem solvente	Lipozyme [RM IM] = 6,0 m-%; RM= 1:4; T= 45ºC; t= 6.0h	SGLH	Qin *et al.* (2011)
Interesterificação	Banha + óleo de soja	Lipozyme TL IM	Continuo PBR	*n*-hexano	T= 60ºC; Tempo de residência= 1.0 h	SGLH	Silva *et al.* (2011)

Legenda/abreviaturas: t = tempo de reacção; T = temperatura; TAG = triacilglicerol; AG = ácido gordo; RM = razão molar TAG:AG; C18:1 = ácido oleico; PPP = Tripalmitina; LIP1 – lipase livre de *Candida rugosa* isoforma 1; [Biocatalisador] = carga do biocatalisador; AGPI = ácidos gordos polinsaturados.

Tabela 2: Lipases não comerciais produzidas no âmbito da Red ENZNUT e utilizadas na produção de SGLH.

Enzima	**Suporte de imobilização**	**Designação**
Lipase 2 de *Yarrowia lipolytica*	Lewatit MP 62	*Y. lipolytica* 1
	Amberlite 96	*Y. lipolytica* 2
	Lewatit K 2629	*Y. lipolytica* 3
	Lewatit VP OC 1065	*Y. lipolytica* 4
	Lewatit VP OC 1026	*Y. lipolytica* 5
	Lewatit MP TP 214	Y. lipolytica *6*
Lipase de *Carica papaya*	Auto-imobilizada no latex da papaia (batch 1)	*LCP*1
	Auto-imobilizada no latex da papaia (batch 2)	*LCP*2
Lipase heteróloga de *Rhizopus oryzae*	Eupergit C	r-LRO

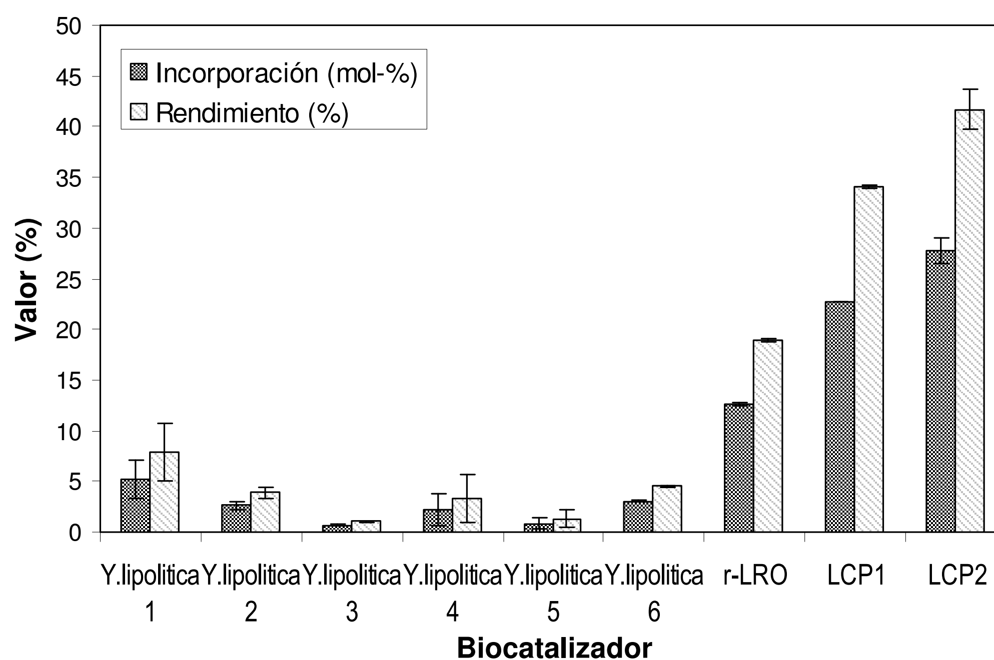

Figura 1: *Incorporação de ácido oleico em tripalmitina e respectivo rendimento molar, após 24h de acidólise, catalisada por diferentes lípases não comerciais (tabela 2), em meio sem solvente, a 60ºC.*

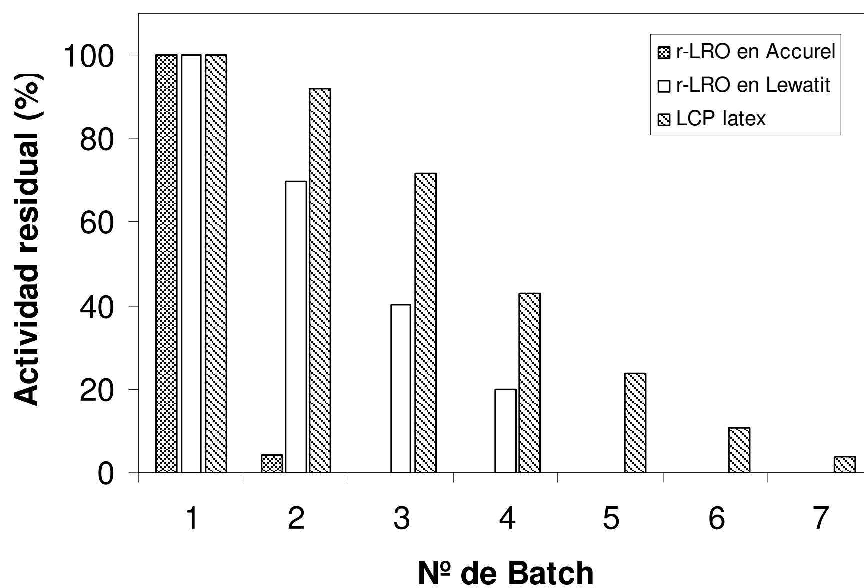

Figura 2: *Ensaios de estabilidade operacional: actividade residual da LCP e da r-LRO imobilizada em Accurel e Lewatit, no final de cada batch de 23h, na reacção de acidólise da tripalmitina com o ácido oleico, à razão molar (TAG:AG) 1:2*

Capítulo III.

Production of Human Milk Fat Substitutes enriched in Omega-3 Polyunsaturated Fatty Acids using Immobilized Commercial Lipases and *Candida parapsilosis* Lipase/Acyltransferase

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Production of human milk fat substitutes enriched in omega-3 polyunsaturated fatty acids using immobilized commercial lipases and *Candida parapsilosis* lipase/acyltransferase

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ABSTRACT

In human milk fat (HMF), palmitic acid (20–30%), the major saturated fatty acid, is mostly esterified at the *sn*-2 position of triacylglycerols, while unsaturated fatty acids are at the *sn*-1,3 positions, conversely to that occurring in vegetable oils.

This study aims at the production of HMF substitutes by enzyme-catalyzed interesterification of tripalmitin with (i) oleic acid (system I) or (ii) omega-3 polyunsaturated fatty acids (omega-3 PUFA) (system II) in solvent-free media. Interesterification activity and batch operational stability of commercial immobilized lipases from *Rhizomucor miehei* (Lipozyme RM IM), *Thermomyces lanuginosa* (Lipozyme TL IM) and *Candida antarctica* (Novozym 435) from Novozymes, DK, and *Candida parapsilosis* lipase/acyltransferase immobilized on Accurel MP 1000 were evaluated. After 24-h reaction at 60 °C, molar incorporation of oleic acid was about 27% for all the commercial lipases tested and 9% with *C. parapsilosis* enzyme. Concerning omega-3 PUFA, the highest incorporations were observed with Novozym 435 (21.6%) and Lipozyme RM IM (20%), in contrast with *C. parapsilosis* enzyme (8.5%) and Lipozyme TL IM (8.2%). In system I, Lipozyme RM IM maintained its activity for 10 repeated 23-h batches while for Lipozyme TL IM, Novozym 435 and *C. parapsilosis* enzyme, linear (half-life time, $t_{1/2}$ = 154 h), series-type ($t_{1/2}$ = 253 h) and first-order ($t_{1/2}$ = 34.5 h) deactivations were respectively observed. In system II, Lipozyme RM IM showed linear deactivation ($t_{1/2}$ = 276 h), while Novozym 435 ($t_{1/2}$ = 322 h) and *C. parapsilosis* enzyme ($t_{1/2}$ = 127 h), presented series-type deactivation. Both activity and stability of the biocatalysts depended on the acyl donor used.

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1. Introduction

In the field of edible oils and fats, research on lipase-catalyzed production of structured lipids (SL) presenting specific functional properties has greatly increased due to the potential benefits of the enzymatic route relatively to chemical processes [1]. Lipases (triacylglycerol acylhydrolases, EC. 3.1.1.3) catalyze ester hydrolysis in aqueous media, but when in organic media at low water activity, they can also catalyze esterification and transesterification reactions [2]. Lipase-catalyzed reactions are carried out under milder conditions (temperature lower than 70 °C, atmospheric pressure) and with a higher selectivity than chemically catalyzed reactions. In addition, the use of 1,3-selective lipases allows to maintain the fatty acids in the *sn*-2 position of the acylglycerols. This is nutritionally desirable and not possible to attain by chemical catalysis.

The synthesis of triacylglycerols (TAG) modified in their fatty acid composition is usually carried out by 1,3-specific lipase-catalyzed acidolysis of an ester (a single TAG, oil or fat) with a free fatty acid (FFA). The term "acidolysis" is classically used to describe a reaction where a fatty acyl group is exchanged through a combination of hydrolysis of a donor ester and esterification of the released alcohol moiety with another free fatty acid.

The human milk fat substitutes obtained by the enzymatic route are among the most important structured lipids, for the food industry. Human milk fat (HMF) contains long-chain fatty acids, namely oleic (30–35%), palmitic (20–30%), linoleic (7–14%) and stearic acids

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(5.7–8%). Unlike in vegetable oils and in cow milk fat, in HMF, palmitic acid, the major saturated fatty acid, is mostly esterified at the *sn*-2 position of the triacylglycerols, while unsaturated fatty acids are at the external positions. The use of vegetable oils and cow milk fat as a substitute of HMF in infant formulas may cause a deficient calcium and fatty acid absorption due to the formation of insoluble calcium soaps with saturated fatty acids released by the action of the 1,3-specific pancreatic lipase [3].

Nowadays, "Betapol™", a commercial structured lipid made of vegetable oils by position-specific enzymatic interesterification, is used as HMF substitute (HMFS) in both premature and term infant formula. This product is manufactured by Lipid Nutrition, Wormerveer, in the Netherlands [4].

In order to produce HMFS that mimic human milk fat, several studies have been carried out using immobilized lipases as catalysts. In the majority of these studies, HMFS were obtained by acidolysis of tripalmitin or lard (rich in palmitic acid) with free fatty acids from different sources.

Thus, HMFS containing palmitic, oleic, stearic and linoleic acid [3], long-chain polyunsaturated and essential fatty acids [5,6], gamma-linolenic acid [7], and omega-3 polyunsaturated fatty acids (omega-3 PUFA) [8], were obtained. The incorporation of omega-3 PUFA in infant formulas, and docosahexaenoic acids (DHA, 22:6n-3) in particular, has known benefits in the development of brain and nervous system of infants [9,10]. HMFS were also obtained by interesterification of (i) tripalmitin with blends of vegetable oils [11,12] or of (ii) lard with soybean oil [13].

Nowadays, the high cost of the commercial non-immobilized and immobilized lipases, together with a low operational stability of the latter, have been recognized as the major constraints to their use in the food industry. To make the enzymatic process competitive, lipases should be reusable and stable.

The high operational stability exhibited by several immobilized lipases in different reaction systems is a key factor in terms of the economical and technological competitiveness with chemical catalysts. The industrial implementation of an enzymatic process depends not only on the catalytic activity of the biocatalyst but also and principally on its operational stability.

The majority of the studies on the production of structured lipids use high cost commercial immobilized lipases. In this study, in order to search for biocatalysts with eventual novel properties as alternative to these commercial biocatalysts, the performance of immobilized *Candida parapsilosis* lipase/acyltransferase, as catalyst for the acidolysis aimed at the production of HMFS, was investigated. Although the free form of this enzyme presents only a slight 1,3-regioselectivity during hydrolysis and transesterification in aqueous media [14], it was included in this study in order to evaluate its potential in acidolysis in organic systems. The lipase/acyltransferase, when in aqueous or in biphasic aqueous/organic media, preferentially catalyzes alcoholysis over hydrolysis [14,15]. We have previously shown that this enzyme was able to catalyze the interesterification of fat blends containing omega-3 PUFA, in solvent-free media, batchwise and in a continuous fluidized-bed reactor. The lipase/acyltransferase presented, at a water activity (a_w) of 0.97, an interesterification activity similar to that exhibited by commercial immobilized lipases at a_w values lower than 0.5 [16,17].

In the present study, the production of HMFS was performed by enzyme-catalyzed acidolysis of tripalmitin with (i) oleic acid (system I) or (ii) omega-3 PUFA (system II), in solvent-free media at 60 °C. Four immobilized enzymes were tested in the same systems: *C. parapsilosis* lipase/acyltransferase and the immobilized commercial lipases from *Rhizomucor miehei* (Lipozyme RM IM), *Thermomyces lanuginosa* (Lipozyme TL IM) and *Candida antarctica* lipase B (Novozym 435). In both reaction systems, the activity and batch operational stability of these enzymes were evaluated in order to select the best biocatalyst.

2. Materials and methods

2.1. Materials

Tripalmitin (95% purity; MW = 807.35), 2′,7′-dichlorofluorescein and methyl myristate standard (>99%) were obtained from Fluka; extra pure oleic acid was from Merck and sodium cholate 99% was from Acros Organics. The commercial concentrate of triacylglycerols rich in omega-3 PUFA, "EPAX 1050TG" (10% eicosapentaenoic acid, EPA, and 50% docosahexaenoic acid, DHA), was a gift from EPAX AS, Lysaker, Norway. Silica-Gel 60 (0.25 mm width, 20 cm × 20 cm) thin layer chromatography (TLC) plates were purchased from Merck. The standards of triolein, diolein (mixed isomers) and monoolein were from Sigma–Aldrich. The other reagents used were *p.a.* and obtained from various sources.

The immobilized thermostable 1,3-selective lipases from *T. lanuginosa* ("Lipozyme™ TL IM") and from *R. miehei* ("Lipozyme™ RM IM"), and the immobilized thermostable preparation of the lipase B from *C. antarctica* ("Novozym™ 435") were kindly donated by Novozymes™, A/S, Bagsvaerd, Denmark. The lipase/acyltransferase from *C. parapsilosis* was produced by overexpression of the corresponding gene in *Pichia pastoris* according to Brunel et al. [18] and immobilized on Accurel MP 1000 (Membrana GmbH, Obernburg, Germany) as previously described [16]. Hog pancreas lipase (30.1 U/mg) was purchased from Fluka.

2.2. Methods

2.2.1. Preparation of free omega-3 PUFA

The preparation of free omega-3 PUFA from "EPAX 1050TG" was carried out according to the method described by Sahín et al. [3]: 25 g of "EPAX 1050TG" were saponified using a mixture of potassium hydroxide (5.75 g), 11 mL of water and 66 mL of 95% (v/v) aqueous ethanol, by refluxing at 100 °C for 60 min, in a flask equipped with a Liebig condenser. Distilled water (50 mL) was added to the saponified mixture and transferred to a separating funnel, where the unsaponifiable matter was extracted by *n*-hexane (2× 100 mL) and discarded. The aqueous layer containing the saponified matter was acidified to pH 1.0 with 3N HCl. The released free fatty acids were extracted with *n*-hexane (50 mL) and dried with anhydrous sodium sulphate. Sodium sulphate was removed by paper filtration and the *n*-hexane was evaporated in a rotavapor at 40 °C and a pressure lower than 200 mbar. A TLC of the obtained FFA was performed (*cf.* 2.2.4.1.) in order to confirm the efficiency of the process. The obtained FFA were stored at −18 °C under nitrogen until use.

2.2.2. Interesterification reaction

Interesterification reactions were performed for 24 h in 20 mL closed thermostated cylindrical batch reactors at 60 °C under magnetic stirring. Two different reaction media were used: (i) 3.90 g tripalmitin and 2.76 g oleic acid (system I) and (ii) 3.90 g tripalmitin and 3.17 g omega-3 PUFA (system II). The amounts of tripalmitin and free fatty acids (FFA) correspond to a molar ratio FFA:tripalmitin of 2:1, calculated on the basis of molecular weights of oleic acid (282.5) and of DHA (328.5), respectively.

A load of 8.9% (w/w tripalmitin) of the immobilized enzyme was added to the reaction medium, after complete melting.

Prior to and after 24 h reaction time, 1 mL samples were taken and the biocatalyst was removed by paper filtration at approximately 70 °C. All samples were stored at −18 °C for subsequent analysis. All the experiments were carried out in triplicate.

For each system, molar incorporation degree (%) was calculated on the basis of molecular weight of oleic acid (system I) and of DHA (system II), respectively.

2.2.3. Batch operational stability tests

In each system, batch operational stability of the biocatalysts was evaluated in consecutive 23 h batches. Interesterification was carried out as previously described (*cf.* 2.2.2.). After each batch, the biocatalyst was removed from the reaction medium by paper filtration and reused in the next batch with fresh medium, under the same reaction conditions. A total of up to 10 batches were performed using the same biocatalyst sample.

The activity of the biocatalyst corresponds to the molar incorporation degree of oleic acid (system I) or omega-3 PUFA (system II) in tripalmitin, observed at the end of each batch. The first batch was used as the reference (100% activity). The residual activity (a_n, %) of the biocatalyst at the end of each batch n ($n = 1, \ldots, 10$) was thus estimated as follows:

$$a_n = \left(\frac{\text{Incorporation}_{\text{Batch-}n}}{\text{Incorporation}_{\text{Batch_1}}} \right) \times 100 \qquad (1)$$

The operational half-life time of the biocatalyst, i.e., the operation time needed to reduce its original activity to 50%, was estimated by the models fitted to the observed deactivation profiles.

The fit of the deactivation models to experimental data was carried out using “solver” add-in from Excel for Windows, version 8.0 SR2, by minimizing the residual sum-of-squares between the experimental data points and those estimated by the respective model, using the following options: Newton method; 100 iterations, precision of 10^{-5}; 5% of tolerance and 0.001 convergence.

2.2.4. Analysis of reaction products

2.2.4.1. Evaluation of incorporation degree. To determine the amount of oleic acid or omega-3 PUFA incorporated in TAG, the following procedure was followed for each sample: 0.15 g of the reaction medium was dissolved in 25 mL of chloroform *p.a.* and 200 μL of this solution was spotted on a continuous layer on a silica gel TLC plate. Elution was carried out in *n*-hexane/diethyl ether/acetic acid (70/30/1.5, v/v/v) as the mobile phase. Plates were sprayed with 0.2% (w/v) 2′,7′-dichlorofluorescein in 95% ethanol and observed under UV at 366 nm. The various groups of compounds (triacylglycerols, free fatty acids, diacylglycerols and monoacylglycerols) were identified by comparison with standards.

The TAG band was scrapped off and methylated, in order to be assayed as fatty acid methyl esters (FAME) by gas chromatography (GC). For methylation, the silica gel containing TAG was mixed with 5 mL of methylation reagent (anhydrous methanol/*n*-hexane/concentrated sulphuric acid; 75/25/1, v/v/v), in a conical flask equipped with a Liebig condenser. This mixture was allowed to boil under reflux for 60 min in a water bath at about 80 °C. Then, 10 mL of distilled water and 10 mL of petroleum ether were added and the mixture was transferred to a separating funnel, vigorously agitated and allowed to settle for phase separation. The organic upper layer was recovered, washed twice with distilled water (2× 10 mL) and dried with anhydrous sodium sulphate. Sodium sulphate was removed by paper filtration, the solution was transferred to a conical-bottom flask and the solvent was evaporated in a rotavapor at 30 °C under a pressure lower than 200 mbar.

The FAME were dissolved in 100 μL of 0.1% (w/v) methyl myristate (internal standard) in *n*-hexane solution and 1 μL of this solution was GC analyzed. A Finnigan TRACE GC Ultra gas chromatograph (Thermo Electron Corporation) equipped with a Thermo TR-FAME capillary column (30 m × 0.25 mm ID × 0.25 μm film), an auto sampler AS 3000 from Thermo Electron Corporation and a flame ionization detector, was used for FAME analysis. Injector (in splitless mode) and detector temperatures were set at 250 °C and 260 °C, respectively. Helium was used as carrier gas at a flow rate of 1.5 mL/min. Air and hydrogen were supplied to the detector at flow rates of 350 mL/min and 35 mL/min, respectively.

For the analysis of samples of system I, the oven temperature program was as follows: 60 °C for 1 min, a temperature increase to 150 °C at 15 °C/min, a plateau at 150 °C for 1 min, followed by temperature increase to 180 °C at 5 °C/min, a plateau at 180 °C for 3 min, an increase in temperature until 220 °C, at a rate of 10 °C/min and a final plateau at 220 °C for 1 min.

For the analysis of samples of system II, the oven temperature program was as follows: 60 °C for 1 min, a temperature increase to 150 °C at 15 °C/min, a plateau at 150 °C for 1 min, followed by temperature increase to 220 °C, at a rate of 5 °C/min and a final plateau at 220 °C for 10 min.

2.2.4.2. Fatty acid composition at the sn-2 position. The fatty acid composition at the *sn*-2 position of the modified triacylglycerols was determined according to the following protocol, adapted from Jennings and Akoh [19]: each sample (1 g of fat) obtained after 24-h enzymatic acidolysis was dissolved in chloroform (5 mL) and 300 μL of this solution was spotted in a continuous layer on a silica gel TLC plate and developed as previously described (*cf.* 2.2.4.1.).

The band corresponding to the TAG fraction was scrapped off, the TAG fraction was extracted by diethyl ether (3× 5 mL) and the solvent evaporated in a rotavapor. TAG were re-suspended in 2 mL 0.1 M Tris–HCl aqueous buffer (pH 8.0) with 0.5 mL of 0.1% (w/v) sodium cholate aqueous solution and 0.2 mL of 22% (w/v) calcium chloride aqueous solution. Pancreatic lipase (50 mg) was added to this mixture and the hydrolysis was carried out at 40 °C. After 5 min, the reaction was stopped by the addition of 1 mL 6N HCl aqueous solution; 3 mL of ethyl ether was added and the mixture was centrifuged for 5 min at 1200 × g. The upper organic layer was recovered, the solvent evaporated, the extract was re-suspended in 300 μL of diethyl ether and spotted in a continuous layer on a silica gel TLC plate and developed. The band corresponding to the *sn*-2 monoacylglycerols was scrapped off, methylated and GC analyzed as previously described (*cf.* 2.2.4.1.).

3. Results and discussion

3.1. Acidolysis activity

All the enzymes were tested as catalysts for both the incorporation of oleic acid or omega-3 PUFA in tripalmitin. Since the hydrolytic activity is not always correlated with interesterification activity [20], the biocatalysts were used at the same weight ratio, in order to give some hints for industrial applications, in terms of process costs estimation [21].

For each system, the molar incorporation degree is presented in Table 1. After 24-h reaction, the incorporation levels of oleic acid were about 27–28%, for all the commercial lipases tested, and only 9% with *C. parapsilosis* enzyme. When omega-3 PUFA were incorporated in tripalmitin, the highest incorporation levels were observed for Novozym 435 (21.6%) and Lipozyme RM IM

Table 1
Average molar incorporation values (%) of oleic acid (system I) or omega-3 PUFA (system II) in tripalmitin, upon 24-h acidolysis of tripalmitin, catalyzed by different biocatalysts (±standard deviation; 3 repetitions).

Biocatalyst	System I	System II
Lipozyme RM IM	27.10 ± 0.35	20.80 ± 1.33
Lipozyme TL IM	27.10 ± 0.07	8.20 ± 0.57
Novozym 435	28.20 ± 0.07	21.60 ± 1.60
Lipase/acyltransferase	9.00 ± 2.13	8.50 ± 2.26

(20.8%); with *C. parapsilosis* lipase/acyltransferase and Lipozyme TL IM, only 8.5 and 8.2% incorporation was detected, respectively. Thus, the immobilized *C. parapsilosis* lipase/acyltransferase was not affected by the replacement of oleic acid with omega-3 PUFA in the reaction medium. Conversely, the immobilized *T. lanuginosa* lipase (Lipozyme TL IM) seemed to present higher affinity for oleic acid than for omega-3 PUFA.

However, in previous studies, Lipozyme TL IM showed to be adequate to catalyze the interesterification of blends of palm stearin, palm kernel oil and concentrates of triacylglycerols rich in omega-3 PUFA ("EPAX 2050TG", rich in DHA, and "EPAX 4510TG", rich in EPA) in solvent-free media [22,23]. Also, a higher selectivity of this biocatalyst for DHA as compared to EPA was previously observed [22]. The commercial immobilized *C. antarctica* lipase B ("Novozym 435") was successfully used as catalyst for the interesterification of palm stearin with a concentrate of triglycerides enriched in omega-3 PUFA and soybean oil ("EPAX 2050TG") in solvent-free media [24]. The different behaviors observed in the present study may be explained by the presence of free oleic acid and free omega-3 PUFA, while in the previous studies these fatty acids were esterified in the triacylglycerol molecules.

Molar incorporation values ranging from 10% to 47% of oleic acid were observed after 24-h acidolysis of tripalmitin with hazelnut fatty acids and stearic acid, in *n*-hexane, catalyzed by Lipozyme RM IM [3]. The highest incorporation value was achieved at 65 °C and at the highest substrate molar ratio tested (1:12:1.5, tripalmitin:hazelnut FA:stearic acid).

HMFS containing gamma-linolenic acid (GLA) was also produced by interesterification of tripalmitin with hazelnut fatty acids and GLA, in *n*-hexane, catalyzed by Lipozyme RM IM and Lipozyme TL IM [7]. Similar results were obtained with both enzymes: to attain both 10% GLA and 45% oleic acid incorporation, the optimal reaction conditions, predicted by response surface methodology, were 55 °C, 24 h reaction time and molar substrate ratio of about 14:1 (moles of total FA/moles of tripalmitin).

When the incorporation of omega-3 PUFA was carried out in a similar system (tripalmitin, hazelnut fatty acids and omega-3 PUFA, in n-hexane), catalyzed by Lipozyme RM IM, the highest incorporation levels of EPA and DHA (5%) and of oleic acid (40%) were predicted to be attained upon 24 h reaction time, at 55 °C, using a substrate ratio (moles of total FFA/moles of tripalmitin) of 12.4:1 [8].

An isoform of *Candida rugosa* lipase (LIP1) and Lipozyme RM IM were also used as catalysts for the interesterification of tripalmitin with oleic acid or methyl oleate in *n*-hexane [25]. In this study, higher oleic acid incorporations were obtained, in general, with methyl oleate as acyl donor, increasing with increasing substrate molar ratio up to 1:3. When oleic acid was used, 26.3% incorporation was obtained with LIP1, at 45 °C, and about 45% with Lipozyme RM IM, at 65 °C, after 24-h reaction and with a substrate ratio of 1:3 (tripalmitin:fatty acid).

It is worthy to notice that the high incorporation values reported in the literature, were obtained using high FFA/tripalmitin molar ratios. These reaction conditions will increase operation costs, namely unconverted substrates recycling and product recovery.

3.2. Fatty acid composition at the sn-2 position

The fatty acids at the *sn*-2 position of the structured TAG obtained after 24-h reaction of tripalmitin with oleic acid, are presented in Table 2.

Table 2
Fatty acid (mole percent) at *sn*-2 position of the TAG obtained upon 24-h interesterification of tripalmitin with oleic acid.

Fatty acid	Lipozyme RM IM	Lipozyme TL IM	Novozym 435	Lipase/acyltransferase
C16:0	79.90	75.60	61.20	87.30
C16:1	0.30	0.70	1.70	0.00
C18:0	3.10	2.80	2.70	6.10
C18:1	16.00	20.60	33.20	6.60
C18:2	0.70	0.30	1.20	0.00

As observed, the *sn*-2 positions of the structured lipids were predominantly occupied by palmitic acid (61.2–87.3 mol%). When the 1,3-specific lipases Lipozyme RM IM and Lipozyme TL IM were used, despite their region-specificity, the incorporation of oleic acid (16 and 20.6 mol%, respectively) in the *sn*-2 positions occurred due to acyl migration during acidolysis. The acyl migration levels were similar to those reported by others in analogous systems and under similar temperature values [3,7,8,25]. The lowest *sn*-2 incorporation level was observed with *C. parapsilosis* lipase/acyltransferase, probably explained by a low acidolysis rate, which results in a low acyl migration. In general, an increase of acyl migration with temperature has been reported [25,26].

The highest incorporation of oleic acid in *sn*-2 position (33.2 mol%) was observed with Novozym 435. In fact, this enzyme is usually not positionally specific towards fatty acid residues in TAG. As reported by the manufacturer, Novozym 435 is a highly versatile catalyst with activity towards a great variety of different substrates and it has been primarily used in the synthesis of optically active alcohols, amines and carboxylic acids, due to its highly enantioselectivity.

3.3. Operational stability tests

In order to select the best biocatalyst for HMFS production, not only the catalytic activity but also batch operational stability was evaluated.

For system I, batch operational stability tests were carried out for the three commercial immobilized biocatalysts, since all of them presented similar interesterification activity. For system II, the operational stability was evaluated for the commercial biocatalysts presenting the highest interesterification activity (Lipozyme RM IM and Novozym 435). In addition, even though the activity of the *C. parapsilosis* lipase/acyltransferase immobilized sample was not high, its operational stability was investigated in both systems, since it had not been tested before.

The residual acidolysis activities of the biocatalysts, at the end of each of the repeated 23-h batches, in systems I and II, are presented in Figs. 1 and 2, respectively. The deactivation models fitted to these results, as well as the respective estimated half-life times, are shown in Table 3.

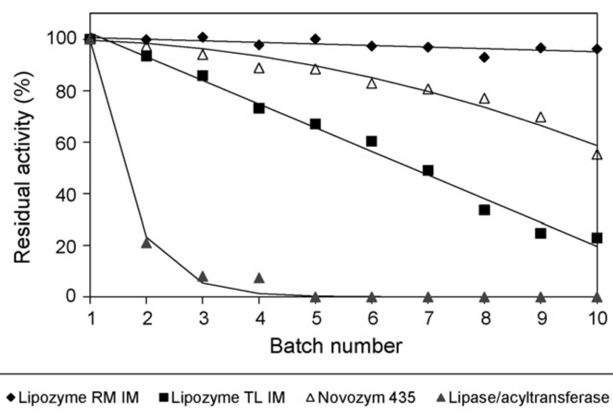

Fig. 1. Batch operational stability test: residual activity of the biocatalysts at the end of each 23-h batch reutilization in the acidolysis of tripalmitin with oleic acid (system I).

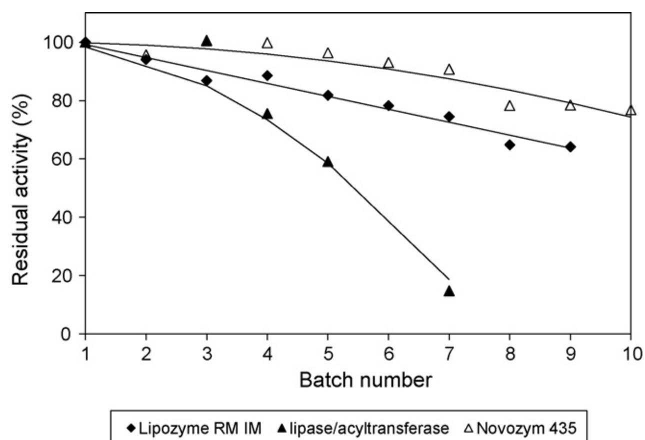

Fig. 2. Batch operational stability test: residual activity of the biocatalysts at the end of each 23-h batch reutilization in the acidolysis of tripalmitin with omega-3 PUFA (system II).

In system I, Lipozyme RM IM maintained its activity for 10 repeated 23-h batches while a linear decrease in Lipozyme TL IM activity was observed (Fig. 1). In analogy with the models fitted to biocatalyst deactivation in continuous bioreactors, the following linear equation could be fitted to the residual activity (a_n) at the end of each consecutive batch, n, for Lipozyme TL IM:

$$a_n = -9.19n + 111.55 \quad (2)$$

(determination coefficient: $R^2 = 0.9886$)

The estimated half-life time of this biocatalyst, $t_{1/2}$, i.e., the operation time required for half the enzyme activity to be lost as a result of deactivation, is about 6.7 batches, corresponding to 154 h operation.

Concerning the immobilized lipase B from *C. antarctica* (Novozym 435), an inactivation parabolic profile was observed for this biocatalyst in system I (Fig. 1). This behavior could be well described by the model of series-type inactivation kinetics proposed by Sadana [27]. The fraction of the original activity (%) of the biocatalyst, a_n, at the end of each batch n, can be given by the following simplified model equation:

$$a_n = 100 - 50k_d n^2 \quad (3)$$

where k_d (batch^{-2}) is the deactivation coefficient. Thus, the deactivation model fitted to the experimental data is given by the following equation where k_d is equal to 0.0082 batch$^{(-2)}$:

$$a_n = 100 - 0.41n^2 \quad (4)$$

The $t_{1/2}$ is given by

$$t_{1/2} = k_d^{(-1/2)} \quad (5)$$

Thus, a half-life time of 11 batches (253 h) was estimated from Eq. (5) for Novozym 435, when used in system I.

In system I, the observed deactivation profile of *C. parapsilosis* lipase/acyltransferase follows a first-order deactivation kinetics model:

$$a_n = A * \ e^{-k_d n} \quad (6)$$

where A is a constant. Therefore, from this model, a half-life time of 1.5 batches (34.5 h) was estimated for this biocatalyst (Eq. (7)):

$$a_n = 428.58 * \ e^{-1.46n} \quad (7)$$

In system II, the replacement of oleic acid (system I) by omega-3 PUFA in reaction media, conducted to changes in operational stability profiles of the biocatalysts (Fig. 2). Lipozyme RM IM, which was very stable in system I, showed a linear activity decrease when reused in system II, in 10 cycles of 23-h each, as follows:

$$a_n = -4.44n + 103.6 \quad (8)$$

($R^2 = 0.9696$)

An estimated half-life time of about 12 batches (276 h) was found for this biocatalyst.

In system II, Novozym 435 presented the highest stability with an estimated half-life time of 14 batches (322 h). As in system I, the inactivation profile of this biocatalyst can be described by Sadana's series-type model [27] with a k_d of 0.00515 batch$^{(-2)}$:

$$a_n = 100 - 0.257n^2 \quad (9)$$

The activity decay observed for the immobilized *C. parapsilosis* lipase/acyltransferase during the consecutive 23-h batches, in system II, could also be described by Sadana's series-type inactivation kinetic model (Eq. (3)) [27], with a k_d of 0.0332 batch$^{(-2)}$:

$$a_n = 100 - 1.66n^2 \quad (10)$$

A half-life time of 5.5 batches (127 h) was estimated for this biocatalyst. The inactivation profile was similar to that observed for Novozym 435 in both systems (Eqs. (4) and (9)).

C. parapsilosis lipase/acyltransferase displayed lower operational stability in the presence of oleic acid (more than 80% loss along the first reutilization) than in the presence of omega-3 PUFA. A difference in operational stability according to the acid used was also observed with the commercial immobilized lipases: Lipozyme RM IM was more stable in the presence of oleic acid (no significant deactivation in 10 batches) than in the presence of omega-3 PUFA (half-life 12 batches), conversely to that observed with Novozym 435. The different behaviors exhibited by the biocatalysts might be due to differences in (i) enzyme sensitivity to by-products accumulating on the immobilization support, including fatty acids oxidation products, or (ii) to enzyme dehydration during the reaction and medium renewal between batches. In fact, the presence of omega-3 PUFA, which are rather prone to oxidation, may explain the lower activity exhibited by Lipozyme RM IM in system II. The negative effect of the presence of hydroperoxides, final oxidation products, phospholipids, chlorophyll and carotenoids and lipid polymers, on lipase stability was also previously observed [23,28–30]. A loss of activity of *C. parapsilosis* lipase/acyltransferase in Accurel MP 1000 was observed in continuous fluidized-bed reactor and during the reutilizations in consecutive batches, due to biocatalyst dehydration [17].

Table 3
Deactivation models and respective half-life times estimated for the biocatalysts used in 10 consecutive 23-h batches in System I and System II (n.d.—not determined).

Biocatalyst	System I (tripalmitin + oleic acid)			System II (tripalmitin + omega-3 PUFA)		
	Deactivation model	Half-life time		Deactivation model	Half-life time	
		Batch no.	h		Batch no.	h
Lipozyme RM IM	No deactivation	No deactivation	No deactivation	Linear	12	276
Lipozyme TL IM	Linear	6.7	154	n.d.	n.d.	n.d.
Novozym 435	Series-type	11	253	Series-type	14	322
Lipase/acyltransferase	First-order	1.5	34.5	Series-type	5.5	127

The decrease in operational stability might also be due to modifications in the protonation state of the enzymes under the influence of the free fatty acids used: pK_a of free fatty acids are known to decrease with unsaturation degree [31–33]. This may explain the higher operational stability of Novozym 435 and *C. parapsilosis* enzyme in the presence of omega-3 PUFA than in the presence of oleic acid. This hypothesis will be further investigated.

4. Conclusions

All the biocatalysts tested presented acidolysis activity aimed at the production of structured lipids adequate to be used as human milk fat substitutes. In system I (tripalmitin and oleic acid), all the immobilized commercial lipases presented similar activities (ca. 27 mol% incorporation) but only Lipozyme RM IM was stable along 10 consecutive 23-h cycles. When oleic acid was replaced by omega-3 PUFA (system II), a decrease in incorporation levels was observed with the commercial lipases tested. The highest incorporation values were obtained with Novozym 435 (21.6 mol%) and Lipozyme RM IM (20.8 mol%). In this system, Novozym 435 presented higher operational stability than when in system I and than Lipozyme RM IM, in system II.

C. parapsilosis lipase/acyltransferase showed similar catalytic activities in both systems, though low incorporation values were obtained (about 9 mol%) in the conditions tested. This enzyme seems to be an adequate catalyst for the acidolysis of tripalmitin with blends of oleic acid and omega-3 PUFA to prepare SL similar to human milk fat. The search for more adequate supports and/or immobilization techniques will be attempted to improve both activity and operational stability of this enzyme.

Concerning the commercial immobilized lipases, only Lipozyme RM IM seems to be adequate for using in reaction media containing blends of oleic acid and omega-3 PUFA.

The results obtained in this study show that the activity and operational stability of the biocatalysts used depend on the acyl donor used.

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Capítulo IV.

Production of human milk fat substitutes by interesterification of tripalmitin with ethyl oleate catalyzed by *Candida parapsilosis* lipase/acyltransferase

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Production of human milk fat substitutes by interesterification of tripalmitin with ethyl oleate catalyzed by *Candida parapsilosis* lipase/acyltransferase

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Abstract

The recombinant lipase/acyltransferase from *Candida parapsilosis* (CpLIP2) immobilized in Accurel MP1000 was used as biocatalyst for the production of human-milk fat substitutes (HMFS). In our previous work, using this biocatalyst for the acidolysis of free oleic acid with tripalmitin, in a solvent-free medium at 60°C, a limited incorporation of oleic acid in triacylglycerols (8.5 mol% in 24h) was observed. In the present study, oleic acid was replaced by ethyl oleate, under the same conditions, and using different molar ratios of ethyl oleate to tripalmitin (2:1 to 8:1). After 4h reaction time, about 30 mol-% incorporation was already observed for all ratios tested. An apparent equilibrium was reached after 12-24 hours, with 32 to 45 mol-% final incorporation, depending on the molar ratio used. The maximum incorporation of oleic acid into triacylglycerols linearly increased with molar ratio. The incorporation levels of oleic acid are similar or even higher than those achieved when high-cost commercial immobilized lipases are used.

Key Words: acyltransferase; *Candida parapsilosis* lipase/acyltransferase; ethyl oleate; human milk fat substitutes; immobilization; interesterification.

1. Introduction

The synthesis of structured triacylglycerols (TAG), modified in their fatty acid composition or in their regio-distribution in the glycerol backbone, is usually carried out by *sn*-1,3 regioselective lipase-catalyzed reactions.

Lipases present recognized advantages over the chemical catalysts, namely operation under mild conditions (normal pressure and temperature below 70ºC) and high selectivity (regio-, stereo-, typo- and substrate selectivities), leading to a decrease in the formation of side products [1].

The *sn*-1,3 regioselectivity of some lipases has been used to produce human milk fat substitutes (HMFS) that mimic the rather unique fatty acid profile of breast milk. Human milk fat (HMF) contains long-chain fatty acids, namely oleic (30-35%), palmitic (20-30%), linoleic (7-14%) and stearic acids (5.7-8%). Unlike in vegetable oils and in cow milk fat, the major saturated fatty acid in HMF, palmitic acid, is mostly esterified at the *sn*-2 position of the triacylglycerols, while unsaturated fatty acids are at the external positions. The use of vegetable oils and cow milk fat as a substitute of HMF in infant formulas may cause a deficient calcium and fatty acid absorption due to the formation of insoluble calcium soaps with saturated fatty acids released by the action of the *sn*-1,3 selective pancreatic lipase [2].

Several studies concerning the synthesis of HMFS obtained by *sn*-1,3 lipase-catalyzed (i) acidolysis of tripalmitin, butterfat or lard (rich in palmitic acid in *sn*-2 position) with free fatty acids (FFA) from different sources [2-8] or (ii) interesterification of tripalmitin with blends of vegetable oils [9, 10] have been reported. Also, several

research groups have focused on multistep reactions for the synthesis of 1,3-dioleoyl-2-palmitoylglycerol (OPO), an important structured TAG in infant formulas [11-14]. Nowadays, two HMFS have been commercialized: "Betapol™" by Lipid Nutrition, Wormerveer, the Netherlands) and, more recently, "InFat™" by Advanced Lipids (Karlshamn, Sweden). Both "Betapol™" and "InFat™" are synthesized by acidolysis reactions between fats with palmitic acid esterified, predominantly, at the *sn*-2 position, such as fractionated palm stearin or tripalmitin, with blends rich in oleic acid. These reactions are catalyzed by sn-1,3 selective lipases such as those from *Rhizomucor miehei* (Lipozyme RM IM) or *Rhizopus oryzae* [15].

Nowadays, the high cost of the commercial non-immobilized and immobilized lipases, together with a low operational stability, have been recognized as the major constraints to their use in the food industry.

In order to search for biocatalysts with eventual novel properties as alternative to the commercial biocatalysts, the performance of immobilized *C. parapsilosis* lipase/acyltransferase (CpLIP2) was investigated. CpLIP2 shows the ability to preferentially catalyze alcoholysis over hydrolysis in biphasic aqueous/organic media [16-18]. This is a peculiar feature of CpLIP2 since it is generally recognized that the transfer of acyl groups between two alcohols results from a hydrolysis reaction followed by a reverse esterification reaction [19].

This enzyme was successfully studied for the production of structured lipids, to be used as margarine fat bases, prepared from the interesterification of blends of vegetable oils with concentrates of TAG rich in omega-3 polyunsaturated fatty acids [20, 21]. Also, CpLIP2 was tested for the ability to catalyze the incorporation of free oleic acid in tripalmitin, aimed at the production of HMFS, as reported in our previous work [7].

In the present study, CpLIP2 was used as biocatalyst in the interesterification reaction between ethyl oleate and tripalmitin, batchwise, for the synthesis of structured triacylglycerols to be used in HMFS. The effect of the molar ratio on the incorporation of oleic acid in TAG was investigated.

2. Materials and Methods

2.1. Materials

Tripalmitin (95% purity), 2',7'-dichlorofluorescein and methyl myristate standard (>99%) were obtained from Fluka; ethyl oleate (98% purity) was from Sigma-Aldrich. Silica-Gel 60 thin layer chromatography (TLC) plates (0.25 mm width, 20 x 20cm) were purchased from Merck. The standards of triolein, diolein (mixed isomers) and monoolein were from Sigma-Aldrich. The other reagents used were *p.a.* and obtained from various sources.

The lipase/acyltransferase from *C. parapsilosis* was produced by overexpression of the corresponding gene in *Pichia pastoris* according to Brunel *et al*. [16].

2.2. Methods

2.2.1. *C. parapsilosis* immobilization procedure

C. parapsilosis lipase/acyltransferase was immobilized in Accurel MP 1000 (Membrana GmbH, Obernburg, Germany) according to the following protocol. One gram of dry

immobilization support was soaked in 7 mL ethanol for 15 min under gentle magnetic stirring. Ethanol was then carefully removed using a micropipette, and the wetted resin was added to 20 mL of enzyme solution (100 mg enzyme). The suspension was gently stirred for 24 h at 20°C. The biocatalyst was then stabilized by covalent crosslinking. For this propose, 25 mL of a 2.5% (v/v) glutaraldehyde aqueous solution was added to the immobilization medium and allow to stand for two hours under stirring. The supernatant was removed by vacuum succion over a glass filter and the immobilized biocatalyst was washed six times with 10 mL of sodium phosphate buffer (20 mM, pH 6.5). The excess of water was discarded under vacuum and the immobilized lipase was stored at 4ºC until use.

2.2.2. Interesterification reaction

Interesterification reactions were performed batchwise in closed thermostated cylindrical batch reactors (20 mL), at 60ºC, under magnetic stirring. The reaction medium consisted of a blend of tripalmitin and ethyl oleate. A load of 5 % (w/w of total lipids) of the immobilized enzyme was added to the reaction medium, after complete melting of the lipid blend.

At different reaction times, 1 mL samples were taken and the biocatalyst was removed by paper filtration at approximately 70ºC. All samples were stored at –18 ºC for subsequent analysis. All the experiments were carried out in triplicate. For each system, molar incorporation degree (%) was calculated on the basis of the molecular weight of oleic acid (282.5 $g.mol^{-1}$).

2.2.3. Evaluation of incorporation degree

To determine the amount of oleic acid incorporated in TAG, the following procedure was used for each sample: 0.15 g of the reaction medium was dissolved in 25 mL of chloroform *p.a.* and 200 µL of this solution was deposited as a continuous line on a silica gel TLC plate. Elution was carried out with petroleum ether/diethyl ether/acetic acid (85/15/1; v/v/v) as the mobile phase. Plates were sprayed with 0.2% (w/v) 2',7'-dichlorofluorescein in 95% ethanol and observed under U.V. at 366 nm. The various groups of compounds (triacylglycerols, free fatty acids, diacylglycerols and monoacylglycerols) were identified by comparison with standards.

The TAG band was scrapped off and methylated, in order to be assayed as fatty acid methyl esters (FAME) by gas chromatography (GC). For methylation, the silica gel containing TAG was mixed with 5 mL of methylation reagent (anhydrous methanol/*n*-hexane/concentrated sulphuric acid; 75/25/1, v/v/v), in a conical flask equipped with a Liebig condenser. This mixture was allowed to boil under reflux for 60 min in a water bath at about 80ºC. Then, 10 mL of distilled water and 10 mL of petroleum ether were added and the mixture was transferred to a separating funnel, vigorously agitated and allowed to settle for phase separation. The organic upper layer was recovered, washed twice with distilled water (2 x 10 mL) and dried with anhydrous sodium sulphate. Sodium sulphate was removed by paper filtration, the solution was transferred to a conical-bottom flask and the solvent was evaporated in a rotating evaporator at 30ºC under a pressure lower than 200 mbar.

FAME were dissolved in 100 µL of 0.1% (w/v) methyl myristate in *n*-hexane solution (internal standard solution) and 1 µL of this solution was GC analyzed. A Finnigan TRACE GC Ultra gas chromatograph (Thermo Electron Corporation) equipped with a

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Thermo TR-FAME capillary column (30 m x 0.25 mm ID x 0.25 μm film), an auto sampler AS 3000 and a flame ionization detector were used for FAME analysis. Injector (in splitless mode) and detector temperatures were set at 250ºC and 260ºC, respectively. Helium was used as carrier gas at a flow rate of 1.5 mL/min. Air and hydrogen were supplied to the detector at flow rates of 350 mL/min and 35 mL/min, respectively.

For the analysis of samples, the oven temperature program was as follows: 60ºC for 1 min, a temperature increase to 150ºC at 15ºC/min, a plateau at 150ºC for 1 min, followed by temperature increase to 180ºC at 5ºC/min, a plateau at 180ºC for 3 min, an increase in temperature until 220ºC, at a rate of 10ºC/min and a final plateau at 220ºC for 1 min.

3. Results and Discussion

Different molar ratios of ethyl oleate to tripalmitin (2:1 to 8:1) were used and their influence on the oleic acid incorporation (mol%) in the TAG, catalyzed by CpLIP2 from *C. parapsilosis* was investigated. After 4h of reaction, about 30 mol% incorporation was observed for all the ratios tested. An apparent equilibrium was reached after 12-24 hours, with 32 to 45 mol% final incorporation depending on the molar ratio used (Fig 1). A linear increase in oleic acid incorporation at equilibrium, with molar ratio, was observed (Fig. 2).

It is generally accepted that an increase of acyl donor/TAG molar ratio has a positive effect on the incorporation of FFA in the glycerol backbone, since it displaces the equilibrium towards the esterification reaction. Nevertheless, high amounts of acyl donors are undesirable due to the cost of recycling of unconverted substrates and product recovery.

Oleic acid incorporation in tripalmitin (31.5 mol-%), achieved after 24 h reaction at a molar ratio of 2:1 (ethyl oleate:tripalmitin), was higher than the obtained values reported in our previous work, where three commercial immobilized lipases (Novozym 435, Lipozyme RM IM and Lipozyme TL IM, from Novozymes, Denmark) and CpLIP2 were used as catalyst for the acidolysis reaction between oleic acid and tripalmitin, in solvent-free media [7]. In fact, similar fatty acid incorporations were attained for all the commercial lipases (*c.a.* 27 mol%) and only a residual value (9 mol%) was reached for CpLIP2.

According to Briand *et al*. [22], CpLIP2 has a high specificity towards esters with long-chain fatty acids and, particularly, for unsaturated fatty acids with a *cis*-9, such as ethyl oleate. Therefore, it is not surprisingly that higher levels of oleic acid incorporation in tripalmitin were achieved when ethyl oleate was used as acyl donor instead of oleic acid.

Similar results were attained when an isoform of *C. rugosa* lipase (LIP1) and Lipozyme RM IM were used as catalysts for the interesterification of tripalmitin with oleic acid or methyl oleate in *n*-hexane [23]. In this study, the highest oleic acid incorporation (37.7 mol-%) was obtained with methyl oleate as acyl donor, at 45ºC and after 24 h reaction time and a substrate molar ratio of 3:1 (methyl oleate:tripalmitin:). When free oleic acid was used, 26.3 mol-% incorporation was obtained with LIP1, at 45ºC, and about 45% with Lipozyme RM IM, at 65ºC, after 24-h reaction and with a substrate ratio of 3:1 (tripalmitin:oleic acid).

Also, ethyl esters, being more volatile than free fatty acids, are more easily recovered from the reaction media by distillation, which leads to less expensive downstream processing.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

Sahín and co-workers [3] synthesized HMFS by acidolysis of tripalmitin with hazelnut oil fatty acids and a concentrate of omega-3 fatty acids, in *n*-hexane, using Lipozyme RM IM as biocatalyst. The optimal conditions predicted by the models, for the targeted total of 5% of EPA and DHA and 40% of oleic acid incorporation, were 12.4 mol/mol (FFA/TAG), 55ºC and 24h.

When the incorporation of gamma-linolenic acid (GLA) was carried out in a similar system (tripalmitin, hazelnut fatty acids and GLA, in *n*-hexane), catalyzed by Lipozyme RM IM and Lipozyme TL IM, identical results were obtained with both enzymes: to attain 10% of GLA and 45% of oleic acid incorporation: the optimal reaction conditions, predicted by response surface methodology, were 55ºC, 24h reaction time and molar substrate ratio of about 14:1 (FFA/TAG) [7].

It is worth noticing that the incorporation values reported in the literature were of the same order or even lower than the values obtained in the present study, despite higher FFA/tripalmitin molar ratios used.

4. Conclusions

This study shows the potentialities of *C. parapsilosis* lipase/acyltransferase as a promising biocatalyst for the synthesis of HMFS, as an alternative to the commercial immobilized lipases usually used. The incorporation levels of oleic acid are similar or even higher than those achieved when high-cost commercial preparation immobilized lipases are used.

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Figure Legends:

Figure 1. Effect of the molar ratio ethyl oleate:tripalmitin on the interesterification kinetics, catalyzed by *C. parapsilosis* lipase/acyltransferase at 60ºC in solvent-free medium.

Figure 2. Effect of the molar ratio ethyl oleate:tripalmitin on the oleic acid incorporation level at interesterification equilibrium, catalyzed by *C. parapsilosis* lipase/acyltransferase at 60ºC in solvent-free medium.

Figure 1

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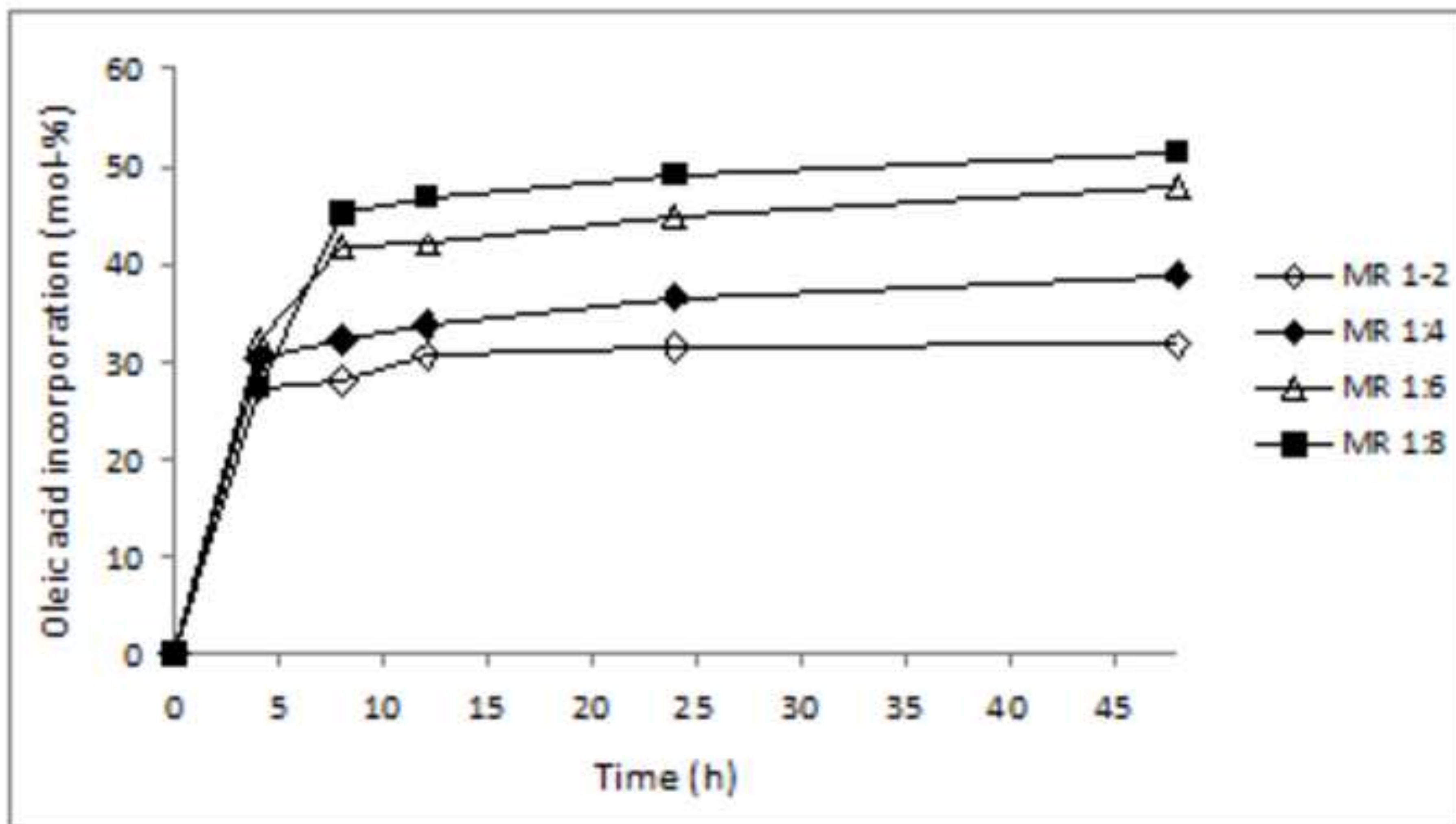

Figure 2

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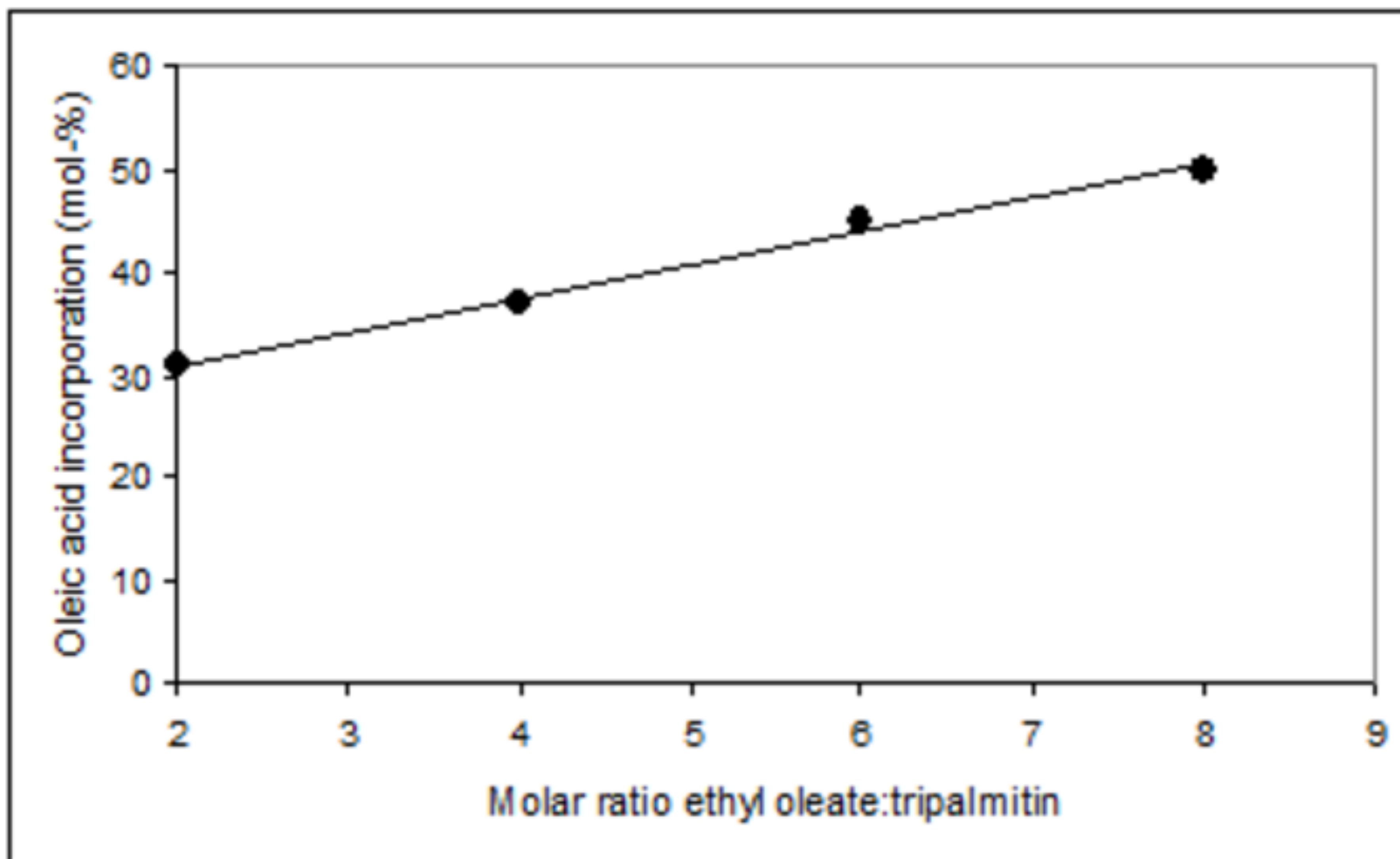

Capítulo V.

Carica papaya latex: a low-cost biocatalyst for human milk fat substitutes production

Tecelão, C., Rivera, I., Sandoval, G., Ferreira-Dias, S.

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Additional Keywords (select from list):	Long-chain polyunsaturated fatty acids, Structured lipids, Infant formula, Response surface methodology, Operational stability

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Carica papaya latex: a low-cost biocatalyst for human milk fat substitutes production

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Summary

This work aims at evaluating the potentialities of *Carica papaya* lipase (CPL) self-immobilized in papaya latex, as biocatalyst for the synthesis of Human Milk Fat Substitutes (HMFS), to be used as a low-cost alternative to commercial lipases. Two different CPL preparations, one extracted from the papaya fruit (CPL I) and the other from petiole leaves (CPL II) of papaya tree, were tested as catalysts for the acidolysis between tripalmitin and (i) oleic acid or (ii) omega-3 polyunsaturated fatty acids, batchwise, at 60ºC, in solvent-free media. After 24h, molar incorporation was higher for oleic acid (22.1 mol-%) when CPL I was used. This biocatalyst was selected for further studies. Response surface methodology was used to model reaction conditions: medium formulation (molar ratio oleic acid:tripalmitin, MR, 1.2:1-6.8:1) and temperature (58-72ºC). Acyl migration decreased with MR increase. In batch operational stability assays at 60ºC, using MR of 2:1 and 6:1, the highest stability was observed for a MR of 2:1.

Practical Applications: The use of this biocatalyst is a feasible way to valorise papaya agro-residues which represent an important environmental problem in producing countries. The obtained results were rather promising since, with this almost zero-cost biocatalyst, it was possible to produce a high added-value product (HMFS). Under optimised conditions, the obtained results were comparable with those obtained with expensive immobilized commercial lipases.

Key Words: Acidolysis*;* *Carica papaya* wastes; human milk fat substitutes; latex; lipase

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1. Introduction

In human milk fat (HMF), the *sn*-2 position of triacylglycerols (TAG) is essentially occupied by saturated fatty acids (70% of all palmitic acid), while unsaturated fatty acids are esterified at *sn*-1 and *sn*-3 positions. This is in contrast to what occurs in vegetable oils and ruminant milk fat, where unsaturated fatty acids are predominantly in *sn*-2 position [1]. The fatty acid profile of HMF has a crucial effect on its digestion and intestinal absorption in the infants. In fact, the presence of palmitic acid in the *sn*-2 position provides a more efficient absorption of palmitic acid, as *sn*-2 monoacylpalmitate [2]. The use of vegetable oils or cow milk fat as a substitute of HMF in infant formulas may cause a deficient calcium and fatty acid absorption, due to the formation of insoluble calcium soaps with saturated fatty acids released by the action of the *sn*-1,3 specific pancreatic lipase [3]. The production of structured lipids resembling human milk fat has been a challenge for the food industry. The synthesis of human milk fat substitutes (HMFS) has been carried out making use of lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3.) as biocatalysts. Lipases are versatile enzymes that naturally catalyse the hydrolysis of acylglycerols at oil/water interfaces but, in non-aqueous media under low water activity, they are also effective catalysts for esterification and interesterification reactions. Lipases present recognized advantages compared to classical chemical catalysts namely operations under mild conditions (normal pressure and temperatures below 70ºC) and high selectivity (regio-, stereo-, typo- and substrate selectivities), leading to a decrease in side products formation [4]. Nowadays, "Betapol™", a commercial structured lipid made of vegetable oils by position-specific enzymatic interesterification, is used as HMFS in both premature and term infant formula. This product is manufactured by Lipid Nutrition, Wormerveer, in the Netherlands (http://www.lipidnutrition.com, May 2011).

In the reported studies, HMFS have been obtained by *sn*-1,3 lipase-catalyzed (i) acidolysis of tripalmitin, butterfat or lard (rich in palmitic acid in *sn*-2 position) with free fatty acids (FFA) from different sources [3, 5-10] or (ii) interesterification of tripalmitin with blends of vegetable oils [11, 12]. Also, several research groups have focused on multistep reactions for the synthesis of 1,3-dioleoyl-2-palmitoylglycerol (OPO), an important structured TAG in infant formulas. Schmid *et al.* [13] performed the highly selective synthesis of OPO by the following two-step process: (i) alcoholysis

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of tripalmitin in dry ethanol catalyzed by *sn*-1,3 regioselective immobilized lipases from *Rhizopus delemar* and from *Rhizomucor miehei* yielding 2-monopalmitin that was isolated by crystallization and (ii) esterified with oleic acid using the same lipases.

Recently, Qin and co-workers [14] prepared OPO also by a two-step process: (i) dry fractionation of leaf lard at a crystallization temperature of 34ºC and (ii) enzymatic acidolysis of the fractionated leaf lard with camellia oil fatty acids catalyzed by Lipozyme RM IM.

Using palm oil as starting material, it was possible to synthesize OPO by a three-step process consisting of: (i) low-temperature fractionation of palm oil FA in acetone, yielding a palmitic acid-rich fraction and an oleic acid-rich fraction, and subsequent selective enzymatic esterification of palmitic acid into ethyl palmitate, followed by (ii) esterification of ethyl palmitate with glycerol, catalyzed by Novozym 435 under vacuum, for the synthesis of tripalmitin and finally (iii) the production of OPO by acidolysis of tripalmitin with oleic acid, catalyzed by Lipozyme IM 60 [15]. Palm stearin, which is a fraction of palm oil, was chemically interesterified and used in lipase-catalyzed acidolysis with a blend of FA from rapeseed, sunflower or palm kernel oils, stearic acid and myristic acid, in solvent free media, for HMFS production [16].

In the majority of these studies high-cost *sn*-1,3 regioselective immobilized commercial lipases, most of them from microbial sources, have been used. Nevertheless, lipases extracted from plants show some advantages over the microbial counterparts, namely the lower cost, the ready availability combined with a wide versatility and stability in organic media [17, 18]. Among them, *Carica papaya* lipase has emerged as a promising biocatalyst for oil and fats restructuring [19-21].

Carica papaya L. is an unbranched tree native from the Central America which is easily adapted to tropical and subtropical climates. Brazil and Mexico are the biggest producers of papaya fruit, followed by Nigeria and India (FAO, 2011). Its genome has been recently sequenced [22]. Due to bacterial and viral diseases, papaya plantations must be remade every 1-2 years, resulting in *ca* 7 million tons of agrowastes worldwide per year. Papaya wastes contain unripe and sick fruits that are rich in hydrolases in their latex, namely papain (an endoprotease), which is water soluble, and lipases that are strongly attached to the insoluble fraction of the latex. Following washing of the papain, the highly hydrophobic lipases remain strongly attached to the insoluble fraction of the latex and therefore, self-immobilized. After drying and milling, the biocatalyst is ready

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for use. The use of *C. papaya* lipase (CPL), recovered from *C. papaya* plantations, is a mean of upgrading these agro-wastes.

The interest on *C. papaya* lipase has greatly increased in recent years. Even though it has been reported since 1935, as probably being a mixture of enzymes, it has not yet been completely characterized so far [23]. It is a versatile "naturally immobilized" biocatalyst successfully tested in the following applications: (i) modification of fats and oils, based on its *sn*-3 stereoselectivity [24, 25] and *sn*-1,3 regioselectivity [20, 26], as well as on its preference for short-chain fatty acids; (ii) esterification and interesterification reactions in organic media, accepting a wide range of acids and alcohols as substrates; (iii) the asymmetric resolution of different non-steroidal anti-inflammatory drugs, and non-natural amino acids and (iv) biopolymers [19, 27].

The use of a commercial extract of CPL as catalyst for the synthesis of HMFS, by interesterification of tripalmitin with fatty acid alkyl esters or low-erucic rapeseed oil fatty acids, in solvent-free media, has been described [26, 28, 29].

The aim of this study was to investigate the potentialities of the non-commercial *Carica papaya* lipase as biocatalyst for the synthesis of HMFS to be used as a low-cost alternative to the commercial immobilized lipases used in the majority of the studies.

Firstly, two enzyme preparations, one extracted from the fruit and the other from the petiole leaves of papaya tree, were tested for the ability to catalyze the acidolysis of tripalmitin with oleic acid or omega-3 polyunsaturated fatty acids (omega-3 PUFA), in solvent-free media.

The biocatalyst that led to the highest incorporation of fatty acids in the glycerol backbone was selected for further investigation. Modelling and optimisation of reaction conditions (medium formulation and temperature) were attempted by response surface methodology. Also, the characterization of modified TAG obtained after 24-h batch reactions of tripalmitin with oleic acid at MR oleic acid:tripalmitin of 2:1 and 6:1, concerning the total fatty acid (mol %) and the fatty acid regio-distribution (mol %), was performed. Batch operational stability tests of the biocatalyst using different molar ratios were also performed. This aspect is of great importance since one of the main constraints to the use of immobilised lipases at industrial scale has been their relatively low operational stability.

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2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Tripalmitin (95% purity; MW= 807.35), 2',7'-dichlorofluorescein and methyl myristate standard (>99%) were obtained from Fluka; extra pure oleic acid (MW= 282.5) was from Merck and the commercial concentrate of triacylglycerols rich in omega-3 PUFA, "EPAX 1050TG" (about 10% eicosapentaenoic acid, EPA, and 50% docosahexaenoic acid, DHA), was a gift from EPAX AS, Lysaker, Norway (http://www.epax.com/filestore/EPAX1050TG2.pdf); Portuguese extra virgin olive oil (0.7 % free fatty acids) was purchased in a local market. Silica-Gel 60 (0.25 mm width, 20 x 20cm) thin layer chromatography (TLC) plates were purchased from Merck. The standards of triolein, diolein (mixed isomers) and monoolein were from Sigma-Aldrich. Hog pancreas lipase (30.1 U/mg) was purchased from Fluka. The other reagents used were *p.a*. and obtained from various sources.

2.1.2. Enzyme preparations

CPL extracts from unripe or sick plants are more active because they contain higher levels of defensive enzymes [19]. Plant material was collected in Cihuatlan, Jalisco (Mexico). CPL I: The latex from unripe or sick fruits was recovered by making longitudinal incisions of 3- 4 mm on fruits. The endo-proteases were washed by out and the strongly hydrophobic lipases remained attached to the solid fraction, self-immobilized. The partial purification was achieved by successive cold (4ºC) water washes and centrifugation at 6,000 r.p.m. for 20 min., followed by freeze-drying milling and sieving (mesh 10).

CPL II: Preparation of the biocatalyst was carried out as for CPLI, but the starting plant material was petiole juice and centrifugation was performed at 10,000 r.p.m for 5 min. The 30-40 mesh freeze-dried powder was kept as biocatalyst.

Although delipidation could be performed by organic solvent washing, CPLI and CPLII were not delipidated because the loss of activity and stability after delipidation.

The methodology followed for CPL I and CPLII preparation was fully described by Rivera *et al.* [30].

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Lipid content and composition of CPLI and CPLII were not determined as their concentrations are negligible compared to those of the substrates and since the reactions samples were accurately analysed by chromatography.

2.2. Methods

2.2.1. Assessment of hydrolytic activity of lipase preparations

The hydrolytic activity of both *C. papaya* lipase preparations, obtained from the fruit (CPL I) or from petiole leaves (CPL II), was assayed using the method described by Soares *et al.* [31], after modification: 50 g of extra virgin olive oil were mixed with 50 g of distilled water and 3.5 g of gum arabic. This mixture was stirred for 10 min. Then, 80 mL of phosphate buffer solution (pH 7.0; 0.1 M) were added and the final emulsion homogenized in an ultraturrax. Nine mL of this emulsion were put in a 20 mL closed thermostated cylindrical batch reactor, at 60 ºC, under magnetic stirring. After 15 min, 0.05 g of enzyme preparation was added and the hydrolysis was allowed to proceed for 5 min. After this time, 20 mL of a solution of ethanol:acetone (1:1, v:v) was added in order to inactivate the enzyme. The amount of free fatty acids (FFA) released during hydrolysis was determined by direct titration with a 0.1 N sodium hydroxide aqueous solution, using phenolphthalein as indicator. In parallel, blank experiments were carried out in the absence of enzyme. Each experiment was performed in triplicate.

The temperature of 60ºC was chosen for hydrolytic activity assays, in order to be able to compare these results with the acidolysis activity values obtained in solvent-free media, at the same temperature. One unit of hydrolytic activity was defined as one micromole of FFA released per minute.

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2.2.2. Preparation of free omega-3 PUFA

In order to obtain free omega-3 PUFA, "EPAX 1050TG" was saponified according to the method described by Sahín *et al.* [3], after modification. A mixture of "EPAX 1050TG" (25g), potassium hydroxide (5.75g), 11 mL of water and 66 mL of 95% (v/v) aqueous ethanol, was added to a flask equipped with a Liebig condenser and maintained in reflux at 100ºC for 60 min. Distilled water (50 mL) was added to the saponified mixture and transferred to a separating funnel, where the unsaponifiable matter was extracted by *n*-hexane (2 x 100 mL) and discarded. The aqueous layer containing the saponified matter was acidified to pH 1.0 with 3N HCl. The released free fatty acids

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were extracted with *n*-hexane (50 mL) and dried with anhydrous sodium sulphate. Sodium sulphate was removed by paper filtration and *n*-hexane was evaporated in a rotavapor at 40ºC and a pressure lower than 200 mbar. A TLC of the obtained FFA was performed (*c.f.* 2.2.6.) in order to confirm the efficiency of the process. The obtained FFA were stored at -18ºC under nitrogen until use.

2.2.3. Interesterification reaction

Interesterification reactions were performed batchwise in closed thermostated cylindrical batch reactors (20 mL), at the desired temperature (c.f. 2.2.4.), under magnetic stirring. The reaction medium consisted of a blend of tripalmitin and oleic acid or omega-3 PUFA. A load of 5 % (w/w of total lipids) of the self-immobilized enzyme was added to the reaction medium, after complete melting. This load is currently used in interesterification experiments without problems of mass transfer limitation [32].

Prior to and at different reaction times, 1 mL samples were taken and the biocatalyst was removed by paper filtration at approximately 70ºC. All samples were stored at –18 ºC for subsequent analysis. All the experiments were carried out in triplicate. For each system, molar incorporation degree (%) was calculated on the basis of the molecular weight of oleic acid or DHA (282.5 and 328.5, respectively).

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2.2.4. Experimental design

Response Surface methodology was used to model and optimize the reaction conditions [33]. A 5-levels central composite rotatable design (CCRD) dictated the experimental conditions, as a function of two factors: temperature (58-72ºC) and reaction medium formulation (molar ratio oleic acid:tripalmitin) (1.2:1-6.8:1).

A total of 11 assays with three replicates of the centre point were generated. Experiments were conducted randomly, according to the methodology described in 2.2.3. The minimum temperature tested was dictated by the melting point of reaction media (melting point of tripalmitin = 66ºC).

The enzyme load was maintained constant at 5% (w/w of total lipids). After 24h reaction, the medium was separated from the enzyme by paper filtration in an oven at approximately 70ºC and stored at -18ºC for subsequent analysis.

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2.2.5. Batch operational stability tests

The operational stability of the biocatalyst was evaluated in consecutive 23 h batches, at 60ºC. Interesterification was carried out as previously described (cf. 2.2.3.), considering two different molar ratios oleic acid:tripalmitin (2:1 and 6:1). After each batch, the biocatalyst was removed from the reaction medium by paper filtration and reused in the next batch with fresh medium, under the same reaction conditions. A total of up to 7 batches were performed using the same biocatalyst sample.

The molar incorporation degree of oleic acid in tripalmitin, observed at the end of each batch, was used as a measure of the activity of the biocatalyst in that batch. The first batch was used as the reference (100% activity). The residual activity (a_n, %) of the biocatalyst at the end of each batch n (n = 1,...,7) was thus estimated as follows:

$$a_n = \left(\frac{Incorporation_{Batch_n}}{Incorporation_{Batch_1}} \right) * 100 \qquad \text{(Eq. 1)}$$

2.2.6. Analysis of reaction products

2.2.6.1. Evaluation of incorporation degree

To determine the amount of oleic acid or omega-3 PUFA incorporated in TAG, the following procedure was followed for each sample: 0.15 g of the reaction medium was dissolved in 25 mL of chloroform *p.a.* and 200 μL of this solution was spotted on a continuous layer on a silica gel TLC plate. Elution was carried out in *n*-hexane/diethyl ether/acetic acid (70/30/1.5; v/v/v) as the mobile phase. Plates were sprayed with 0.2% (w/v) 2',7'-dichlorofluorescein in 95% ethanol and observed under U.V. at 366 nm. The various groups of compounds (triacylglycerols, free fatty acids, diacylglycerols and monoacylglycerols) were identified by comparison with standards.

The TAG band was scrapped off and methylated, in order to be assayed as fatty acid methyl esters (FAME) by gas chromatography (GC). For methylation, the silica gel containing TAG was mixed with 5 mL of methylation reagent (anhydrous methanol*n*-hexane/concentrated sulphuric acid; 75/25/1, v/v/v), in a conical flask equipped with a Liebig condenser. This mixture was allowed to boil under reflux for 60 min in a water bath at about 80ºC. Then, 10 mL of distilled water and 10 mL of petroleum ether were

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added and the mixture was transferred to a separating funnel, vigorously agitated and allowed to settle for phase separation. The organic upper layer was recovered, washed twice with distilled water (2 x 10 mL) and dried with anhydrous sodium sulphate. Sodium sulphate was removed by paper filtration, the solution was transferred to a conical-bottom flask and the solvent was evaporated in a rotavapor at 30ºC under a pressure lower than 200 mbar.

FAME were dissolved in 100 µL of 0.1% (w/v) methyl myristate (internal standard) in *n*-hexane solution and 1 µL of this solution was GC analyzed. A Finnigan TRACE GC Ultra gas chromatograph (Thermo Electron Corporation) equipped with a Thermo TR-FAME capillary column (30 m x 0.25 mm ID x 0.25 µm film), an auto sampler AS 3000 from Thermo Electron Corporation and a flame ionization detector, was used for FAME analysis. Injector (in splitless mode) and detector temperatures were set at 250ºC and 260ºC, respectively. Helium was used as carrier gas at a flow rate of 1.5 mL/min. Air and hydrogen were supplied to the detector at flow rates of 350 mL/min and 35 mL/min, respectively.

For the analysis of samples from the system of acidolysis of tripalmitin with oleic acid, the oven temperature program was as follows: 60ºC for 1 min, a temperature increase to 150ºC at 15ºC/min, a plateau at 150ºC for 1 min, followed by temperature increase to 180ºC at 5ºC/min, a plateau at 180ºC for 3 min, an increase in temperature until 220ºC, at a rate of 10ºC/min and a final plateau at 220ºC for 1 min.

For the analysis of samples from the system of acidolysis of tripalmitin with omega-3 PUFA, the oven temperature program was as follows: 60ºC for 1 min, a temperature increase to 150ºC at 15ºC/min, a plateau at 150ºC for 1 min, followed by temperature increase to 220ºC, at a rate of 5ºC/min and a final plateau at 220ºC for 10 min.

2.2.6.2. Fatty acid composition in the *sn*-2 position

The fatty acid composition in the *sn*-2 position of the modified triacylglycerols was determined according to the following protocol, adapted from Sahín *et al.* [6, 7]: each sample (1g of fat) obtained after 24-h enzymatic acidolysis was dissolved in chloroform (5 mL) and 300 µL of this solution was spotted in a continuous layer on a silica gel TLC plate and developed as previously described (c.f. 2.2.6.1.).

The band corresponding to the TAG fraction was scrapped off, the TAG fraction was extracted by diethyl ether (3 x 5 mL) and the solvent evaporated in a rotavapor. TAG were re-suspended in 2 mL 0.1 M Tris-HCl aqueous buffer (pH 8.0) with 0.5 mL of

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0.1% (w/v) sodium cholate aqueous solution and 0.2 mL of 22% (w/v) calcium chloride aqueous solution. Pancreatic lipase (50 mg) was added to this mixture and the hydrolysis was carried out at 40ºC. After 5 min, the reaction was stopped by the addition of 1 mL 6N HCl aqueous solution; 3 mL of ethyl ether was added and the mixture was centrifuged for 5 min at 1200 g. The upper organic layer was recovered, the solvent evaporated, the extract was re-suspended in 300 μL of diethyl ether and spotted in a continuous layer on a silica gel TLC plate and developed. The band corresponding to the *sn*-2 monoacylglycerols was scrapped off, methylated and GC analyzed as previously described (c.f. 2.2.6.1.).

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2.2.7. Statistical analysis

The software "Statistica ™", version 5, from Statsoft, Tulsa, USA, was used to analyze the results of the CCRD and the results of FA composition of structured TAG (total FA and in the *sn*-2 position).

Both linear and quadratic effects of each factor under study, as well as their linear interaction, on acidolysis reaction were calculated. Their significance was evaluated by analysis of variance. A three-dimensional surface, described by a second-order polynomial equation, was fitted to the experimental values of the CCRD. First- and second-order coefficients of this equation were estimated from the experimental data by using the statistical principle of least squares.

The goodness of fit of the model was evaluated by the determination coefficients (R^2) and adjusted R^2 (R^2_{adj}). The R^2 value provides a measure of how much of the variability in the observed response values can be explained by the experimental factors and their interactions. R_{adj}^2 is an unbiased estimate of the coefficient of determination and is always smaller than R^2. High values of both R^2 and R^2_{adj} suggest a good fit of the model to the experimental data. In practice, R^2 should be at least 0.75 or greater; values above 0.90 are considered to be very good [33].

Concerning the statistical analysis of FA composition of structured TAG, ANOVA of molar values was carried out, using LSD *pos hoc* multiple comparison tests.

2.2.8. Validation of the model

In order to validate the polynomial model fitted to the experimental data points and to investigate when the reaction equilibrium was reached, a time-course was carried out at 60ºC for 48h, using a molar ratio oleic acid:tripalmitin of 6:1 (cf. 2.2.3.), The

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incorporation value obtained after 24 h reaction time was compared with that predicted by the model.

3. Results and Discussion

3.1. Hydrolytic activity and fatty acid selectivity in acidolysis

The hydrolytic activity of both enzyme preparations from *Carica papaya*, one obtained from the fruit (CPL I) and the other extracted from petiole leaves (CPL II) of papaya tree, was determined at 60ºC using virgin olive oil as substrate. The enzymes from the fruit and from petiole leaves presented hydrolytic activities of 387 U/g (std= 56.6) and 207 U/g (std= 28.3), respectively.

These enzymes were used as catalysts for the acidolysis of tripalmitin with oleic acid or omega-3 PUFA, at 60ºC, using 2:1 mole ratio (free fatty acids:tripalmitin). For each system, the molar incorporation degree is presented in Table 1. After 24 h reaction, the highest levels were observed with CPL I (22.1 mol-%), when oleic acid was used as the acyl donor. The biocatalyst was less efficient when omega-3 PUFA were incorporated in tripalmitin, reaching only 8.4 mol-% incorporation. With CPL II, only residual incorporation of oleic acid was attained (3.2 mol-%) and no incorporation of omega-3 PUFA was detected. These results show that hydrolytic activity is not directly related with the acidolysis activity of the lipases, as previously reported [34, 35].

When CPL II was previously tested as catalyst for the hydrolysis of PNP esters, a preference towards long chain fatty acids was observed (unpublished data). Conversely, CPL I usually prefers short chain fatty acids over long chain fatty acids [25]. Lower hydrolytic and acidolysis activities of CPL II, observed in the present study, may be explained by a thermal deactivation of this enzyme.

Therefore, *Carica papaya* lipase extracted from the fruit (CPL I) was selected for further experiments on acidolysis of tripalmitin with oleic acid. With this enzyme, a time-course reaction was performed under the same operational conditions (Fig 1): quasi-equilibrium was attained after 30 h reaction with oleic acid incorporation of about 24.1 mol-%.

The oleic acid incorporation in tripalmitin (22.1 mol-%), achieved after 24 h reaction using CPL I, was not much lower than the obtained values, reported in our previous work (*c.a.* 27 mol-%), where three commercial immobilized lipases (Novozym 435, Lipozyme RM IM and Lipozyme TL IM, from Novozymes, Denmark) were used. The

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incorporation of omega-3 PUFA in tripalmitin, catalysed by CPL I, was similar to that obtained when Lipozyme TL IM (*ca* 8% mol-%) was used [9].

The observed preference of CPL towards oleic acid instead of omega-3 PUFA is in accordance with the reported typoselectivity of this lipase in the esterification of free fatty acids with 1-butanol. In these studies, CPL presented the ability to discriminate against fatty acids with *cis*-4 (e.g., docosahexaenoic acid, DHA; C22:6 omega-3), and *cis*-6 (e.g., gamma linolenic acid, C18:3 omega-6), while acyl exchanges of *cis*-5 fatty acid (e.g., eicosapentaenoic acid, C20:5 omega-3) or *cis*-9 (e.g., oleic acid, C18:1 omega-9, or alpha linolenic acid, C18:3 omega-3) were preferred [36]. In fact, DHA was the major fatty acid present in "EPAX 1050TG", used as the source of omega-3 PUFA in this study.

Also, the preference of CPL towards FFA over alkyl esters as acyl donors, has been demonstrated in several transesterification reactions [25, 37]. Moreover, studies concerning the selectivity of CPL towards different fatty acid alkyl esters in the interesterification of tripalmitin, have been reported [28, 29].

CPL was also used as catalyst for the interesterification between tripalmitin and ethyl esters (C2 to C14 saturated acids and C18:1), in solvent free media at 63ºC (66ºC in the cases of ethyl myristate and ethyl oleate), using a substrate ratio of 2:1 (ethyl ester: tripalmitin). After 24 h reaction, the incorporation of the acyl moieties increased with time and chain length of the ethyl ester, except for ethyl oleate [29].

In the interesterification of tributyrin with unsaturated TAG, in *n*-hexane, catalysed by crude papain, triolein reacted faster than polyunsaturated TAG such as trilinolein and tri-α-linolein [38].

3.2. Modelling reaction conditions

In order to model acidolysis of tripalmitin with oleic acid, catalysed by CPL I, a set of 11 experiments following a CCRD as a function of both temperature (T) and MR was performed. The obtained results are presented in Table 2. The effect of enzyme load on oleic acid incorporation degree was not investigated since (i) using a crude *Carica papaya* latex extract, some variation was expected on enzymatic activity with variety, geographic localization and plantation [39]; and (ii) the optimized values for temperature and molar ratio do not depend on the amount of enzyme used. Therefore, the optimization of latex load must be carried out for each extract used.

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To investigate the role of the factors T and MR on the acidolysis reaction, linear and quadratic effects of each factor, as well as their linear interaction on the oleic acid incorporation (mol-%) values were calculated (Table 3). Only the variable MR showed a significant positive linear effect on oleic acid incorporation. Quadratic negative effect was observed for the variable temperature, indicating a convex response surface. Although no significant effects for the interaction T x MR and for MR quadratic term were detected, these variables could not be neglected for oleic acid incorporation. In fact, variables that exhibited a significant effect ($p<0.05$) and those having a confidence range smaller than the value of the effect (data not shown), were included in the following second-order polynomial equation that describes the response surface [33]:

$$Inc(mol-\%) = 6.940 + 0.004T^2 + 12.322MR - 0.167MR^2 - 0.139T * MR \qquad \text{(Eq. 2)}$$

Thus, oleic acid incorporation values can be fitted to a three-dimensional surface described by a second-order polynomial model as a function of T and MR (Eq. 2, Fig.2). The high values of both R^2 (0.863) and R^2_{adj} (0.771) of this model suggest a close agreement between the experimental data and the theoretical values predicted by the model.

No optimal conditions were observed in the response surface for the oleic acid incorporation. Therefore, only the identification of the region corresponding to the best response can be achieved.

As stated before, substrate molar ratio has a positive effect on the response. Thus, a higher MR leads to an increase in oleic acid incorporation. This effect was more pronounced at lower temperatures which may suggest a possible inactivation of the biocatalyst at high T. However, the temperature is a key variable in this process since it is necessary to ensure values higher than 60ºC in order to prevent medium solidification. For T around 60-65 ºC, incorporation levels of oleic acid of about 40 mol-% were observed (Table 2) when oleic acid was used in stoichiometric excess.

Identical levels for oleic acid incorporation were attained in previous studies using high cost commercial immobilized *sn*-1,3 selective lipases.

Sahín and co-workers [7] synthesized HMFS by acidolysis of tripalmitin with hazelnut oil fatty acids and a concentrate of omega-3 fatty acids, in *n*-hexane, using Lipozyme RM IM as biocatalyst. The optimal conditions predicted by the models, for the targeted

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total of 5% of EPA and DHA and 40% of oleic acid incorporation, were as follows: 12.4 mol/mol (FFA/TAG), 55ºC and 24h.

When the incorporation of gamma-linolenic acid (GLA) was carried out in a similar system (tripalmitin, hazelnut fatty acids and GLA, in *n*-hexane), catalysed by Lipozyme RM IM and Lipozyme TL IM, identical results were obtained with both enzymes: to attain 10% of GLA and 45% of oleic acid incorporation: the optimal reaction conditions, predicted by response surface methodology, were 55ºC, 24h reaction time and molar substrate ratio of about 14:1 (FFA/TAG) [6].

In fact, the incorporation values reported in the literature were obtained using high FFA/tripalmitin molar ratios. It is generally accepted that increasing ratios of acyl donor/TAG result in higher incorporation of fatty acids into the TAG, by displacement of reaction equilibrium towards product formation. Nevertheless, high amounts of free fatty acids are undesirable since it increases operation costs due to recycling of unconverted substrates and product recovery.

The selected operational conditions, based on the response surface, must take into account not only the conjugation of factors that increase oleic acid incorporation but also processing constraints and operation costs. Considering the balance of these different factors, it was reasonable to fix reaction temperature at 60ºC and molar ratio oleic acid:tripalmitin at 6:1 as the selected conditions for further enzymatic assays.

3.3. Validation of the second-order model for oleic acid incorporation

To investigate the applicability of the second-order model describing oleic acid incorporation, a time course experiment was carried out, at 60ºC using a molar ratio oleic acid: tripalmitin of 6:1 (Fig. 3).

The oleic acid incorporation value attained after 24h reaction was compared to the theoretical value predicted by the model. The incorporation level of oleic acid obtained after 24 h reaction time was only 32.1 mol-%, in contrast with 39.2mol-% dictated by the model and observed in experiment 2 (41.5 mol-%) of CCRD (Table 2). However, it was possible to achieve the theoretical value at the end of 48 h reaction time (Fig. 3). This difference might be due to the fact that *Carica papaya* latex is a crude enzymatic extract, containing several compounds such as proteases, inhibitive polyphenol oxidases and lipases in variable amounts. Both protein content and biocatalytic activity of crude CPL preparation in proteolysis, hydrolysis and interesterification reactions, depend on several factors such as the variety and the geographic location of plantation. According

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to Caro and co-workers [39], for several crude latices obtained from different papaya varieties, no correlation between protease activity of the latex and its lipase activity was observed.

3.4. Operational stability assays

The low operational stability of immobilized commercial lipases, together with their high prices, have been considered as the most important drawbacks to their applications at industrial scale. Several parameters may affect the decrease of enzyme activity throughout the reaction, namely the water content, the presence of oxidation products, the bioreactor configuration and the operation mode [40, 41]. Therefore, it is important to select the biocatalyst taking into account, not only the catalytic activity, but also its maintenance along the reaction.

The operational stability of CPL I was evaluated under the experimental conditions stated by the model, *i.e.*, 60ºC and MR oleic acid: tripalmitin of 6:1, for 23 h repeated batches. Also, operational stability assay was carried out using lower oleic acid concentration, (MR of 2:1, oleic acid:tripalmitin), in order to investigate the effect of the amount of free fatty acids in reaction media on CPL I stability.

The residual acidolysis activities of the biocatalyst at the end of each batch are presented in Fig. 4. After the second 23 h batch, the catalyst retained only 44.6% of its initial activity and after four consecutive runs only 2.3% was attained, when a MR of 6:1 was used. In the experiments with a MR of 2:1, a higher operational stability was observed: at the end of the third consecutive batch, the biocatalyst still retained 71.5% of its initial activity and after seven consecutives 23 h batches, a residual activity of 3.7% was still observed.

The lower operational stability exhibited by CPL I in the reaction medium containing a MR of 6:1, might be due to enzyme sensitivity to free fatty acids, leading to a modification in the protonation state of the biocatalyst [42].

The effect of biocatalyst recycling was studied on the catalytic activity of CPL in interesterification reactions between tributyrin and hydrogenated soybean oil, at 65ºC. The results of the recycling experiments showed a linear decrease in CPL activity so that, after eight 24 h consecutive runs, the CPL catalyst retained about 50% of its initial interesterification activity [20]. It is worthy to notice that during interesterification reactions, the amount of free fatty acids in reaction medium is much lower than during acidolysis.

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The application of CPL as biocatalyst in consecutive batches was also evaluated for the synthesis of terpene esters in organic media [43]. After three 8 h consecutive batches, the biocatalyst retained only 51% of its original activity. When these naturally immobilized CPL particles were coated with ionic liquid, in an attempt to overcome the negative effects caused by the presence of organic solvent and acetaldehyde in the reaction media, the operational stability was significantly improved. The ionic liquid-coated CPL retaining about 90% of the initial activity, after fifteen 8 h consecutive batches [43].

Considering operational stability tests performed with commercial immobilized biocatalysts in the acidolysis between tripalmitin and oleic acid, at 60ºC, considerably higher stability values were observed when compared to the values exhibited by CPL I [9]. The reusability of Lipozyme RM IM was investigated in the acidolysis of interesterified palm stearin with a blend of FA aimed at the production of HMFS. In this experiment, the immobilized lipase could be reused for, at least, seventeen 3.4 h recycling cycles without significant loss of initial activity [16].

However, in spite of the lower operational stability of CPL I, comparing with the commercial preparations, the low-cost of crude CPL together with the valorisation of an agro-waste to be used in the synthesis of a high-value product, make the crude extract of CPL a suitable biocatalyst for HMFS production.

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3.5. Characterization of HMFS

The FA composition and FA positional distribution in the structured TAG, obtained after 24-h batch reaction of tripalmitin with oleic acid at MR (oleic acid:tripalmitin) of 2:1 and 6:1 at 60ºC, were evaluated (Table 4).

The structured triacylglycerols mainly contain palmitic and oleic acids and smaller amounts of palmitoleic acid. As previously observed (c.f. 3.2.), oleic acid incorporation in TAG increased with MR (28.4 and 41.5 mol-%, for MR of 2:1 and 6:1, respectively). This results in structured TAG with significantly different global and region FA composition (LSD post-hoc comparison test at a p value ≤ 0.05).

The *sn*-2 positions of the structured lipid are predominantly occupied by palmitic acid (73.9 and 78.9 mol-%, for MR of 2:1 and 6:1, respectively). Despite the region-specificity of CPL I, the incorporation of oleic acid in the *sn*-2 position occurred (26.1 and 21.6 mol-%, of the FA esterified in this position, for MR of 2:1 and 6:1). This is

due to acyl migration during acidolysis. Higher molar ratio conducted to a lower acyl migration, as previously reported [14, 44].The acyl migration levels were similar to those reported by others in analogous systems and under similar temperature values [3, 6, 7, 9, 45]. The structured lipids produced in the present study can be used as HMFS, since they present high levels of palmitic acid mainly esterified at the *sn*-2 position.

4. Conclusions

This study shows the potentialities of *Carica papaya* lipase, extracted from the fruit of papaya tree, as a promising low-cost biocatalyst for the synthesis of human milk fat substitutes and as an alternative to the commercial immobilized *sn*-1,3 selective lipases usually used. The incorporation levels of oleic acid are similar to those achieved when high-cost commercial immobilized lipase preparations are used.

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Figure Legends:

Figure 1. Time-course of oleic acid incorporation obtained in the acidolysis reaction of tripalmitin and oleic acid (MR =2:1, oleic acid:tripalmitin), catalyzed by CPL I.

Figure 2. Response surface fitted to the oleic acid incorporation values as a function of temperature and substrate molar ratio (oleic acid:tripalmitin), after 24h acidolysis catalyzed by CPL I

Figure 3. Validation of the second-order model for oleic acid incorporation: time course of oleic acid incorporation obtained in the acidolysis reaction of tripalmitin and oleic acid (MR=6:1), at 60 ºC, catalyzed by CPL I.

Figure 4. Batch operational stability tests: residual activity of CPL I, at the end of each 23h batch reutilization, in the acidolysis of tripalmitin with oleic acid, at MR of 2:1 and 6:1.

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Table 1: Average incorporation values (mol-%) of oleic acid or omega-3 PUFA in tripalmitin, upon 24-h acidolysis reaction catalysed by *Carica papaya* lipase extracted from the fruit (CPL I) or from the petiole leaves (CPL II) (± standard deviation; 3 repetitions). (n.d.- not determined).

Acyl donor type	**CPL I**	**CPL II**
Oleic acid	22.1 ± 0.25	3.20 ± 0.00
Omega-3 PUFA	8.74 ± 0.00	n.d.

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Table 2. CCRD followed in the experiments as a function of temperature (T) and the molar ratio (MR) of oleic acid/tripalmitin used and respective values of oleic acid incorporation (mol-%), after 24 h reaction, catalysed by CPL I.

Experiment nº.	T (ºC)	MR (oleic acid/tripalmitin)	Oleic acid incorporation (mol-%)
1	60	2:1	28.4
2	60	6:1	41.5
3	70	2:1	28.3
4	70	6:1	34.3
5	58	4:1	28.0
6	72	4:1	33.0
7	65	1.2:1	29.9
8	65	6.8:1	38.7
9	65	4:1	37.1
10	65	4:1	38.4
11	65	4:1	39.0

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Table 3. Effects and respective *p* levels (values between brackets) of temperature (T) and molar ratio (MR) of oleic acid/tripalmitin used in CCRD and respective linear interactions on the values of oleic acid incorporation (mol-%), after 24h reaction, catalysed by CPL I.

Factor	**Oleic acid incorporation (mol-%)**
T (linear term)	-0.074 (0.970)
T (quadratic term)	-7.34 (0.022)
MR (linear term)	7.93 (0.008)
MR (quadratic term)	-3.48 (0.179)
T * MR	-3.57 (0.230)

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Table 4: Fatty acid composition and its distribution in the structured TAG obtained upon 24-h acidolysis of tripalmitin with oleic acid (MR oleic acid:tripalmitin of 2:1 and 6:1), at 60ºC, catalysed by CPL I. (± standard deviation; 3 repetitions). (n.d.- not determined).

For each acid, in each group (total, *sn*-2) different letters mean that the results are significantly different at a p value ≤ 0.05.

sn-1,3 (mol %) = [3 x total (mol %) – *sn*-2 (mol %)]/2 [16].

	MR 2:1			MR 6:1		
Fatty Acid	**Total**	***sn*-2**	***sn*-1,3**	**Total**	***sn*-2**	***sn*-1,3**
C16:0	70.6 [a] ± 0.339	73.9 [a] ± 0.415	69.0	55.8 [b] ± 3.01	78.9 [b] ± 3.12	44.2
C16:1	0.975 [e] ± 0.955	n.d.	1.46	1.55 [e] ± 1.64	n.d.	2.32
C18:1	28.4 [c] ±0.615	26.1 [c] ± 2.81	29.6	41.5 [d] ± 0.163	21.6 [d] ± 2.81	51.5

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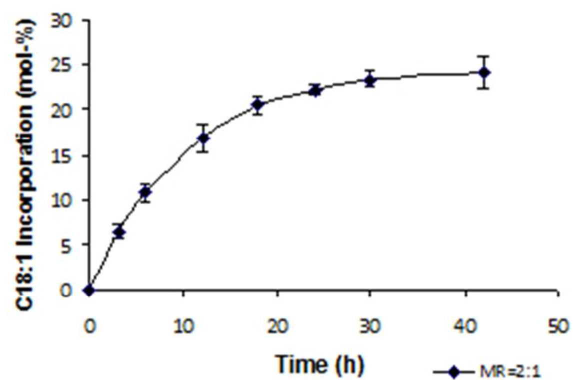

Time-course of oleic acid incorporation obtained in the acidolysis reaction of tripalmitin and oleic acid (MR =2:1, oleic acid:tripalmitin), catalyzed by CPL I.
82x83mm (96 x 96 DPI)

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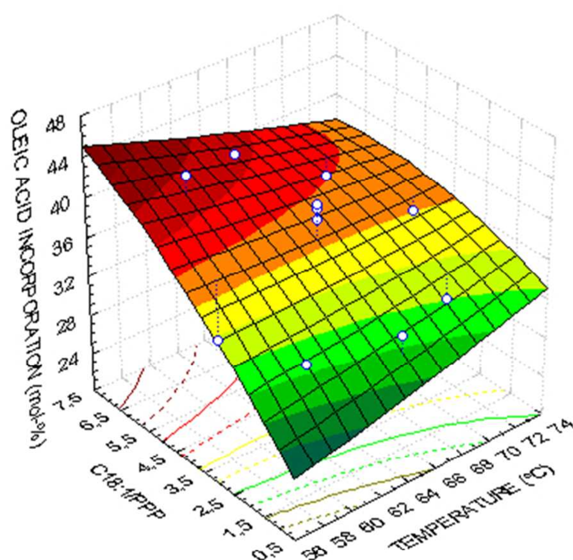

Figure 2. Response surface fitted to the oleic acid incorporation values as a function of temperature and substrate molar ratio (oleic acid:tripalmitin), after 24h acidolysis catalyzed by CPL I
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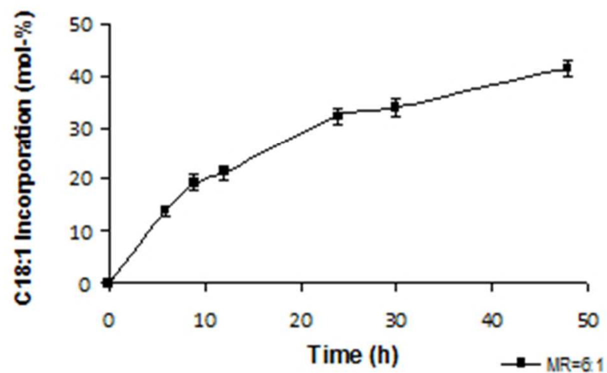

Validation of the second-order model for oleic acid incorporation: time course of oleic acid incorporation obtained in the acidolysis reaction of tripalmitin and oleic acid (MR=6:1), at 60 ºC, catalyzed by CPL I.
87x83mm (96 x 96 DPI)

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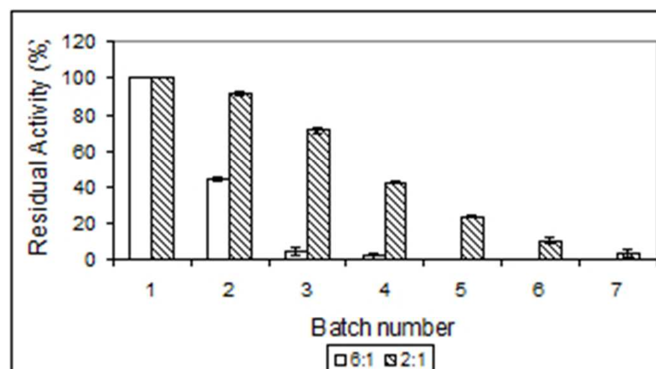

Batch operational stability tests: residual activity of CPL I, at the end of each 23h batch reutilization, in the acidolysis of tripalmitin with oleic acid, at MR of 2:1 and 6:1.
89x83mm (96 x 96 DPI)

Review

Capítulo VI.

Immobilized heterologous *Rhizopus oryzae* lipase: a feasible biocatalyst for the production of human milk fat substitutes

Tecelão, C.,Guillén, M., Valero, F., Ferreira-Dias, S.

Biochemical Engineering Journal

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Manuscript Draft

Manuscript Number:

Title: Immobilized heterologous Rhizopus oryzae lipase: a feasible biocatalyst for the production of human milk fat substitutes

Article Type: Full Length Article

Keywords: Acidolysis; Human milk fat substitutes; Immobilization; Recombinant lipase; Rhizopus oryzae; Operational stability.

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Corresponding Author's Institution: Instituto Superior de Agronomia

First Author: Carla Tecelão, MSc

Order of Authors: Carla Tecelão, MSc; Marina Guillén; Francisco Valero; Suzana Ferreira-Dias, PhD

Abstract: This work aims at evaluating the potentialities of a heterologous Rhizopus oryzae lipase (rROL) as a feasible biocatalyst, to be used as an alternative to commercial lipases, for the synthesis of Human Milk Fat Substitutes (HMFS). This enzyme was immobilized in three different supports (Accurel® MP 1000, Eupergit® C and Lewatit® VP OC 1600) and tested as catalysts for the acidolysis between tripalmitin and oleic acid, batchwise, at 60ºC, in solvent-free media.
The highest molar incorporations of oleic acid in the triacylglycerols were achieved with rROL in Lewatit® VP OC 1600 and Accurel® MP 1000, although the highest immobilization yields were observed with Eupergit® C.
Quasi-equilibrium was attained after 6h or 12h reaction, with about 30 mol-% and 22 mol-% of oleic acid incorporation, when rROL immobilized in Lewatit and Accurel were used, respectively.
Also, 23h batch operational stability tests were performed: half-life times (t1/2) of 34.5h and 64.0h were achieved for rROL immobilized in Accurel® MP 1000 and in Lewatit® VP OC 1600, respectively.
An important increase in the operational stability was observed for rROL immobilized in Lewatit after rehydration at the end of each batch (t1/2=202h). Thus, the loss of activity may be explained by a progressive dehydration occurring along the reaction.

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Immobilized heterologous *Rhizopus oryzae* lipase: a feasible biocatalyst for the production of human milk fat substitutes

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Abstract

This work aims at evaluating the potentialities of a heterologous *Rhizopus oryzae* lipase (rROL) as a feasible biocatalyst, to be used as an alternative to commercial lipases, for the synthesis of Human Milk Fat Substitutes (HMFS). This enzyme was immobilized in three different supports (Accurel® MP 1000, Eupergit® C and Lewatit® VP OC 1600) and tested as catalysts for the acidolysis between tripalmitin and oleic acid, batchwise, at 60ºC, in solvent-free media.

The highest molar incorporations of oleic acid in the triacylglycerols were achieved with rROL in Lewatit® VP OC 1600 and Accurel® MP 1000, although the highest immobilization yields were observed with Eupergit® C.

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Also, 23h batch operational stability tests were performed: half-life times ($t_{1/2}$) of 34.5h and 64.0h were achieved for rROL immobilized in Accurel® MP 1000 and in Lewatit® VP OC 1600, respectively. An important increase in the operational stability was observed for rROL immobilized in Lewatit after rehydration at the end of each batch ($t_{1/2}$=202h). Thus, the loss of activity may be explained by a progressive dehydration occurring along the reaction.

Key Words: Acidolysis*;* Human milk fat substitutes; Immobilization; Recombinant lipase; *Rhizopus oryzae*; operational stability

1. Introduction

The synthesis of structured lipids resembling human milk fat (HMF) has been carried out using lipases (E.C. 3.1.1.3., triacylglycerol acylhydrolases) as biocatalysts. Lipases are water soluble enzymes that catalyze the hydrolysis of acylglycerols at oil/water interfaces but, in non-aqueous media under low water activity, they are also efficient catalysts for esterification and interesterification reactions. Lipases present recognized advantages over the chemical catalysts: they act under mild operation conditions (atmosphere pressure and temperatures below 70ºC) and present high selectivity (regio-, stereo-, typo- and substrate selectivity), leading to a decrease in the formation of side products [1].

The *sn*-1,3 regioselectivity of some lipases has been used to produce human milk fat substitutes (HMFS) that mimic the rather unique triacylglycerol (TAG) profile of breast milk fat. The HMF is characterized by the prevalence of saturated fatty acids (70% of palmitic acid) at the *sn*-2 position of TAG while unsaturated fatty acids are esterified at *sn*-1 and *sn*-3 positions. This is in contrast to what occurs in vegetable oils and ruminant milk fat, where unsaturated fatty acids are predominantly in the internal position of TAG [2]. The pancreatic lipase plays an important role on the digestion process, releasing the fatty acids from the positions *sn*-1 and *sn*-3 of TAG that are, subsequently, absorbed in the small intestine as free fatty acids (FFA), together with the monoacylglycerols. The absorbability of the fatty acids depends on their chain length. In fact, longer chain saturated fatty acids (C12:0 to C18:0) tend to form insoluble calcium soaps that are less well absorbed than medium-chain (C6:0 to C10:0) and unsaturated fatty acids. Also, the fatty acids at the *sn*-2 position are preserved during the digestive process and they are better absorbed as *sn*-2 monoacylglycerols [3]. Therefore, the presence of palmitic acid in the *sn*-2 position provides a more efficient absorption of this acid as *sn*-2 monoacylpalmitate, avoiding the formation of insoluble calcium soaps and consequent problems of constipation and calcium deficiency in the infants [4].

Nowadays, "Betapol™" and "InFat™" are examples of commercial HMFS manufactured by biocatalytic processes. "Betapol™" is produced by Lipid Nutrition, Wormerveer, in the Netherlands (http://www.lipidnutrition.com, December 2011) and, more recently, "InFat™", by Advanced Lipids (http://www.advancedlipids.com, December 2011). Both "Betapol™" and "InFat™" are synthesized by acidolysis reactions between fats containing palmitic acid predominantly esterified at the *sn*-2

position, such as fractionated palm stearin or tripalmitin, with blends of free fatty acids rich in oleic acid. These reactions are catalyzed by *sn*-1,3 selective lipases such as *Rhizomucor miehei* lipase and *Rhizopus oryzae* lipase [5].

Several research groups have been working on the synthesis of HMFS obtained by *sn*-1,3 lipase-catalyzed (i) acidolysis of tripalmitin, butterfat, fractionated palm stearin or lard (rich in palmitic acid in *sn*-2 position) with free fatty acids (FFA) from different sources [4, 6-12] or (ii) interesterification of tripalmitin with blends of vegetable oils [13, 14]. Also, some attention has been drawn to multistep reactions for the synthesis of 1,3-dioleoyl-2-palmitoylglycerol (OPO), an important structured TAG in infant formulas [15-17].

Nowadays, the high cost of commercial lipases, together with a low operational stability, has been recognized as the main constraints to their use in the Food Industry. The use of stable and active immobilized lipases is a way to make enzymatic process competitive [18].

The methods usually followed to immobilize lipases are based on adsorption processes using more or less hydrophobic supports, exploring the interfacial activation of lipases on hydrophobic interfaces at low ionic strength [19].

The properties of the support material used for enzyme immobilization, namely the chemical composition, the particle size, and the mechanical stability, have a great influence on the activity and stability of the biocatalyst preparation [20]. Several hydrophobic supports have been described as suitable for lipase immobilization. Among them, polypropylene-based supports (such as Accurel MP 1000 and EP 100) have been extensively studied for their useful features, namely a high porosity and a large surface area, that provide a high capacity for enzyme immobilization [20-23]. Lewatit, a methacrylate-styrenedivinylbenzene resin, is the support used in a commercial immobilized preparation of *Candida antarctica* (Novozym 435, Novozymes, Denmark), that has been used in many applications [10, 24]. The commercial product Eupergit C is amongst the most used matrices for enzyme immobilization and consists on a microporous resin with epoxy-activated acrylic beads [25, 26].

The extracellular lipase from the filamentous fungus *Rhizopus oryzae* immobilized on different supports shows recognized potentialities as biocatalyst for lipid restructuring, due to its *sn*-1,3 regioselectivity, namely for the synthesis of human milk fat substitutes [27] and low-caloric triacylglycerols [28].

The use of recombinant lipases is an effective strategy to increase the productivity of an enzyme bioprocess production. In fact, recombinant *Rhizopus oryzae* lipase (rROL), cloned and expressed in the methylotrophic yeast *Pichia pastoris* showed a 40-fold higher specific activity compared with commercial native ROL [29].

The aim of this study was to investigate the potentialities of the immobilized rROL as biocatalyst for the synthesis of HMFS, to be used as an alternative to the commercial lipases used in the majority of the studies.

The rROL was immobilized in three different hydrophobic carriers (Accurel® MP 1000, Eupergit® C and Lewatit® VP OC 1600) and tested as catalyst for the acidolysis of tripalmitin with oleic acid, in solvent-free media, in order to select the most suitable immobilization support. Also, batch operational stability tests of the selected biocatalysts were performed.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Accurel® MP 1000 was acquired from Membrana, GmbH (Obernburg, Germany), Lewatit® VP OC 1600 was purchased from Bayer (Leverkusen, Germany) and Eupergit® C was kindly donated by Röhm GmbH & Co., Degussa (Darmstadt, Germany). Table 1 shows some properties of these supports. Tripalmitin (95% purity; MW= 807.35), 2',7'-dichlorofluorescein and methyl myristate standard (>99%) were obtained from Fluka; extra pure oleic acid (MW= 282.5), glutaraldehyde (25% aqueous solution) and silica-Gel 60 (0.25 mm width, 20 x 20cm) thin layer chromatography (TLC) plates were purchased from Merck. The Bradford protein assay kit was acquired from Bio-Rad. The standards of triolein, diolein (mixed isomers) and monoolein were from Sigma-Aldrich. Portuguese extra virgin olive oil (0.7 % free fatty acids) was purchased in a local market. The other reagents used were *p.a.* and obtained from various sources.

2.1.2. Enzyme preparations

rROL was produced in *Pichia pastoris* following the procedure described by Arnau *et al* [30]. This lipase was produced by a fed-batch cultivation of a recombinant *Pichia pastoris* strain using methanol as inducer. The biomass was separated from the culture broth by centrifugation and microfiltration. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system equipped, with an Omega membrane of 10 KDa cut-off, was subsequently dialyzed against 10 mM Tris-HCl buffer at pH 7.5 and finally lyophilized.

2.2. Methods

2.2.1. rROL immobilization procedures

rROL was immobilized in three different supports: Accurel® MP 1000, Lewatit® VP OC 1600 and Eupergit® C.

The immobilization procedure for rROL in Accurel® MP 1000 started by wetting 1g of the support with 7.0 mL of ethanol during, approximately, 15 min. The ethanol was then carefully removed, using a micropipette, and the wetted resin was added to 50 mL of phosphate buffer solution (0.1M, pH 7.0) containing an appropriate amount of dissolved lipase (0.15-0.50 g). To select the immobilization support, an amount of 0.15 g of rROL was used in 50 mL of phosphate solution per gram of support. To choose the most appropriate ratio lipase powder:support, different amounts of rROL in the phosphate buffer solution (0.15-0.50 g), corresponding to ratios from 15 % to 50 %, were tested. In batch operational stability tests, 0.25 g of rROL were used per gram of support. The immobilization was carried out at room temperature, overnight, under gentle stirring. After that, the beads were recovered by vacuum filtration and incubated with 25 mL of phosphate buffer solution (0.1M, pH 7.0) containing 2.5% (v/v) glutaraldeyde aqueous solution for 2h, under slow mixing. The beads were, once again, recovered by vacuum filtration and washed twice with 50 mL of phosphate buffer solution (0.1M, pH 7.0) in order to remove the free enzyme. The immobilized lipase was dried under vacuum for approximately 10 min and stored at 4ºC until use.

The protocol followed for immobilization rROL in Lewatit® VP OC 1600 was similar to the formerly described for Accurel® MP 1000 except that the support was not previously wetted with ethanol.

The immobilization of rROL in Eupergit® C followed the conventional method previously described, which involves the direct enzyme binding on support via oxirane

groups [26]. Unmodified Eupergit® C (1g) was incubated with 0.20 g of recombinant lipase in 5 cm^3 of sodium phosphate buffer solution (0.1 M, pH 7.0) at 20ºC, with no agitation, for 96h. Afterwards, the beads were recovered by vacuum filtration and washed twice (2 x 25 cm^3) with phosphate buffer solution (0.1 M, pH 7.0).

2.2.2. Determination of immobilization yield

The immobilization yield was determined by quantifying the unbound protein content in the supernatant before and after immobilization. Protein content was evaluated according to the Bradford method [31]. A standard curve was obtained for rROL. The yield of immobilization (η) was estimated according to the following equation:

$$\eta = \frac{P_0 - P_1}{P_0} \times 100 \qquad \text{(Eq. 1)}$$

where P_0 is the enzyme amount present in the buffer solution before the immobilization support was added and P_1 is the residual amount of enzyme present in the supernatant after immobilization as well as in the subsequent washing solutions.

2.2.3. Assessment of hydrolytic activity of lipase preparations

The hydrolytic activity was assayed for (i) the initial lipase suspension, (ii) the supernatant after immobilization and (iii) for rROL immobilized in the supports tested, according to the method described by Soares *et al*. [32], after modification: 50 g of extra virgin olive oil were mixed with 50 g of distilled water and 3.5 g of gum arabic. This mixture was stirred for 10 min. Then, 80 mL of phosphate buffer solution (0.1 M, pH 7.0) were added and the final emulsion homogenized in an ultraturrax. Nine mL of this emulsion were put in a 20 mL closed thermostated cylindrical batch reactor, at 30 ºC, under magnetic stirring. After 15 min, 0.3 g of immobilized enzyme, or 10 mL of the initial lipase suspension or of the final lipase suspension recovered after immobilization, was added and the hydrolysis was allowed to proceed during 5 min (immobilized preparation) or 10 min respectively (supernatant solution). Afterwards, 20 mL of a solution of ethanol:acetone (1:1, v:v) was added in order to inactivate the enzyme. The amount of free fatty acids (FFA) released during hydrolysis was determined by direct titration with a 0.1 N sodium hydroxide aqueous solution, using phenolphthalein as

indicator. In parallel, blank experiments were carried out in the absence of enzyme. Each experiment was performed in triplicate.

One unit of hydrolytic activity (U) was defined as the amount of enzyme that catalyzes the hydrolysis of olive oil releasing one micromole of FFA per minute.

The loss of activity due to rROL immobilization (expressed in U) was estimated based on equation 2

$$Activity\ loss = A_i - A_E - A_s \qquad \text{(Eq. 2)}$$

where A_i is the initial suspension activity, A_E is the immobilized enzyme activity and A_s is the supernatant activity after immobilization.

2.2.4. Interesterification reaction

Interesterification reactions were performed batchwise in closed thermostated cylindrical batch reactors (20 mL), at 60ºC, under magnetic stirring. The reaction medium consisted of a blend of 3.90 g of tripalmitin and 2.76 g of oleic acid (molar ratio oleic acid: tripalmitin of 2:1). In each experiment, 0.33 g of immobilized rROL (rROL in Accurel® MP 1000, in Lewatit® VP OC 1600 or in Eupergit C)., corresponding to 5 % (w/w) of the reaction medium, were added to the reaction medium, after complete melting. Prior to, and at different reaction times, 1 mL samples were taken and the biocatalyst was removed by paper filtration. All the samples were stored at −18ºC for subsequent analysis. All the experiments were carried out in triplicate. For each system, molar incorporation degree (%) was calculated on the basis of the molecular weight of oleic acid (282.5).

2.2.5. Batch operational stability tests

The operational stability of the biocatalyst was evaluated in consecutive 23 h batches for rROL immobilized in Accurel® MP 1000 and for rROL in Lewatit® VP OC 1600. These immobilized enzymes were prepared by using 0.25 g of rROL in the initial phosphate solution per gram of support (referred as enzyme load of 25 %). Interesterification was carried out as previously described (cf. 2.2.4). After each batch, the biocatalyst was removed from the reaction medium by paper filtration and reused in

the next batch with fresh medium, under the same reaction conditions. A total of up to 2 batches (for rROL in Accurel) and 4 batches (for rROL in Lewatit) were performed using the same biocatalyst sample.

In parallel, similar 8 consecutive batches were carried out using rROL immobilized in Lewatit that was rehydrated at the end of each batch. Rehydration was performed by adding 50 mL of phosphate buffer solution (0.1M, pH 7.0) to the enzyme preparation that was subsequently filtered under vacuum and reused in the subsequent batch.

The molar incorporation degree of oleic acid in tripalmitin, observed at the end of each batch, was used as a measure of the activity of the biocatalyst in that batch. The first batch was used as the reference (100% activity). The residual activity (a_n, %) of the biocatalyst at the end of each batch n (n = 1,...,8) was thus estimated as follows:

$$a_n = \left(\frac{Incorporation_{Batch_n}}{Incorporation_{Batch_1}} \right) * 100 \qquad \text{(Eq. 3)}$$

2.2.6. Analysis of reaction products

To determine the amount of oleic acid incorporated in TAG, the following procedure was followed for each sample: 0.15 g of the reaction medium was dissolved in 25 mL of chloroform *p.a.* and 200 µL of this solution was spotted on a continuous layer on a silica gel TLC plate. Elution was carried out in *n*-hexane/diethyl ether/acetic acid (70/30/1.5; v/v/v) as the mobile phase. Plates were sprayed with 0.2% (w/v) 2',7'-dichlorofluorescein in 95% ethanol and observed under U.V. at 366 nm. The various groups of compounds (triacylglycerols, free fatty acids, diacylglycerols and monoacylglycerols) were identified by comparison with standards.

The TAG band was scrapped off and methylated, in order to be assayed as fatty acid methyl esters (FAME) by gas chromatography (GC). For methylation, the silica gel containing TAG was mixed with 5 mL of methylation reagent (anhydrous methanol/*n*-hexane/concentrated sulphuric acid; 75/25/1, v/v/v), in a conical flask equipped with a Liebig condenser. This mixture was allowed to boil under reflux for 60 min in a water bath at about 80ºC. Then, 10 mL of distilled water and 10 mL of petroleum ether were added and the mixture was transferred to a separating funnel, vigorously agitated and allowed to settle for phase separation. The organic upper layer was recovered, washed

twice with distilled water (2 x 10 mL) and dried with anhydrous sodium sulphate. Sodium sulphate was removed by paper filtration, the solution was transferred to a conical-bottom flask and the solvent was evaporated in a rotavapor at 30ºC under a pressure lower than 200 mbar.

FAME were dissolved in 100 µL of 0.1% (w/v) methyl myristate (internal standard) in *n*-hexane solution and 1 µL of this solution was GC analyzed. A Finnigan TRACE GC Ultra gas chromatograph (Thermo Electron Corporation) equipped with a Thermo TR-FAME capillary column (30 m x 0.25 mm ID x 0.25 µm film), an auto sampler AS 3000 from Thermo Electron Corporation and a flame ionization detector, was used for FAME analysis. Injector (in splitless mode) and detector temperatures were set at 250ºC and 260ºC, respectively. Helium was used as carrier gas at a flow rate of 1.5 mL/min. Air and hydrogen were supplied to the detector at flow rates of 350 mL/min and 35 mL/min, respectively.

The oven temperature program was as follows: 60ºC for 1 min, a temperature increase to 150ºC at 15ºC/min, a plateau at 150ºC for 1 min, followed by temperature increase to 180ºC at 5ºC/min, a plateau at 180ºC for 3 min, an increase in temperature until 220ºC, at a rate of 10ºC/min and a final plateau at 220ºC for 1 min.

3. Results and Discussion

3.1. Selection of immobilization supports

In this work, rROL immobilized in Accurel® MP 1000, Eupergit® C or in Lewatit® VP OC 1600 was used as catalyst for the acidolysis reaction between tripalmitin and oleic acid in the absence of a solvent. The selection of the immobilization support was carried out taking into account (i) the immobilization yield (c.f. 2.2.2.), (ii) the hydrolytic activity of the immobilized lipase in each support (c.f. 2.2.3.) and (iii) the interesterification activity (c.f. 2.2.4.).

Immobilization yields and interesterification activities of the immobilized preparations are shown in Fig. 1. Concerning immobilization yields, Eupergit® C was the support that enabled the coupling of a higher amount of enzyme (86.0%), followed by Accurel® MP 1000 (67.2%) and Lewatit® VP OC 1600 (63.5%).

The enzyme immobilization yields are strongly dependent on the properties of the support. In fact, a solid support has a limited area available for protein molecules

adsorption due to intrinsic characteristics such as the surface area, the number of sites accessible for binding, the porosity and the pore size. Therefore, it is important to analyze some properties of the supports tested (Table 1).

Eupergit C is a microporous immobilization matrix containing epoxy-activated acrylic beads with density of oxirane groups of 600 μmol/g dry beads [25]. Mateos *et al*. [33] proposed a two binding step mechanism for the immobilization process of enzymes on multifunctional supports, such as in the case of Eupergit C. Initially, the enzyme is physically adsorbed on the carrier by hydrophobic interactions that brings amino and thiol groups on the surface of the enzyme in close proximity to the dense layer of oxirane groups on the support. In the following step, these groups react with the oxirane groups by nucleophilic attack that enables the establishment of very stable C-N and C-S covalent bonds.

Both Accurel MP 1000 and Lewatit VP OC 1600 are hydrophobic supports. The immobilization is established by physical adsorption between the hydrophobic surfaces surrounding the active centre of the enzyme and the hydrophobic groups of the matrix, with a consequent stabilization of the open form of the lipase. Glutaraldehyde is added after immobilization to promote stable crosslinks between the lipase and the matrix. In fact, this crosslinking agent has a widespread application due to its commercial availability, low cost, and high reactivity with amine groups at neutral pH [34].

Although Eupergit® C was the support that conducted to the highest immobilization yield, the lowest oleic acid incorporation was observed with rROL in this support (4.2 mol-%) (Fig 1). In fact, rROL immobilized in Lewatit® VP OC 1600 leads to the highest oleic acid incorporation (18.4 mol-%), followed by the Accurel MP® 1000 preparation (15.7 mol-%).

The obtained results may be due to the fact that enzymes are usually immobilized on Eupergit® C through their different groups (amino, sulfhydryl, hydroxyl, phenolic) that can block the substrate accessibility to the active site, or may establish multipoint-binding or can even lead to the enzyme denaturation [26]. The use of glutaraldehyde on the immobilization procedures for rROL in Accurel and Lewatit may promote the formation of enzyme aggregates with eventual enzyme stabilization.

A great loss in the hydrolytic activity of rROL after immobilization was observed for all the supports tested, being more pronounced for rROL immobilized in Accurel and in Lewatit (Table 2), probably due to mass transfer limitations. In fact, Accurel and

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

Lewatit are macroporous supports with a large internal surface area available for enzyme immobilization, within the deep pore structured, which limits the access of the lipase to the substrate. This problem is more evident in an oil/water interface reaction system, since the size of emulsion droplets may exceed the mean diameter pore of the support, with consequent low retained activity [35]. Also, changes in lipase conformation after immobilization or modification in the lipase microenvironment owing to interactions between the support and the enzyme could lead to the loss of catalytic activity after immobilization [36].

No relationship between the activity of the lipases in water solution and the activity in the acidolysis reaction was observed. Similar results were attained in previous works [37-39].

Since rROL immobilized in Lewatit® VP OC 1600 or in Accurel® MP 1000 presented the best performance regarding oleic acid incorporation (mol-%) on tripalmitin, both carriers were selected for further experiments. It is worth to notice that these supports have similar prices (Table 1).

3.2. Selection of enzyme loading in the supports

The selection of an appropriate enzyme loading in the support is a crucial issue in the development of a cost-effective biocatalyst.

In order to choose the most appropriate ratio of lipase powder in the initial immobilization solution/support (w/w), different rROL loadings (15, 25 and 50 wt%) were tested in Accurel® MP 1000 and in Lewatit® VP OC 1600 (c.f. 2.2.1.). The immobilized lipase preparations were then used as catalysts for the acidolysis reaction between tripalmitin and oleic acid (MR 1:2), at 60ºC for 24 h. The values of oleic acid incorporation in TAG and immobilization yields are presented in Fig. 2.

Both supports exhibited similar patterns concerning immobilization yields and oleic acid incorporation (mol-%) in TAG. It can be seen that when using more than 0.25 g of rROL in the initial solution per gram of support, during the immobilization procedure, only a moderate increase in oleic acid incorporation was observed with an increase in loading, probably due to mass transfer limitation and/or to the absence of free sites for enzyme adsorption (Fig 2). In fact, this increase in loading is accompanied by a decrease in immobilization yield. Therefore, the real amounts of immobilized protein were 0.09, 0.11 and 0.16 g/g of Lewatit VP OC 1600 and 0.10, 0.11 and 0.20 g/g of

Accurel® MP 1000, respectively when solutions containing 0.15, 0.25 and 0.50 g of enzyme per gram of support were used. Thus, 0.25 g of rROL in the solution per gram of support (initial load of 25 w-%) was selected for further experiments.

3.3. Time-course reactions

Time-course reactions were performed with rROL immobilized in Accurel MP 1000 and in Lewatit VP OC 1600 at the selected enzyme load/support of 25 wt (Fig 3).

The reaction catalyzed by rROL in Lewatit was faster (incorporation of 8.34 mol-%/h) and lead to higher levels of oleic acid incorporation than with rROL immobilized in Accurel (incorporation of 2.52 mol-%/h). In fact, a quasi-equilibrium was attained after 6h reaction for rROL in Lewatit with an oleic acid incorporation of about 30 mol-% while with rROL in Accurel, only after 12h, 22 mol-% incorporation of C18:1 was obtained.

The oleic acid incorporation in tripalmitin (*c.a* 30 mol-%), achieved at equilibrium using rROL immobilized on Lewatit® VP OC 1600, is slightly higher than the obtained values reported in our previous work (*c.a*. 27 mol-%), where three commercial immobilized lipases (Novozym 435, Lipozyme RM IM and Lipozyme TL IM, from Novozymes, Denmark) were used [10]. Also, the incorporation level obtained with rROL in Lewatit is higher than the value attained in our previous study performed with a non commercial extract of *Carica papaya* latex as biocatalyst for the acidolysis of tripalmitin with oleic acid (*c.a*. 22 mol-%) [39].

Esteban *et al*. [27] investigated the production of TAG rich in palmitic acid at *sn*-2 and oleic acid at *sn*-1,3 positions by acidolysis catalyzed by the lipase DF from *Rhizopus oryzae* immobilized on Accurel MP 1000. The maximum oleic acid incorporation at the extreme positions of TAG was 50 (mol-%), in experiments carried out in the presence of hexane at 37ºC [27].

Sahín and co-workers [8] synthesized HMFS by acidolysis of tripalmitin with hazelnut oil fatty acids and a concentrate of omega-3 fatty acids, in *n*-hexane, using Lipozyme RM IM as biocatalyst. The optimal conditions predicted by response surface polynomial models, for the targeted total of 5% of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and 40% of oleic acid incorporation, were as follows: 12.4 mol/mol (FFA/TAG), 55ºC and 24h.

When the incorporation of gamma-linolenic acid (GLA) was carried out in a similar system (tripalmitin, hazelnut fatty acids and GLA, in *n*-hexane) by the same group [7],

catalyzed by Lipozyme RM IM and Lipozyme TL IM, similar results were obtained with both enzymes to attain 10% of GLA and 45% of oleic acid incorporation: the optimal reaction conditions, predicted by response surface methodology, were 55ºC, 24h reaction time and molar substrate ratio of about 14:1 (FFA/TAG).

It is worth noting that the incorporation values reported in the literature were obtained using high FFA/tripalmitin molar ratios. It is generally accepted that increasing ratios of acyl donor/TAG result in higher incorporation of fatty acids into the TAG, by displacement of reaction equilibrium towards product formation. Nevertheless, high amounts of free fatty acids are undesirable since it increases operation costs due to recycling of unconverted substrates and product recovery.

3.4. Operational stability assays

The use of immobilized lipases at industrial scale requires the maintenance of the biocatalyst activity along the reaction time in order to ensure the economical viability of the process. Several parameters may affect the decrease of enzyme activity throughout the reaction, namely the water content, the presence of oxidation products, the bioreactor configuration and the operation mode [40, 41].

In order to select the best immobilized biocatalyst for HMFS, batch operational stability tests were performed for rROL immobilized in Accurel MP 1000 and in Lewatit VP OC 1600. The influence of enzyme rehydration, between consecutive batches, on the operational stability of rROL in Lewatit was also evaluated.

The residual acidolysis activities at the end of each of the repeated 23-h batches are presented in Fig. 4. The deactivation models and equations fitted to these results, as well as the respective estimated half-life times, are shown in Table 3.

For both rROL immobilized in Accurel and in Lewatit, a linear inactivation kinetic model was observed with estimated half-life times ($t_{1/2}$) of 34.5 h and 64 h of operation, respectively.

Since rROL immobilized in Lewatit conducted to the most promising results, further operational stability tests were carried out in an attempt to enhance the biocatalyst stability (c.f. 2.2.5). Thus, after each 23h batch, the biocatalyst was rehydrated with phosphate buffer solution and reused. In this condition, important changes on the operational stability profile of the biocatalyst were achieved: rROL immobilized in Lewatit with rehydration showed an inactivation parabolic profile. This behavior could be well described by the model of series-type inactivation kinetics proposed by Sadana

[42]. The fraction of the original activity (%) of the biocatalyst, a_n, at the end of each batch *n*, can be given by the following simplified model equation:

$$a_n = 100 - 50k_d n^2 \quad \text{(Eq. 4)}$$

where k_d (batch^{-2}) is the deactivation coefficient. The $t_{1/2}$ is given by:

$$t_{1/2} = k_d^{-1/2} \quad \text{(Eq. 5)}$$

Thus, a half-time of 8.8 batches (202 h) was estimated from Eq. 5 for rROL immobilized in Lewatit with rehydratation. The inactivation of the biocatalyst may result from its dehydration since an increase on oleic acid incorporation was observed after hydration of biocatalyst in consecutive batches.

Similar behavior was observed for the lipase/acyltransferase from *Candida parapsilosis* immobilized in Accurel MP 1000, when used as catalyst for the interesterification reaction of fat blends containing omega-3 polyunsaturated fatty acids in a continuous fluidized-bed reactor. When no water and extra water was added to the reaction medium, the obtained half-lives of this biocatalyst were 10 h and 18 h, respectively [43].

4. Conclusions

This study shows the potentialities of the recombinant *Rhizopus oryzae* lipase as biocatalyst for the synthesis of HMFS as an alternative to the commercial immobilized *sn*-1,3 selective lipases usually used. With rROL immobilized in Lewatit VP OC 1600, faster acidolysis of tripalmitin with oleic acid and similar incorporation levels of oleic acid to those achieved when high-cost commercial preparation immobilized lipases are used, were observed. The operational stability of this immobilized preparation increased with hydration between each consecutive 23-h batch, from a half-life of 64 to 202 h.

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Figure Legends:

Figure 1. Oleic acid incorporation obtained by acidolysis between tripalmitin and oleic acid, catalyzed by rROL immobilized in three different supports: Eupergit® C, Accurel® MP 1000 and Lewatit® VP OC 1600.

Figure 2. The effect of rROL load in Lewatit® VP OC 1600 and in Accurel® MP 1000 on (i) oleic acid incorporation in tripalmitin and on (ii) immobilization yield.

Figure 3. Time-course acidolysis reaction of tripalmitin and oleic acid catalyzed by rROL immobilized in Lewatit® VP OC 1600 (▲) and in Accurel® MP 1000 (◊).

Figure 4. Batch operational stability tests: residual activity of r-ROL immobilized in Accurel® MP 1000 (☐), Lewatit® VPOC 1600 (◊) and in Lewatit® VPOC 1600 with rehydration (△), at the end of each 23h batch reutilization, in the acidolysis of tripalmitin with oleic acid.

Table Legends:

Table 1. Characteristics of the immobilization supports tested: Accurel® MP 1000, Eupergit® C and Lewatit® VP OC 1600. [a]According to Tufvesson *et al*. [23]; [b]According to Boller *et al*. [25]; (n.a.-not available).

Table 2. Loss of hydrolytic activity after immobilization on the supports tested: Accurel® MP 1000, Eupergit® C and Lewatit® VP OC 1600 [a]Estimated according to equation 2.

Table 3. Deactivation models and equations and respective half-life times estimated for r-ROL immobilized in Accurel® MP 1000 and in Lewatit® VP OC 1600 with and without hydration.

Table 1: Characteristics of the immobilization supports tested: Accurel® MP 1000, Eupergit® C and Lewatit® VP OC 1600 ([a]according to Tufvesson *et al*. [23]; [b]according to Boller *et al*. [25]; n.a.-not available).

Name	Matriz	Specific area (m^2/g)	Mean pore diameter (Å)	Mean diameter (μm)	Cost (€/kg)
Accurel MP 1000[a]	Polypropylene	70	2000	50-1000	40
Eupergit C[b]	epoxy-activated acrylic beads	n.a.	1000	100-250	n.a.
Lewatit VP OC 1600[a]	DVB-crosslinked methacrylate polymer	130	15	315-1000	41

Table 2: Loss of hydrolytic activity after immobilization on the supports tested: Accurel® MP 1000, Eupergit® C and Lewatit® VP OC 1600 ([a]Estimated according to equation 2).

Immobilization support	**Initial suspension activity (U)**	**Immobilized rROL activity (U)**	**Supernatant activity (U)**	**Loss of activity[a]**	
				(U)	**(%)**
Accurel® MP 1000	1340	84.7	63.5	1191.8	88.9
Eupergit® C	1072	178	300	594	55.4
Lewatit® VP OC 1600	1340	35.5	199.0	1105.5	82.5

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9 **Table 3.** Deactivation models, equations and respective half-life times estimated for r-ROL immobilized in Accurel® MP 1000 and in Lewatit®
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11 VP OC 1600 with and without hydration.
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Immobilization support	Deactivation model	Model equation	Half-life time (h)
Accurel® MP 1000	Linear	$a_n = -95.87n + 195.9$	34.5
Lewatit® VP OC 1600 without hydration	Linear	$a_n = - 6.94\,n + 124.9$	64.0
Lewatit® VP OC 1600 with hydration	Series-type	$a_n = 100 - 0.65n^2$	202.0

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Figure 1
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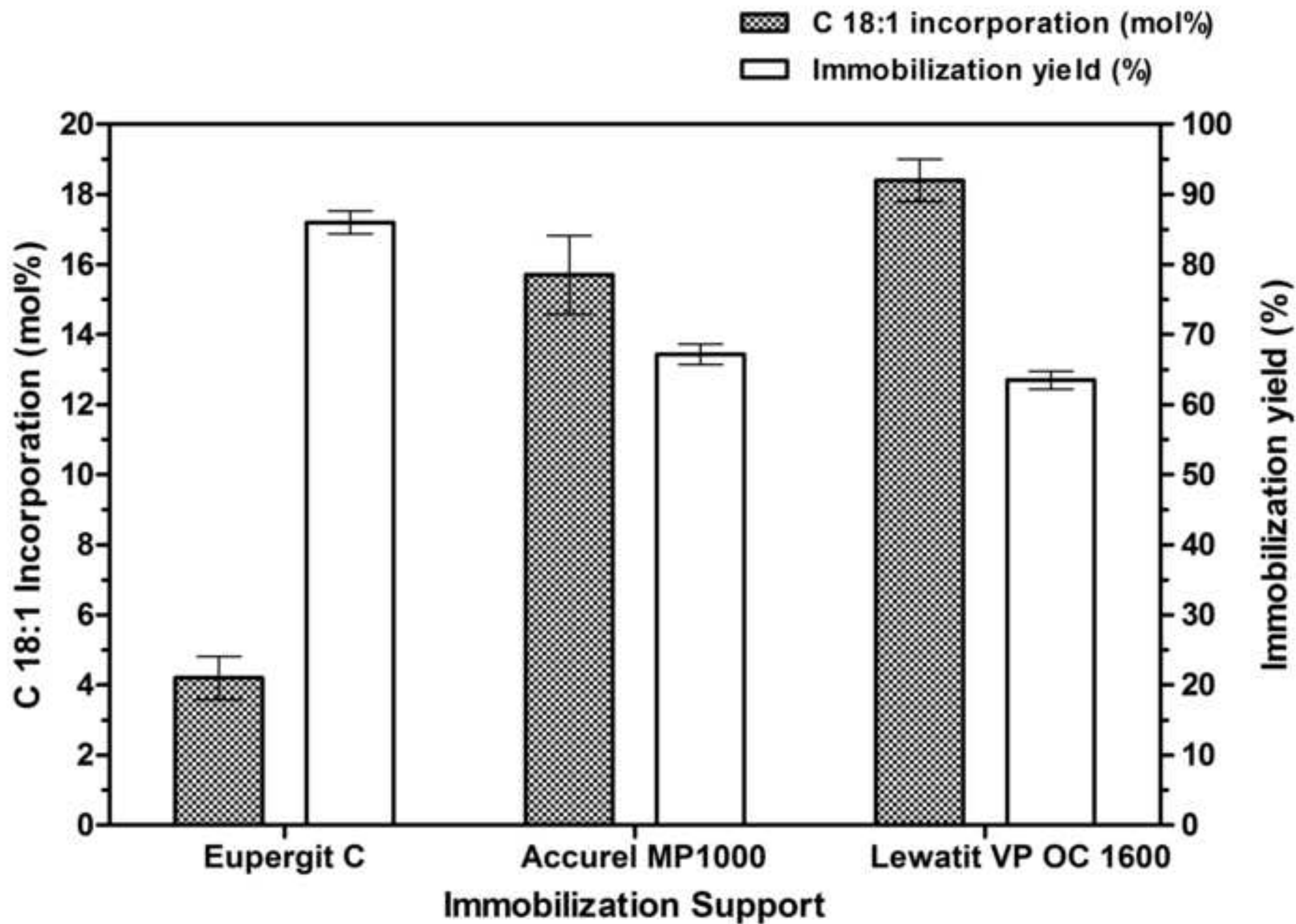

Figure 2

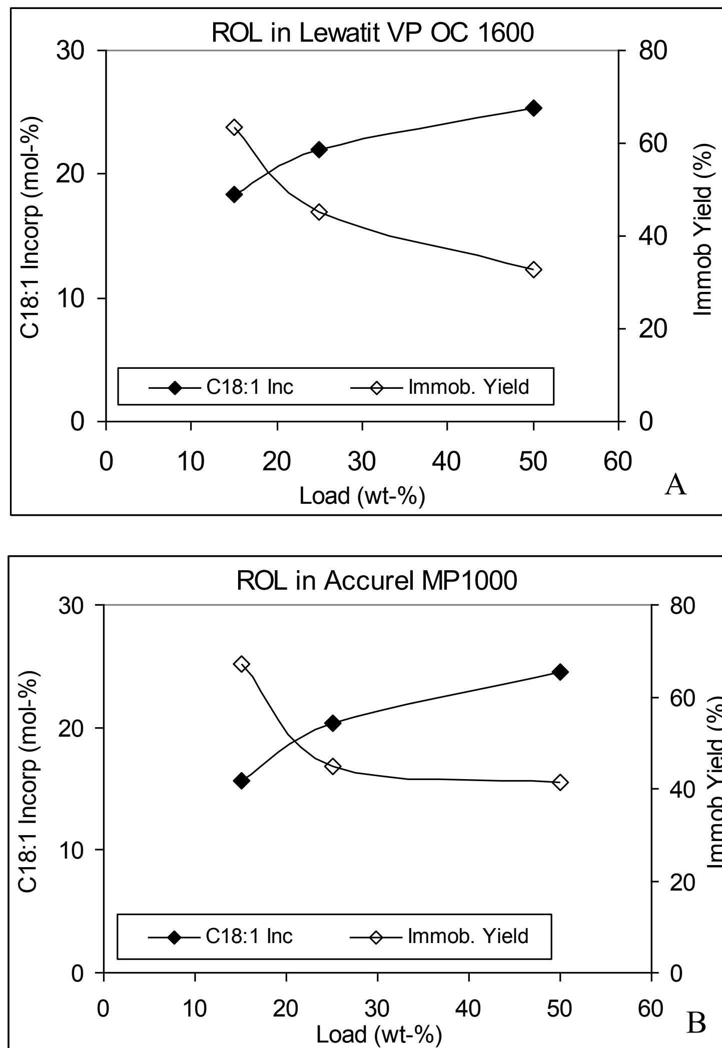

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Figure 3

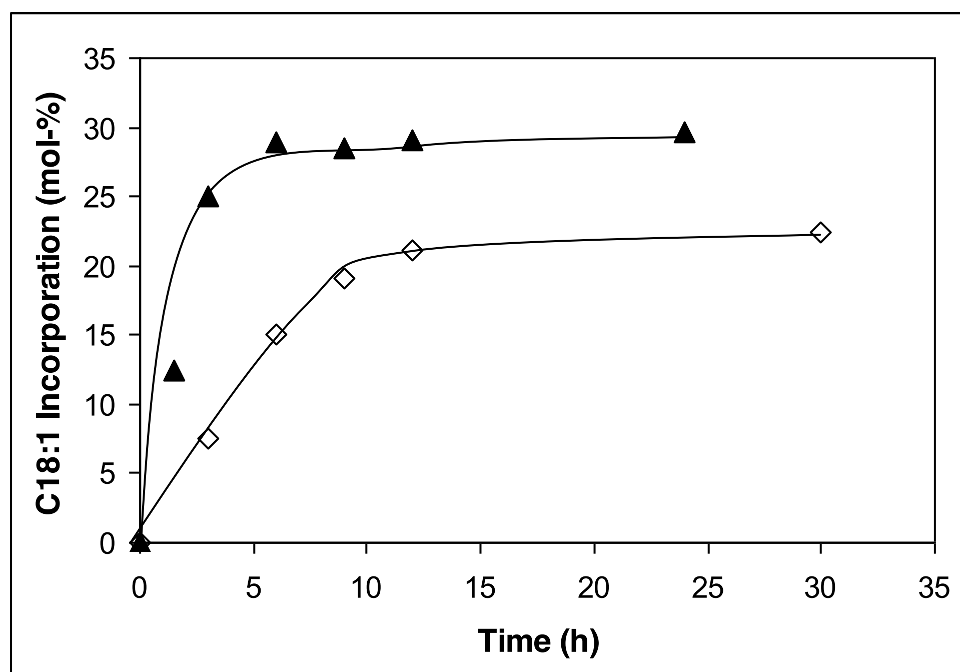

Figure 3. Time-course acidolysis reaction of tripalmitin and oleic acid catalyzed by rROL immobilized in Lewatit® VP OC 1600 (▲) and in Accurel® MP 1000 (◊).

Figure 4

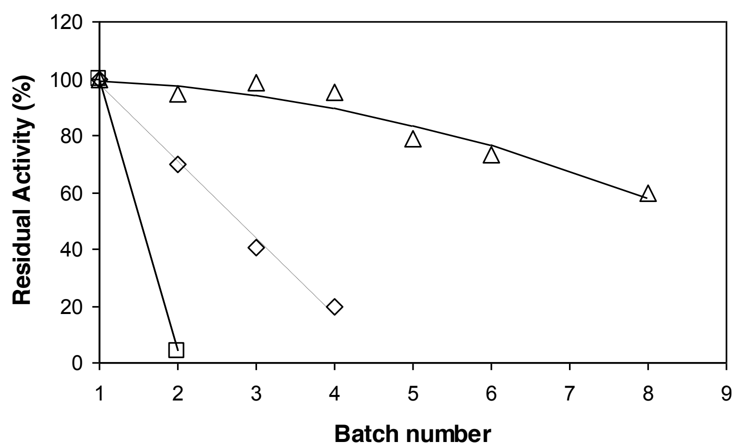

Figure 4. Batch operational stability tests: residual activity of r-ROL immobilized in Accurel® MP 1000 (□), Lewatit® VPOC 1600 (◊) and in Lewatit® VPOC 1600 with rehydration (△), at the end of each 23h batch reutilization, in the acidolysis of tripalmitin with oleic acid.

Capítulo VII.

Conclusões e Perspectivas Futuras

7.1. Conclusões Gerais

O trabalho desenvolvido no âmbito desta tese demonstrou a possibilidade de produção de sucedâneos de gordura de leite humano (SGLH), por interesterificação enzimática, para posterior incorporação em fórmulas de leite infantil.

Parte substancial deste estudo centrou-se na caracterização da reacção de acidólise entre a tripalmitina e o ácido oleico (C18:1), catalisada por diferentes lipases *sn*-1,3 selectivas, num reactor descontínuo, em meio isento de solvente, com o intuito de sintetizar *sn*-1,3-dioleil-2-palmitoilglicerol, um TAG importante na composição da gordura de leite materno.

As lipases comerciais de *Candida antarctica* ("Novozym 435"), de *Thermomyces lanuginosa* ("Lipozyme TL IM") e de *Rhizomucor miehei* ("Lipozyme RM IM") testadas conduziram a valores similares de incorporação de C18:1 (cerca de 27 mol-%), após 24 h de reacção.

Na perspectiva de implementação do sistema reaccional em grande escala é importante assegurar a manutenção da actividade catalítica da lipase ao longo do tempo de reacção. Assim, a estabilidade operacional do biocatalisador foi um aspecto fulcral desta investigação, avaliando-se a incorporação molar de C18:1 em ensaios descontínuos de reutilização sucessiva. Verificou-se que a "Lipozyme RM IM" não apresentou perda de actividade catalítica ao longo de 230h de operação. Por seu turno, a "Lipozyme TL IM" revelou ser o biocatalisador mais instável, registando-se um perfil linear de inactivação enzimática, que se traduziu por um tempo de meia vida ($t_{1/2}$) de cerca de 154h. No que respeita à Novozym 435, foi possível ajustar um modelo de inactivação tipo série com valor para $t_{1/2}$ de 253h.

Actualmente, as preparações de lipases comerciais são vendidas a preços muito elevados, o que condiciona a sua aplicação em processos de síntese, que se pretendem competitivos sob o ponto de vista económico. Nesta perspectiva, avaliaram-se diferentes preparações de lipases não comerciais, com valor económico substancialmente inferior, como biocatalisadores com potencial interesse para a síntese de SGLH.

Investigou-se a utilização da lipase/aciltransferase recombinante de *Candida parapsilosis* (CpLIP2) imobilizada em Accurel MP 1000, que exibe a característica peculiar de catalisar preferencialmente a reacção de alcoólise face à hidrólise em sistema bifásico aquoso/orgânico, como catalisador da interesterificação da tripalmitina com o ácido oleico em meio orgânico. Obteve-se uma incorporação pouco expressiva de ácido oleico em tripalmitina, cerca de 9 mol-%, após 24h de reacção.

Com o intuito de aumentar a incorporação de C18:1 nos TAG, testou-se o oleato de etilo como dador de grupo acilo, em alternativa ao ácido oleico, nas mesmas condições operacionais, dada a elevada especificidade desta lipase para esteres de ácidos gordos de cadeia longa com insaturação *cis*-9. Testaram-se diferentes razões molares oleato de etilo/tripalmitina (2:1 a 8:1) e após 4h de reacção, observaram-se incorporações de C18:1 de 30 mol-% para todas as razões molares testadas. Atingiu-se um equilíbrio aparente após 12-24h de reacção, com incorporações entre 32 e 45 mol-% dependendo da razão molar estudada. Observou-se um aumento linear da incorporação de C18:1 nos TAG com o aumento da razão molar. Estes resultados obtidos com CpLIP2 são muito interessantes, uma vez que os valores de incorporação conseguidos são da mesma ordem de grandeza ou superiores aos publicados para lipases comerciais.

Estudou-se também a preparação da lipase de *Carica papaya* (LCP) auto-imobilizada no látex, extraída de agro-resíduos de plantações de papaia. A lipase extraída do fruto da papaia (LCP I), conduziu a valores de incorporação de C18:1 idênticos aos observados para as preparações de lipases comerciais (cerca de 22 mol-%), nas mesmas condições operacionais. Todavia, quando se utilizou a preparação enzimática extraída do pecíolo das folhas da papaia (LCP II), apenas se observaram valores residuais de incorporação de C18:1. Estes resultados promissores obtidos com a LCP I impulsionaram uma série de estudos no sentido de avaliar a actuação deste biocatalisador. Nesse sentido, recorreu-se à metodologia da superfície de resposta para modelar e optimizar as condições reaccionais. Os ensaios de modelação da reacção de acidólise entre a tripalmitina e o ácido oleico foram estabelecidos de acordo com uma matriz central compósita rotativa em função dos factores temperatura e razão molar AG/TAG. Os resultados experimentais foram ajustados a uma superfície tridimensional descrita por um polinómio de segundo grau, que é função da temperatura e da razão molar. Não foi possível estabelecer um valor óptimo para a incorporação molar de C18:1 em função das condições operacionais, mas apenas identificar uma

região correspondente à melhor resposta. Assim, seleccionou-se a razão molar 6:1 (AG/TAG) e temperatura de 60ºC como as condições operacionais para validação do modelo. A comparação do valor experimental obtido após 24h de reacção foi cerca de 18% inferior ao valor teórico previsto pelo modelo. Esta discrepância pode ser sustentada pelo facto de a preparação enzimática de *Carica papaya* ser um extracto bruto heterogéneo que contém, para além de lipases, proteases, oxidases e outros compostos em quantidades variáveis. Além disso, a actividade catalítica da LCP é fortemente condicionada por factores como a localização geográfica da plantação de papaia bem como pela variedade do fruto de onde é extraída. Estudou-se também a estabilidade operacional de LCP em meios reaccionais formulados com duas razões molares distintas, 2:1 e 6:1 (AG/TAG), e em condições operacionais idênticas às estabelecidas para os ensaios reaccionais com as lipases comerciais. A inactivação enzimática foi mais pronunciada em presença do meio com a maior razão molar. Tal pode indiciar uma sensibilidade da enzima à maior concentração de AG livres no meio reaccional, os quais podem conduzir à modificação no estado de protonação do biocatalisador e à sua inactivação.

Caracterizaram-se os TAG obtidos após a reacção de acidólise, no que respeita à composição e à regiodistribuição dos AG no glicerol. Os TAG estruturados contêm, essencialmente, ácido oleico e palmítico e vestígios de ácido palmitoleico. As posições *sn*-2 dos lípidos estruturados são predominantemente ocupadas por ácido palmítico. Apesar da regioespecificidade de todas as lipases testadas, observou-se em todas as situações a incorporação molar de ácido oleico na posição central do TAG, como resultado da migração de grupos acilo durante a reacção de acidólise. A incorporação mais elevada de ácido oleico na posição *sn*-2 foi observada quando a "Novozym 435" foi utilizada como biocatalisador (33,2 mol-%). Este resultado vem corroborar estudos já publicados que consideram que esta enzima é, frequentemente, não regioselectiva. Os níveis de migração de grupos acilo registados para as restantes lipases comerciais e para a LCP foram similares (entre 16,0 e 26,1 mol-% de ácido oleico na posição *sn*-2). Observou-se que o aumento da razão molar de ácido oleico se traduziu num decréscimo da migração de grupos acilo para a LCP. Os TAG obtidos por acidólise catalisada pela lipase/aciltransferase recombinante de *Candida parapsilosis* (CpLIP2) imobilizada em Accurel MP 1000, revelaram o menor valor de migração de ácido oleico para a posição

sn-2 (6,6 mol-%), o que pode ser explicado pela baixa velocidade de reacção de acidólise.

Estudou-se também a lipase heteróloga de *Rhizopus oryzae* (r-LRO), expressa numa estirpe mutante de *Pichia pastoris*, como potencial biocatalisador para a síntese do TAG estruturado OPO. A r-LRO imobilizada em Accurel® MP 1000, Eupergit® C ou em Lewatit® VP OC 1600 foi utilizada na reacção de acidólise entre a tripalmitina e o ácido oleico, em meio livre de solvente. Esta vertente do trabalho incidiu sobre (i) a selecção do suporte de imobilização mais adequado para a r-LRO, (ii) estudos de carga enzima/suporte nos suportes mais apropriados e (iii) ensaios de estabilidade operacional do biocatalisador às condições seleccionadas em (i) e (ii).

A selecção do suporte de imobilização teve em consideração (i) o rendimento de imobilização, (ii) a actividade de interesterificação e (iii) a actividade hidrolítica da lipase imobilizada em cada suporte. O rendimento de imobilização foi superior para a r-LRO em Eupergit® C (86,0 %) comparativamente à sua imobilização em Accurel® MP 1000 (67,2%) ou em Lewatit® VP OC 1600 (63,5%). As características do suporte de imobilização condicionam fortemente o rendimento de imobilização, dada a sua área limitada para adsorção de moléculas de proteína associada a características intrínsecas da matriz de imobilização como a área superficial, o número de locais acessíveis para estabelecer ligações à enzima, assim como a porosidade e as dimensões do poro.

No que respeita à actividade de interesterificação, observou-se uma maior incorporação de C18:1 com a r-LRO imobilizada em Lewatit® VP OC 1600 (18,4 mol-%), seguida da r-LRO em Accurel® MP 1000 (15,7 mol-%). A menor incorporação de C18:1 alcançada com a r-LRO em Eupergit® C (4,2 mol-%) pode explicar-se pelo facto de este suporte microporoso estabelecer ligações covalentes estáveis com a enzima que podem dificultar o acesso do substrato ao seu centro activo. A imobilização de r-ROL em Lewatit® VP OC 1600 e em Accurel® MP 1000 ocorreu por adsorção física entre a superfície hidrofóbica que circunda o centro activo da enzima e os grupos hidrofóbicos da matriz seguida de estabilização por reticulação com glutaraldeído, o que promoveu a formação de agregrados enzimáticos com provável estabilização da enzima.

Observou-se uma perda considerável da actividade hidrolítica de r-LRO após imobilização em todos os suportes testados, tendo sido mais pronunciada para r-LRO em Accurel e em Lewatit. Concluiu-se não existir uma relação entre a actividade da lipase em meio aquoso e na reacção de acidólise.

Considerando que os melhores resultados de incorporação molar de C18:1 em tripalmitina foram alcançados para r-LRO imobilizada em Lewatit® VP OC 1600 e em Accurel® MP 1000, ambas as matrizes foram seleccionadas para estudos de optimização de carga de biocatalisador/suporte. A selecção apropriada da carga de enzima no suporte assume uma importância crucial no custo do processo, dado o preço tendencialmente elevado das enzimas comercializadas. Testaram-se cargas de 15, 25 e 50% referente à razão entre a massa de r-LRO, presente na solução inicial de imobilização, e a massa de suporte (Accurel® MP 1000 e em Lewatit® VP OC 1600). Verificou-se que ambos os suportes exibiram padrões similares no que concerne aos rendimentos de imobilização e à incorporação molar de C18:1 nos TAG. Seleccionou-se a carga de 0,25 g de r-LRO na solução inicial de imobilização para 1 g de suporte (25%, m/m) para estudos posteriores.

Estudou-se ainda a incorporação de C18:1 em tripalmitina ao longo de 30h de reacção para r-LRO. Verificou-se que a reacção foi substancialmente mais rápida com a r-LRO imobilizada em Lewatit (incorporação de 8,34 mol-%/h) do que com a r-LRO em Accurel (incorporação 2,52 mol-%/h). Com efeito, atingiu-se um estado de *quasi-equilibrium* para r-LRO em Lewatit após 6h de reacção, registando-se uma incorporação de C18:1 de cerca de 30 mol-%, enquanto que para r-ROL em Accurel apenas se atingiu uma incorporação de 22 mol-% após 12h de reacção.

Por fim, implementaram-se ensaios de estabilidade operacional com reutilizações sucessivas do biocatalisador em ensaios de 23h cada. Obtiveram-se $t_{1/2}$ de 34,5h e 64,0h para r-LRO imobilizada em Accurel® MP 1000 e em Lewatit® VP OC 1600, respectivamente. Observou-se um aumento considerável da estabilidade operacional de r-LRO em Lewatit após re-hidratação do biocatalisador no final de cada utilização em descontínuo ($t_{1/2}$=202h), que indicia que a perda progressiva de actividade esteja associada à desidratação do biocatalisador.

Investigou-se também a síntese de SGLH enriquecidos em ácidos gordos polinsaturados ómega-3 (ómega-3 PUFA), pela comprovada importância que apresentam no desenvolvimento do sistema nervoso do recém-nascido, bem como na massa e mineralização ósseas. Para o efeito, estudou-se a reacção de acidólise entre a tripalmitina e um concentrado de PUFA rico em ácido docosa-hexenóico (DHA), "EPAX 1050 TG", a 60ºC, em meio livre de solvente. Após 24h de reacção a incorporação mais expressiva de ómega-3 PUFA observou-se quando as lipases

"Novozym 435" (21,6 mol-%) e "Lipozyme RM IM" (20,8 mol-%) foram utilizadas como biocatalisadores. Registaram-se níveis de incorporação com a lipase/aciltransferase de *Candida parapsilosis* (8,5 mol-%), a "Lipozyme TL IM" (8,2 mol-%) e a lipase de *Carica papaya* (8,7 mol-%) consideravelmente mais baixos do que os obtidos com o ácido oleico.
Os ensaios de estabilidade operacional, realizados em ensaios consecutivos de 23h cada, indicaram uma desactivação linear para a "Lipozyme RM IM" ($t_{1/2}$ = 276 h), enquanto a "Novozym 435" ($t_{1/2}$ = 322 h) e a lipase de *Candida parapsilosis* ($t_{1/2}$ = 127 h) apresentaram um modelo de inactivação tipo série.

Em suma, as lipases não comerciais testadas demonstraram ser biocatalisadores de elevado potencial para a síntese de SGLH, como alternativa às lipases comercializadas. Todavia, terá de ser feito um investimento no sentido de aumentar a estabilidade destes biocatalisadores com o intuito de implementar processos economicamente viáveis à escala industrial.

7.2. Perspectivas de Trabalho Futuro

O estudo desenvolvido no âmbito desta tese coloca a possibilidade de se perspectivarem trabalhos futuros, que poderão basear-se nas seguintes sugestões:

- Testar novas misturas reaccionais, a submeter a interesterificação: banha (como fonte de ácido palmítico) com outros óleos vegetais (*e.g.* óleo de bolota, azeite) ou concentrados de triacilgliceróis ricos em ómega-3 PUFA comerciais com composições diferentes em EPA e DHA.
- Testar outras preparações lipásicas imobilizadas, no sentido de encontrar preparações com elevada actividade de interesterificação e elevada estabilidade operacional.
- Optimizar as condições reaccionais no sentido de minimizar as migrações dos grupos acilo para a posição *sn*-2 e as reacções degradativas de hidrólise e oxidação lipídica.
- Investigar os factores determinantes da desactivação enzimática, com vista ao seu controlo e à reactivação completa do biocatalisador (e.g. desidratação, inactivação por produtos de oxidação).
- Optimizar a imobilização de lipases com o objectivo de encontrar suportes de imobilização que minimizem a desidratação e/ou inactivação das preparações lipásicas durante a sua utilização.
- Implementar os sistemas optimizados em descontínuo em reactores contínuos, de modo a seleccionar a melhor configuração e modo operatório, em termos de controlo das migrações dos grupos acilo para a posição *sn*-2, das reacções degradativas dos lípidos e da estabilidade operacional dos biocatalisadores.
- Desenvolver estudos de recuperação dos SGLM por adsorção selectiva, fraccionamento ou destilação;
- Avaliar a digestibilidade dos SGLM *in vitro*.