Université de Toulouse

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :
Institut National des Sciences Appliquées de Toulouse (INSA Toulouse)

Discipline ou spécialité :

Ingénieries Microbienne et Enzymatique

Présentée et soutenue par :
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le : vendredi 14 décembre 2012

Titre :

Lipase-Catalyzed Purification and Functionalization of Omega-3 Polyunsaturated Fatty Acids and Production of Structured Lipids

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Titre: Purification et fonctionnalisation d'acides gras polyinsaturés Oméga-3 par des lipases et production de lipides structurés

Spécialité: Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries
Filière: Ingénieries Microbienne et Enzymatique
Année: 2012 **Lieu:** Universidade Técnica de Lisboa

RESUME:

Les lipases sont des enzymes ubiquitaires présentant un grand intérêt industriel. Leurs applications sont diverses dans la pharmacie, la chimie fine, la santé, l'agro-alimentaire, les cosmétiques, l'environnement et l'énergie, entre autre. L'intérêt de ces enzymes a conduit à caractériser ces enzymes, à mieux comprendre leur mécanisme réactionnel et leur cinétique, et à établir des méthodes efficaces de production en système d'expression homologue et hétérologue. Plus récemment, l'ingénierie enzymatique permet d'améliorer les caractéristiques des enzymes telles que l'activité, la sélectivité, la thermo-stabilité et la tolérance à des pH extrêmes et aux solvants organiques.

Ce projet de thèse s'est fixé deux objectifs principaux: premièrement, la purification et la fonctionnalisation d'acides gras poly-insaturés de type Omega-3 (PUFAs), et spécialement l'acide *cis*-4, 7, 10, 13, 16, 19-docosahexaénoique (DHA) et deuxièmement la production de lipides structurés. Le DHA présente des propriétés anti-thrombose et anti-inflammatoire qui permettent de réduire les facteurs de risque de l'arthrite, du cancer, de maladies cardiovasculaires, de l'asthme, du diabète et de la maladie d'Alzheimer.

Un premier objectif fut de produire une molécule pharmaceutique, le nicotinyl DHA ester, actuellement en essai clinique pour le traitement des arythmies cardiaques. Le co-substrat du DHA est le nicotinol (3-hydroxymethylpyridine), un alcool appartenant au groupe de la pro-vitamine B. Après absorption, il est rapidement converti en acide nicotinique (Vitamine B3) qui possède la propriété de décroitre les acides gras libres dans le plasma, les triglycérides, et d'augmenter dans le plasma la concentration des lipoprotéines bénéfiques. La trans-esterification enzymatique entre l'ester éthylique du DHA et le nicotinol a été optimisée dans le but de synthétiser un ester présentant les propriétés cumulatives des deux réactants. Après la sélection de l'enzyme optimale (lipase immobilisée de *Candida antarctica*; Novozyme 435) et le choix du milieu réactionnel (milieu sans solvant), le procédé a été optimisé. Une conversion supérieure à 97 % a été obtenu en 4 heures avec 45 $g.L^{-1}$ d'enzyme. Dans ces conditions, une productivité de 4.2 g de produit $.h^{-1}$.g d'$enzyme^{-1}$ a été obtenue.

Ce projet nécessite une haute pureté en DHA. Un procédé de purification enzymatique a été choisi car cela permet de travailler dans des conditions à faible température ce qui est un pre-requis car le DHA est sensible à l'oxydation. Les lipases sont capables de discriminer entre les acides gras en fonction de la longueur de chaine et du degré d'insaturation. Les lipases agissent par résolution cinétique, en réagissant plus efficacement avec les acides gras saturés et mono-insaturés qu'avec les PUFAs résistants. Il reste toujours d'un grand intérêt de découvrir des enzymes spécifiques pour la purification du DHA. La lipase YLL2 de *Yarrowia lipolytica* apparait comme un bon candidat car elle est homologue à une des lipases les plus efficaces, la lipase de *Thermomyces lanuginosus*. YLL2 a permis d'obtenir une discrimination très efficace, Les raisons de la sélectivité de l'enzyme ont été identifiées : il s'agit du positionnement de la double liaison la plus proche de la fonction carboxylique. La concentration en DHA la plus élevée a été obtenue avec YLL2 (73%) avec un pourcentage de récupération du DHA-EE de 89%. YLL2 est par conséquent l'enzyme décrite la plus efficace pour la purification du DHA.

Devant le grand intérêt de cette enzyme pour la purification du DHA, la mutagénèse ciblée dans le site actif a été utilisée pour améliorer la sélectivité de cette enzyme. L'analyse de la structure 3D et les alignements avec des lipases homologues a permis de choisir les cibles de mutagénèse dirigée. Les acides aminés cibles ont été changés de manière à restreindre ou élargir le site actif. De ce premier screening de variantes deux positions ont permis d'améliorer la spécificité de l'enzyme, les positions I100 et V235. Finalement la saturation de ces 2 positions a été réalisée et les performances de ces variantes analysées.

Le dernier objectif de la thèse était la production de lipides structurés (SL) par acidolysis enzymatique entre l'huile d'olive vierge et les acides caprylic ou capric utilisant la lipase YLL2 immobilisé. Le SL obtenu devrait être riche en acide oléique à la position *sn*-2 tandis que les C8:0 et C10:0 devraient être principalement estérifiés aux positions *sn*-1,3. YLL2 immobilisé sur Accurel 1000 a été testé dans un système sans solvant. La réaction d'acidolysis d'huile d'olive avec C8:0 ou C10:0 catalysé par YLL2 immobilisé a été optimisée avec la méthodologie de surface de réponse (RSM).

MOTS CLES:
Lipase, *Yarrowia lipolytica*, Omega-3, huile de poisson, purification, DHA, mutagenèse, sélectivité, lipids structurés, immobilisation.

Ecole doctorale : Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries (SEVAB).
Unité de recherche : Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés (UMR CNRS 5504, UMR INRA 792) de l'INSA de Toulouse.

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Title: Lipase-catalyzed purification and functionalization of Omega-3 polyunsaturated fatty acids and production of structured lipids

Speciality: Ecological Sciences, Veterinary, Agronomics and Bioengineering
Field: Enzymatic and Microbial Engineering
Year: 2012 **Place:** Universidade Técnica de Lisboa

ABSTRACT:

Lipases are ubiquitous enzymes, widespread in nature. Their applications are extended to a wide variety of industries including pharmacy, fine chemistry, health, food, cosmetics, environment and energy, among others. The variety of lipases applications led to increased research to characterize them and better understand their kinetics and reaction mechanisms and to establish methods for lipase production in homologous and heterologous expression systems. Enzymatic engineering allowed the improvement of lipase characteristics such as activity, selectivity, thermostability and tolerance to extreme pH and organic solvents. Enzyme selectivity improvement is one of the most interesting characteristics that can be changed by enzymatic engineering.

This thesis project studies the use of lipases for two main objectives: lipase-catalyzed purification and functionalization of Omega-3 polyunsaturated fatty acids (PUFAs), especially *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) and production of structured lipids. DHA presents anti-thrombotic and anti-inflammatory properties that reduce risk factors of arthritis rheumatoid, cancer, cardiovascular diseases, myocardial infarction, bronchial asthma, inflammatory intestinal diseases, diabetes and Alzheimer's disease.

DHA was used for the synthesis of a pharmaceutical molecule, the nicotinyl DHA ester, tried in clinical assay for the treatment of cardiac arrhythmia. The co-substrate of the reaction was nicotinol (3-hydroxymethylpyridine), an alcohol from the group B pro-vitamin. After absorption, it is rapidly converted into nicotinic acid (Vitamin B3) that presents the ability to substantially decrease plasma free fatty acid, triglyceride, VLDL and LDL levels and to raise the plasma concentration of protective HDL (high density lipoproteins). The enzymatic trans-esterification of DHA ethyl esters with nicotinol was optimised in order to synthesise an ester presenting the cumulative properties of the two reactants. After enzyme (immobilized lipase from *Candida antarctica*; Novozym 435) and reaction medium (solvent-free system) selection, the process was optimised. A conversion to nicotinyl-DHA superior to 97 % was obtained in 4 hours using 45 $g.L^{-1}$ of enzyme. In these conditions, a productivity of 4.2 g of product $.h^{-1}.g$ of $enzyme^{-1}$ was obtained.

This project requires DHA of high purity. Enzymatic purification was chosen for the production of DHA concentrates since this method enables the purification to be operated under mild conditions, which is preferable since DHA is susceptible to oxidation. Lipases are able to discriminate between fatty acids in function of their chain length and saturation degree in three types of reactions: hydrolysis, trans-esterification, and esterification. Lipases act by kinetic resolution, reacting more efficiently with the bulk of saturated and mono-unsaturated fatty acids than with the more resistant PUFAs. The objective was the discovery of more specific enzymes for DHA purification. The lipase Lip2 from *Yarrowia lipolytica* (YLL2) appears as a good candidate since it is homologous to one of the most efficient lipase, the lipase from *Thermomyces lanuginosus*. YLL2 enables a high discrimination to be obtained, enzyme selectivity being principally due to the positioning of the double-bond the closest from the carboxylic group. The highest concentration of DHA was obtained with YLL2 (73%) with a recovery percentage of DHA-EE of 89%. YLL2 is consequently the most efficient described lipase for DHA purification.

Further research was carried out using site directed mutagenesis to improve YLL2 from *Y. lipolytica*. Using its three dimensional structure and alignment with homologous lipases, targets for site directed mutagenesis were chosen in the active site. Chosen amino acids were substituted by two amino acids of different sizes. From the screening of variants two positions with promising specificities where chosen, positions I100 and V235. Finally saturation of both positions and the analysis of their performances in the selected reactions were carried out.

The last objective studied in the thesis was the production of structured lipids (SL) by enzymatic acidolysis between virgin olive oil and caprylic or capric acids using immobilized Lip2 from *Y. lipolytica*. The SL obtained should be rich in oleic acid at the *sn-2* position while C8:0 and C10:0 should be mainly esterified at the *sn-1,3* positions. Lip2 from *Y. lipolytica* immobilized on Accurel MP 1000 was tested in a solvent-free system. In addition, the acidolysis reaction of olive oil with C8:0 or C10:0 catalyzed by immobilized YLL2 was optimized by response surface methodology (RSM).

KEY WORDS:

Lipase, *Yarrowia lipolytica*, Omega-3, fish oils, purification, DHA, mutagenesis, selectivity, structured lipids, immobilization.

List of publications

Publication 1:

Lipases: An Overview.

Leticia Casas-Godoy, Sophie Duquesne, Florence Bordes, Georgina Sandoval and Alain Marty, in Lipases and Phospholipases, *Methods and Protocols in the series: Methods in Molecular Biology, Vol. 861, Sandoval, Georgina (Ed.),* 2012.

Publication 2 :

Enzymatic trans-esterification of a highly concentrated long chain ω3 polyunsaturated fatty acid ethyl ester with a group B pro-vitamin alcohol for prevention and treatment of cardiovascular diseases.

Leticia Casas Godoy, Etienne Séverac, Laurence Tarquis, Nadine Chomarat, Sophie Duquesne and Alain Marty. Submitted to *Enzyme and Microbial Technology*.

Publication3:

***Yarrowia lipolytica* Lipase Lip2: an efficient enzyme for the production of DHA Ethyl Esters Concentrates**

Leticia Casas-Godoy, Rungtiwa Piamtongkam, Warawut Chulalaksananukul and Alain Marty. Submitted to *Biocatalysis and Biotransformation.*

Publication 4:

Site directed mutagenesis improved specificity of Lip2 from *Yarrowia lipolytica* towards DHA ethyl ester purification.

Leticia Casas-Godoy, Marlène Cot, Sophie Duquesne and Alain Marty. *In preparation.*

Publication 5:

Rationally engineered mono and double substituted variants of *Yarrowia lipolytica* lipase for DHA ethyl ester purification.

Leticia Casas-Godoy and Alain Marty. *In preparation.*

Publication 6:

Optimization of medium chain length fatty acid incorporation into olive oil catalysed by immobilized Lip2 from *Yarrowia lipolytica*.

Leticia Casas-Godoy, Alain Marty, Georgina Sandoval, Suzana Ferreira-Dias. Submitted to *Biochemical Engineering Journal.*

Oral communications

Communication 1:

Synthesis of a nicotinyl DHA ester for prevention and treatment of cardiovascular diseases: enzyme and process optimisation.

Club Bioconversion en Synthèse Organique CBSO, June 2012. Leticia Casas Godoy, Etienne Séverac, Sophie Duquesne, Laurence Tarquis, Nadine Chomarat, Alain Marty.

Poster communications

Poster1:

Hidrólisis de esteres etílicos de aceites de pescado y producción de concentrados de Omega-3 utilizando lipasas de *Candida rugosa*.

II Congreso de la SOLABIAA, December 2010. Leticia Casas Godoy, Rungtiwa Piamtongkam, Sophie Duquesne, Georgina Sandoval, Warawut Chulalaksananukul, Alain Marty.

Poster 2 :

Synthesis of a nicotinol DHA ester for prevention and treatment of cardiovascular diseases.

Congres annuel de la société française de biochimie et biologie moléculaire SFBBM, October 2011. Leticia Casas Godoy, Etienne Séverac, Sophie Duquesne Laurence Tarquis, Nadine Chomarat, Alain Marty.

Poster 3 :

Synthesis of a nicotinol DHA ester for prevention and treatment of cardiovascular diseases: enzyme and process optimisation.

10th Euro Fed Lipid Congress, Fats, Oils and Lipids: from Science and Technology to Health, September 2012. Leticia Casas Godoy, Etienne Séverac, Sophie Duquesne Laurence Tarquis, Nadine Chomarat, Alain Marty.

Poster 4:

Production of Docosahexaenoic Acid and Eicosapentaenoic Acid Ethyl Esters Concentrates by Enzymatic Hydrolysis.

10th Euro Fed Lipid Congress, Fats, Oils and Lipids: from Science and Technology to Health, September 2012. Leticia Casas Godoy, Warawut Chulalaksananukul, Rungtiwa Piamtongkam and Alain Marty.

Acknowledgments

It is difficult to start since there are so many people that contributed in different ways during this process. Now that my thesis is ready is time to say thank you to all the people that helped me during this last four years.

First of all I would like to express my appreciation and gratitude to my supervisors Professor Alain Marty, Professor Suzana Ferreira-Dias and Professor Georgina Sandoval, for their advices, support, guidance and encouragement throughout this research. Thank you for giving me the possibility to complete this research.

The accomplishment of this work was only possible with the technical and scientific support of all the members of the team EAD1 from the Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés (LISBP) de l'INSA de Toulouse, France to whom I sincerely thank. I also want to express my sincere appreciation to all the current and past members of the Lipase team, Sophie Duquesne, Florence Bordes, Marlène Cot, Mélusine Bressy, Marie Guicherd, Muchalin Meunchan, Guillaume Nars, Ann Rungtiwa, Olivier Galy and Etienne Severac for their support, assistance and guidance all the way through my time in the laboratory.

To Géraldine Bastien, Niina Aro-Kärkkäinen, Muchalin Meunchan, Ann Rungtiwa, César Aceves, Tran Thu Hoai, Ines Benhsan, Ly Tran, Miguel DeAbreu and Beau Theerachat thank you for you kindness and friendship during our time working in Toulouse. To my friends Tandy, Ana, David, Angel, Fam. Bonilla, Fam. Parra and all the Mexican community in Toulouse, thank you for your friendship and for all the good times spend together.

I would like to thank the members of the reading committee and members of the jury Thierry Chardot, Catherine Humeau, Marilia Mateus, Eric Dubreuq, Francisco Valero, Manuela Fonseca and Margarida Tome for reading my thesis and spending value time on it.

I would like to acknowledge the Mexican institutions “Consejo Nacional de Ciencia y Tecnología”, CONACYT and “Secretaria de Educación Publica”, SEP, for their financial support during my thesis and to CIATEJ for allowing me to begin in the world of research.

Finally I would like to express my deepest gratitude to my family. Thank you mom, dad, Arturo and Ceci, for your love, support and dedication. Thank you for always listening to me even when you did not understand. I also want to thank them for encouraging me to pursuing this degree.

Summary

Summary

List of Abbreviations

ALA	α-linolenic acid
AN	*Aspergillus niger*
C10:0	Capric acid
C8:0	Caprylic acid
CAL	*Candida antarctica*
CC	Candida *cylindracea*
CLO	Cod liver oil
CV	*Chromobacterium viscosum*
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EFAs	essential fatty acids
EPA	Eicosapentaenoic acid
ETA	Eicosatrienoic acid
E-DHA	Ethyl docosahexaenoate
E-EPA	Ethyl eicosapentaenoate
FA	Fatty acids
FFA	Free fatty acids
GC	*Geotrichum candidum*
GMO	Genetically modified organism
GRAS	Generally Recognized As Safe
HDL	High density lipoproteins
L, LCFA	Long-chain fatty acids,
LDL	Low density lipoproteins
Lip2	Lipase 2 from *Yarrowia lipolytica*
Lip7	Lipase 7 from *Yarrowia lipolytica*
Lip8	Lipase 8 from *Yarrowia lipolytica*
LLL	Long chain triacylglycerols
M, MCFA	Medium-chain fatty acids.
MAG	Monoacylglycerol
MHO	Menhaden oil
MLM	SL with MCFA in the *sn*-1 and *sn*-3 position and LCFA in the *sn-2* position
MMM	Medium chain triacylglycerols
O	Oleic acid
P	Palmitic acid

PUFA	Polyunsaturated fatty acids
POP	1,3-dipalmitoyl-2-oleoyl-glycerol
POS	1(3)-palmitoyl-3(1)-stearoyl-2-oleoyl-glycerol
PS	*Pseudomonas* sp
RM	*Rhizomucor miehei*
RN	*Rhizopus niveus*
RO	*Rhizopus oryzae*
RSO	Refine sardine oil
S, SCFA	Short-chain fatty acids and
St	Stearic acid
SBO	Sea blubber oil
SL	Structured lipids
SLS	SL with SCFA in the *sn*-1 and *sn*-3 position and LCFA in the *sn-2* position
SOS	1,3-distearoyl-2-oleoyl-glycerol
STD	Stearidonic acid
TG	Triacylglycerol
VLDL	Very low density lipoproteins
ω-3 PUFA	Omega-3 polyunsaturated fatty acids

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Chapter I: Literature Review

Publication 1, Part I: Lipases

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Part III: Omega-3 polyunsaturated fatty acids

Part IV: Structured Lipids

Introduction

Introduction

Lipases are ubiquitous enzymes, widespread in nature. Their first applications were in the food industry, mainly for the production of dairy products. Lipases were first isolated from bacteria in the early nineteenth century and the associated research continuously increased due to the particular characteristics of these enzymes. Since then, their applications have extended to a wide variety of industries including pharmaceutical, fine chemistry, health, cosmetics, environmental and bioenergy, among others. The variety of lipases applications led to increased research to characterize them and better understand their kinetics, reaction mechanisms and selectivities. Later, continuous research established methods for lipase production in homologous and heterologous expression systems. Understanding how lipases work encouraged researches to improve these enzymes in function of their industrial applications. Enzymatic engineering allowed the improvement of lipases characteristics such as activity, selectivity, thermostability and tolerance to extreme pH and organic solvents. This technique changes the enzyme at a molecular level, modifying one or several characteristics at the same time. Enzyme selectivity improvement is one of the most interesting characteristics that can be changed by enzymatic engineering. Enzymes improvements can be achieved by two different approaches, rational engineering or directed evolution. The rational approach is based on the analysis of the relationships structure-function of the biocatalyst, enabling the selection of targets for site directed mutagenesis, for example the amino acids in the active site of the enzyme. This technique requires the knowledge of the three dimensional structure of the enzyme and a complete study and comprehension of the molecular level mechanisms involved. Directed evolution is an approach that selects a biocatalyst with improved properties from a library of enzyme variants produced randomly by engineering. This method does not require knowledge of the structural or molecular properties of the enzyme, but a high throughput screening method is required to allow fast testing of a large library of variants.

This thesis project studies the use of lipase for two main objectives: lipase-catalyzed purification and functionalization of Omega-3 polyunsaturated fatty acids (PUFAs), especially *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) and production of structured lipids. DHA presents anti-thrombotic and anti-inflammatory properties that reduce risk factors of arthritis rheumatoid, cancer, cardiovascular diseases, myocardial infarction, bronchial asthma, inflammatory intestinal diseases, diabetes and Alzheimer's disease.

The final objective of this work is to develop a process for the production of a pharmaceutical molecule, the nicotinyl ester of DHA, which could be used in prevention and treatment of

cardiovascular diseases. The main property of this molecule is to be a cardiac antiarrhythmic agent. The co-substrate of the reaction is nicotinol (3-hydroxymethylpyridine), an alcohol from the group B pro-vitamin. After absorption, nicotinol is rapidly converted into nicotinic acid (Vitamin B3). Nicotinic acid has the ability to substantially decrease plasma free fatty acid, triglyceride, VLDL and LDL (very low and low density lipoproteins) levels and to raise the plasma concentration of protective HDL (high density lipoproteins). For a pharmaceutical purpose, the use of DHA of high purity is crucial. The main source of DHA is fish oil which contains around 25% DHA.

The selectivity of lipases was studied to produce Omega-3 polyunsaturated fatty acid concentrates rich in DHA. Enzymatic purification was chosen for the production of concentrates since this method enables the purification to be operated under mild conditions, which is preferable since DHA is susceptible to oxidation. Lipases are able to discriminate between fatty acids in function of their chain length and/or saturation degree in three types of reactions: hydrolysis, trans-esterification, and esterification. Lipases act by kinetic resolution, reacting more efficiently with the bulk of saturated and mono-unsaturated fatty acids than with the more resistant PUFAs. Indeed, the 5 and 6 double bonds, in EPA (*cis*-5, 8, 11, 14, 17-eicosapentaenoic acid) and DHA respectively, enhance steric hindrance in the active site of the lipases.

A second objective was the discovery of more specific enzymes for PUFAs purification, such as Lip2 from *Yarrowia lipolytica*, which can be compared with the lipases identified in the bibliography as efficient, *Thermomyces lanuginosus* lipase and the lipases from *Candida rugosa*. These lipases were studied by comparing their ability to concentrate DHA-EE in the ester fraction by hydrolysing a tuna oil ethyl ester mixture (FOEE) with a high reaction yield.

It is possible that these lipases will not be sufficiently active and selective to fulfil industrial requests, DHA purity higher than 85% with high yields of DHA recovery. In consequence, it will be considered to improve the selectivity of the best enzyme using enzyme engineering tools.

The last objective studied in the thesis was the production of structured lipids (SL) by enzymatic acidolysis between virgin olive oil and caprylic or capric acids using immobilized Lip2 from *Y. lipolytica*. The SL obtained should be rich in oleic acid at the *sn-2* position while C8:0 and C10:0 should be mainly esterified at the *sn-1,3* positions. Lip2 from *Y. lipolytica* immobilized on Accurel MP 1000 was tested in a solvent-free system. In addition, the acidolysis reaction of olive oil with C8:0 or C10:0 catalyzed by immobilized YLL2 was

optimized by response surface methodology (RSM) as a function of the molar ratio free fatty acids/triacylglycerols (FFA/TAG), temperature and reaction time.

This manuscript is organized in three chapters. The first chapter, the literature review is divided in four parts:

- Publication 1, Part I: Lipases: An Overview, in Lipases and Phospholipases, *Methods and Protocols in the series: Methods in Molecular Biology, Vol. 861, Sandoval, Georgina (Ed.),* 2012.
- Part II: Lip2 from *Yarrowia lipolytica.*
- Part III: Omega-3 polyunsaturated fatty acids.
- Part IV: Structured Lipids.

The second chapter presents the results, written in the form of research articles:

- Publication 2: Enzymatic trans-esterification of a highly concentrated long chain ω-3 polyunsaturated fatty acid ethyl ester with a group B pro-vitamin alcohol for prevention and treatment of cardiovascular diseases.
- Publication 3: *Yarrowia lipolytica* lipase Lip2: an efficient enzyme for the production of DHA ethyl esters concentrates.
- Publication 4: Site directed mutagenesis improved specificity of Lip2 from *Yarrowia lipolytica* towards DHA ethyl ester purification.
- Publication 5: Optimization of medium chain length fatty acid incorporation into olive oil catalysed by immobilized Lip2 from *Yarrowia lipolytica.*

The third chapter presents the general conclusion and perspectives for future work.

Chapter I: Literature Review

Publication 1

Part I: Lipases

Lipases

1. Definition of lipases

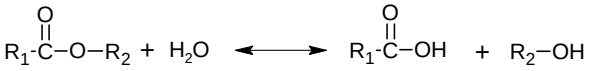
Lipases are serine hydrolases defined as triacylglycerol acylhydrolases (E.C. 3.1.1.3) and should be differentiated from esterases (E.C. 3.1.1.1) by the nature of their substrates. Indeed, the first criteria used to distinguish these two types of enzymes, i.e. activation by the presence of an interface, also called “interfacial activation”, was found unsuitable for the classification of such enzymes as some lipases did not exhibit such phenomenon. Prominent cases of this phenomenon are Lip4 from *Candida rugosa* (Tang et al., 2001) and *C. antarctica B* (Uppenberg et al., 1994). Moreover, lipases and esterases consensus motifs described by ProSite database (Hofmann et al., 1999) are very close. Therefore, lipases were later defined as enzymes capable of hydrolyzing carboxyl esters of long-chain acylglycerol (≥10 carbon atoms), while esterases hydrolyze carboxyl esters of short-chain acylglycerol (≤ 10 carbon atoms). Nevertheless, as both enzymes show a broad substrate specificity, both criteria should be considered (Verger, 1997; Chahinian et al., 2002). Fojan et al. also proposed a novel approach to distinguish between esterases and lipases based on the study of the amino acid composition and protein surface electrostatic distribution (Fojan et al., 2000). Cutinases usually catalyze the hydrolysis of ester bonds in cutine polymers, but, as they are also capable of hydrolyzing long chain and short chain triglycerides without requirement of interfacial activation, they are considered as intermediates between lipases and esterases. This last, cutinases, will not be discussed in this review.

2. Reaction catalyzed by lipases

2.1 Hydrolysis reaction

Lipases naturally catalyze the hydrolysis of the ester bond of tri-, di- and mono- glycerides into fatty acids and glycerol (*Figure 1*). Nevertheless, as shown in *Figure 1*, they are also active on a broad range of substrates. In all cases, the reaction is carried out at the interface of a biphasic system reaction. This biphasic system results from the presence of an immiscible organic phase, containing the hydrophobic substrate, in water.

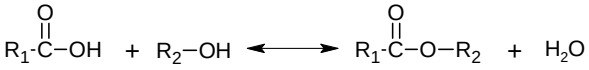
I. Hydrolysis



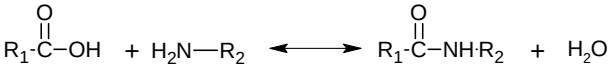
II. Synthesis

a Esterification

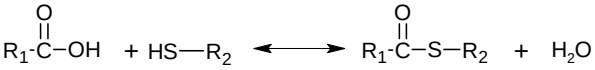
Esterification



Amidation

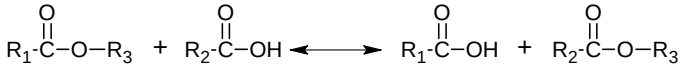


Thioesterification

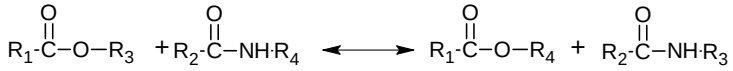


b Transesterification

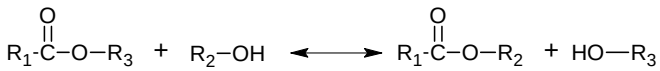
Acidolysis reaction



Aminolysis reaction



Alcoholysis reaction



Interesterification reaction

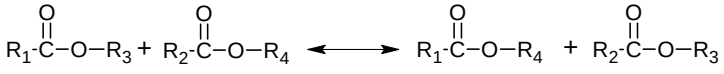


Figure 1. Reactions catalyzed by lipases.

2.2 Synthesis reactions

Lipases, in thermodynamic favorable conditions (i.e. low water activity), also catalyze a large variety of synthesis reactions which can be classified in two main types of reactions, *i.e.* esterification and transesterification (Reis et al., 2009). As shown on *Figure 1*, esterification is the reaction where a fatty acid is linked, through the action of the enzyme, to an alcohol by a covalent bond, producing an ester and releasing a water molecule. Thio-esterification and amidation are similar reactions but with a thiol or an amine as substrates. Transesterification groups alcoholysis, acidolysis, aminolysis and interesterification reactions.

Usually, these synthesis reactions occur in a medium with low thermodynamic water activity, the thermodynamic activity being a measure of the molecule availability in a solvent. The medium then consists in a free-solvent system (molten medium) or in an organic solvent.

Finally, lipases are also capable of expressing other annex activities such as phospholipase, lysophospholipase, cholesterol esterase, cutinase or amidase activities, (Svendsen, 2000).

3. Sources of lipases, physiologic role and regulation of the expression

First lipases were isolated by Eijkmann from *Bacillus prodigiosus, B. pyocyaneus* and *B. fluorescens*, currently known as *Serratia marcescens, Pseudomonas aeruginosa* and *P. fluorescens, respectively* (Eijkmann, 1901). Nowadays, it is recognized that lipases are produced by various organisms, including animals, plants and microorganisms (Vakhlu and Kour, 2006). Most animal lipases are obtained from the pancreas of cattle, sheep, hogs and pigs. Unfortunately lipases extracted from animal pancreas are rarely pure enough to be used in the food industry. For example, pig pancreatic lipase is polluted by trace amounts of trypsine which generate a bitter taste (Vakhlu and Kour, 2006). Other impurities include animal viruses and hormones. Therefore, due to the ease of production and abundance, most studied and industrially used lipases are obtained from microbial sources. Moreover, compared to bacterial lipases, lipases from GRAS (Generally Recognized As Safe) yeast sources are widely accepted and used in several industries including food processing (Vakhlu and Kour, 2006). Some of the major lipases used in industrial processes will be discussed in section 6.

Due to the importance and wide variety of lipases applications, different techniques have been developed in order to isolate lipases from various sources. Due to their ability to use fat as the only carbon source, microorganisms producing lipases were isolated from food

spoilage, where they are responsible for the flavor change of dairy products such as cheese, or from oily environments (sewage, rubbish dump sites and oil mill effluent). For instance, a cold adapted lipase was isolated from a *Pseudomonas sp.* strain (Choo et al., 1998) by screening soil samples from Alaska directly on solid media plates. With the same method, a thermostable lipase from *Geobacillus zalihae* was isolated from a palm oil mill effluent by inoculation into an enriched liquid medium containing olive oil as carbon source (Rahman et al., 2007). More recently new lipases were successfully isolated from the lipolytica proteome of subcutaneous and visceral adipocytes (Schicher et al., 2010), as well as from activated sludge (Nabarlatz et al., 2010). Nevertheless, this direct method can not be applied to uncultivable organisms and do not allow the isolation of the gene encoding the lipase.

For the last ten years, new methods were developed to allow the discovery of lipase genes. These metagenomic approaches are applicable to uncultivable organisms. These include the screening of DNA libraries, created from lipase-producing microorganisms by PCR with degenerate oligonucleotides complementary to lipase gene conserved regions as probe (Bell et al., 2002). An alternative to this method is functional metagenomic, *i.e.* the cloning of a so-called metagenome isolated from environmental DNA and its expression in a host for further screening of its hydrolytic activity (Henne et al., 2000; Zuo et al., 2010). An increasing number of methods, that will be discussed in chapter 3, have been developed to allow the detection of lipolytic activities (Hasan et al., 2009).

Lipases are known to have several physiological functions. In eukaryotes they are key components of lipid and lipoprotein metabolism (Sharma et al., 2001). As so, they are produced in the digestive system to hydrolyze absorbed triglycerides. Their production would be activated by a hormone sensitive regulation system when the energy demand increases, thus initiating the degradation of reserve triglycerides. In insects, lipases are mainly found in muscles, plasma, digestive organs and salivary glands (Pahoja and Sethar, 2002). In plants, lipases are mainly located in seeds, as part of the energy reserve tissues, and carry out the hydrolysis of reserve triglycerides necessary for the seed germination and further growth of the plant (Adlercreutz et al., 1997). Lipases in plants also have an important role in the metabolism, rearrangement and degradation of chlorophyll and the ripening of fruits (Tsuchiya et al., 1999). Besides, they were also postulated to play a defensive role since their production was found to be induced in the presence of pathogens (Stintzi et al., 1993). Microorganisms use the production of extracellular lipases in order to hydrolyze the triglycerides in the media and facilitate the ingestion of lipids.

Lipase expression in microorganisms is mainly regulated by environmental factors, as an extracellular response to a medium deprived in nutriments. Their production will therefore be activated by a cell density regulation system when the microorganism reaches the stationary phase or at the beginning of the growth phase in order to use the stored lipids (Olukoshi and Packter, 1994; Wagner and Daum, 2005). In most microorganisms the presence of lipids and fatty acids as carbon sources induce the production of these extracellular enzymes, thus allowing them to grow on spoiled soil.

4. Structure and catalytic mechanism

The first lipase structures were obtained from *Rhizomucor miehei* (Brady et al., 1990) and the pancreatic human lipase (Winkler et al., 1990). Nowadays, several hundreds of lipase sequences are listed in databases and amongst one hundred three dimensional lipases structures are available in the Protein Data Base (http://www.rcsb.org/pdb/home/). However these one hundred structures represent lipases of only thirty one organisms, since the same lipase can have several structures in different conformations or with different substrates. These include fungal lipases such as those from *Thermomyces lanuginosus* (Derewenda et al., 1994b), *Rhizopus oryzae* and *niveus* (Derewenda et al., 1994b; Kohno et al., 1996), *C. antarctica* Lipase B and Lipase A (Uppenberg et al., 1994; Ericsson et al., 2008), *C. rugosa* (Grochulski et al., 1993b), *Geotrichum candidum* (Schrag and Cygler, 1993), *Penicillium camembertii* and *expansum* (Derewenda et al., 1994a; Bian et al., 2010) and *Yarrowia lipolytica* (Bordes et al., 2010). As well, the known structures of bacterial lipases include those from *B. subtilis* (van Pouderoyen et al., 2001), *Pseudomonas sp.* (Angkawidjaja et al., 2007), *P. aeruginosa* (Nardini et al., 2000), *P. cepacia* (Kim et al., 1997; Schrag et al., 1997), *P. glumae (Noble et al., 1993), Chromobacterium viscosum* (Lang et al., 1996), *G. thermocatenulatus* (Carrasco-Lopez et al., 2009), *G. stearothermophilus* (Jeong et al., 2002; Tyndall et al., 2002), *G. zalihae* (Matsumura et al., 2008), *Photobacterium sp* (Jung et al., 2008), *S. marcescens* (Meier et al., 2007), *Staphylococcus hyicus* (Tiesinga et al., 2007), and *Streptomyces exfoliatus* (Wei et al., 1998). In addition, the structures of *Archaeoglobus fulgidus* lipase (Chen et al., 2009), bovine bile lipase (Wang et al., 1997), dog (Roussel et al., 1998a), horse (Bourne et al., 1994), rat (Roussel et al., 1998b) and Guinea pig (WithersMartinez et al., 1996) pancreatic lipase have been obtained. Lipases from *C. rugosa*, *C. antarctica, P. aeruginosa T. lanuginosus* and *C. viscosum* have a wide variety of industrial applications, (cf. 6).

Table 1. Summary of the structural data available for some extensively studied lipases.

Lipase	PDB entry	Catalytic triad	Oxyanion hole	Lid	Reference
Burkholderia cepacia	1OIL 2LIP 3LIP 4LIP 5LIP 1HQD 1YS1 1YS2	S87, D264, H286	L17,Q88	Y129-L149	(Kim et al., 1997) (Schrag et al., 1997) (Lang et al., 1998) (Luic et al., 2001) (Mezzetti et al., 2005)
Candida antarctica B	1TCA 1TCB TCC 1LBS 1LBT 3ICV 3ICW	S105, D187, H224	T40, Q106	-	(Uppenberg et al., 1994) (Uppenberg et al., 1995) (Qian et al., 2009)
Candida rugosa	1CRL 1TRH 1LPN 1LPO 1LPP 1LPM 1LPS	S209, E341, H449	G124, A210	E66-P92	(Grochulski et al., 1993b) (Grochulski et al., 1994c) (Grochulski et al., 1994a) (Cygler et al., 1994)
Rhizomucor miehei	1TGL 3TGL 4TGL 5TGL	S144,D203,H257	S82,L145	S83-P96	(Brady et al., 1990) (Brzozowski et al., 1992) (Derewenda et al., 1992a) (Brzozowski et al., 1991)
Rhizopus delemar	1TIC_A,B	S145, D204, H257	T83, L146	N84-F95	(Derewenda et al., 1994b)
Thermomyces lanuginosus	1TIB 1DT3 1DT5 1DTE 1DU4 1EIN 1GT6	S146, D201, H258	S83, L147	R84-F95	(Derewenda et al., 1994b) (Brzozowski et al., 2000) (Yapoudjian et al., 2002)
Human pancreatic lipase	1N8S 2PVS 2OXE	S153, H264, D177	F78, L154	-	(Vantilbeurgh et al., 1992) (Eydoux et al., 2008)

Structurally speaking, lipases are characterized by a common α / β hydrolase fold and a conserved catalytic triad. Most lipases also possess the consensus motif G-X1-S-X2-G. From their structures and the residues forming the oxyanion hole (amino acids of the lipase active site that stabilize the reaction intermediate) and catalytic triad, microbial lipases and esterases can be grouped in fifteen superfamilies and thirty two homologous families (Pleiss et al., 2000a). These structural elements will be discussed below. *Table 1* gives a summary of the structural data available for some extensively studied lipases.

4.1 The α / β hydrolase fold

The study of lipases three-dimensional structures showed the presence of a conserved alpha/beta-hydrolase fold, which is widely expanded in hydrolytic enzymes of different origins, such as proteases, haloalkane dehalogenases, acetylcholinesterases, dienelactone hydrolases and serine carboxypeptidases (Jaeger et al., 1999). The alpha/beta-hydrolase fold is generally composed of a central, parallel β-sheet of eight beta-strands, with only the second strand antiparallel (β2). Strands β3 to β8 are connected by α helices arranged on the sides of the central β sheet (*Figure 2*).

Some variations of the α / β fold were found in several lipases. The variations of the fold consist in differences in the amount of α helices, β sheets, loops length and architecture of the substrate binding sites (Pleiss et al., 1998; Jaeger et al., 1999; van Pouderoyen et al., 2001).

Lipases are also characterized by the presence of disulphide bridges that give the enzyme stability and are often important for their catalytic activity.

4.2 The catalytic triad

The catalytic triad, which is conserved among lipases, consists in a serine as nucleophile, an aspartate/glutamate as the acidic residue and a histidine (Brady et al., 1990; Winkler et al., 1990). It is similar to the one observed in serine proteases but with a different order in the sequence (Ollis et al., 1992). In the alpha/beta-hydrolase fold the catalytic serine is located after the sheet β5 and before the following α-helix, the aspartate or glutamate is found after the β7 sheet and the histidine is located in a loop after the β8 sheet (Derewenda et al., 1992b). Recently, a new subclass of esterase/lipase was reported, in which the G-X1-S-X2-G consensus sequence containing the catalytic serine is replaced by a GDSL sequence located closer to the N-terminus (Akoh et al., 2004).

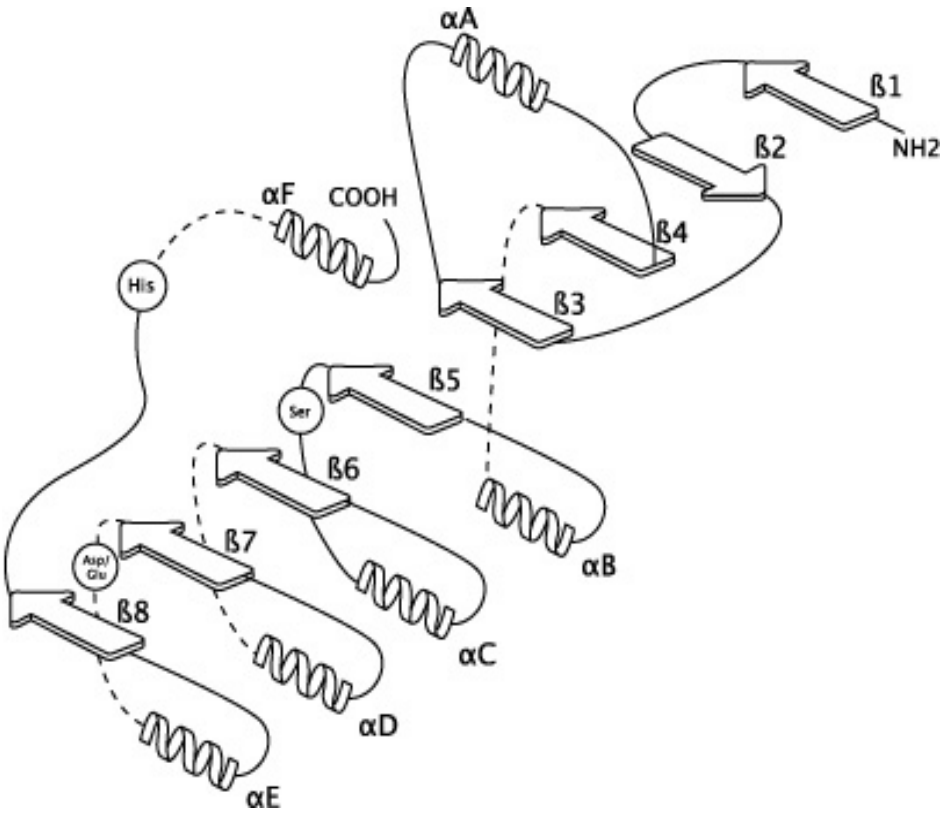


Figure 2. The alpha/beta-hydrolase fold where α- helices are represented by spirals and β strands are indicated by arrows. The active-site residues are shown as circles. Adapted from (Jaeger et al., 1999).

4.3 The oxyanion hole

The tetrahedral intermediate formed during the catalytic mechanism of lipases is stabilized by the presence of hydrogen bonds with two amino acids that form the so-called lipase oxyanion hole. These aminoacids stabilize the intermediate through hydrogen bonds between their backbone amide proton and the oxygen of the substrate carbonyl group (see section 4.6) (Pleiss et al., 2000a).

The first residue of the oxyanion hole is located in the N-terminal part of lipases, in the loop between the strand β3 and the αA helix. Depending on the sequence surrounding this first residue, Pleiss et al. (Pleiss et al., 2000a) identified two types of oxyanion holes: *G**X*** and *GG**G**X*, which are shown in *Figure 3.* The second residue of the oxyanion hole is the X2 residue of the consensus sequence G-X1-S-X2-G, located after strand β5 in the structurally conserved nucleophilic elbow common to all lipases. The oxyanion hole can either be preformed in the closed conformation without the geometrical modification produced during the opening of the lid, or only formed upon the opening of the lid (see section 4.4).

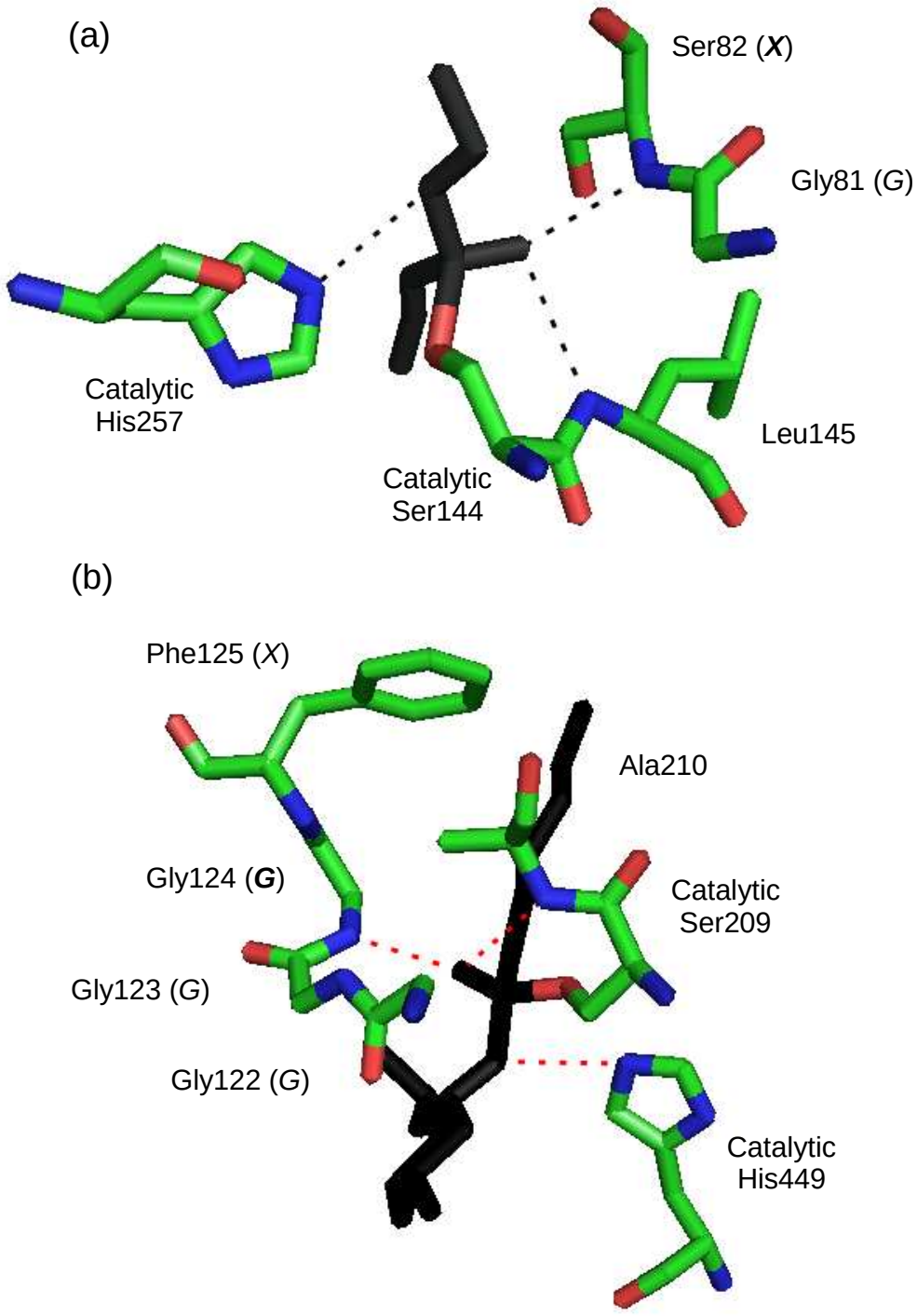


*Figure 3. Two types of oxyanion holes. (a) G**X** type in R. miehei lipase (PDB entry 4TGL): diethylphosphonate stabilized by hydrogen bonds with S82 and Leu145. (b) GG**G**X type in C. rugosa lipase (PDB entry 1LPM): (1R)-menthyl hexyl phosphonate stabilized by hydrogen bonds with G124 and Ala210. Substrate is shown in black and hydrogen bonds are schematized by dotted lines.*

The type of oxyanion hole plays an important role in the specificities of lipases toward their substrates. Indeed, lipases with the *G**X*** type usually hydrolyze substrates with medium and long carbon chain length, while the *GG**G**X* type is found in short length specific lipases and carboxylesterases. Fungal lipases have the oxyanion hole type *G**X,*** where ***X*** is either a serine or threonine, and in most cases they possess a third amino acid, aspartic or asparagine, which also contributes to stabilize the oxyanion hole through a hydrogen bond (Pleiss et al., 2000a).

A third type of oxyanion hole, type *Y*, was identified by Fischer et al. (Fischer et al., 2006). In type *Y* the oxyanion hole is formed by the hydroxyl group of a strictly conserved tyrosine side chain. This type is found in lipase A from *C. antarctica* (family abh38) and few esterases such as cocaine esterases (Pleiss, 2009; Widmann et al., 2010) .

GDSL enzymes do not have the so-called nucleophilic elbow, and their oxyanion hole seems to have a particular structure: the catalytic Ser serves as a proton donor in the oxyanion hole, together with a highly conserved glycine and asparagine. This tri-residue constituted oxyanion hole was proposed to compensate for the lack of hydrogen bond of the intermediate with the catalytic histidine (Akoh et al., 2004).

4.4 Lipases α-helical loop, the lid

The resolution of the first three-dimensional structures of lipases from *Rhizomucor miehei* and human pancreatic lipase (Brady et al., 1990; Winkler et al., 1990) enabled the identification of a lid over the active site. The lid is composed of one or more α helices, joined to the main structure of the enzyme by a flexible structure. It is a mobile element, which uncovers the active site in the presence of a lipid-water interface, generating a conformational change and thus enabling the access of the substrate to the active site (Derewenda and Derewenda, 1991; Grochulski et al., 1993a; Grochulski et al., 1994b; Brzozowski et al., 2000). This mechanism, known as interfacial activation, explains the non Michaelis-Menten behavior observed with most lipases. Indeed, lipase activity increases dramatically when the substrate concentration is high enough to form micelles and emulsions (Fickers et al., 2008; Reis et al., 2009), and thus gives sigmoid curves when the reaction initial rate is plotted against the substrate concentration. When the interface is absent, the entrance to the active site is blocked and the enzyme is inactive. *Figure 4* shows *R. miehei* lipase in its opened and closed conformation. The lid in its closed conformation obstructs the entrance of the substrate, diethyl phosphonate, while the open lid allows

access to the active site (Moore et al., 2001). *Table 1* gives the amino acids that form the lid of the lipases from *B. cepacia*, *C. rugosa*, *R. miehei*, *Rhizopus delemar* and *T. lanuginosus*.

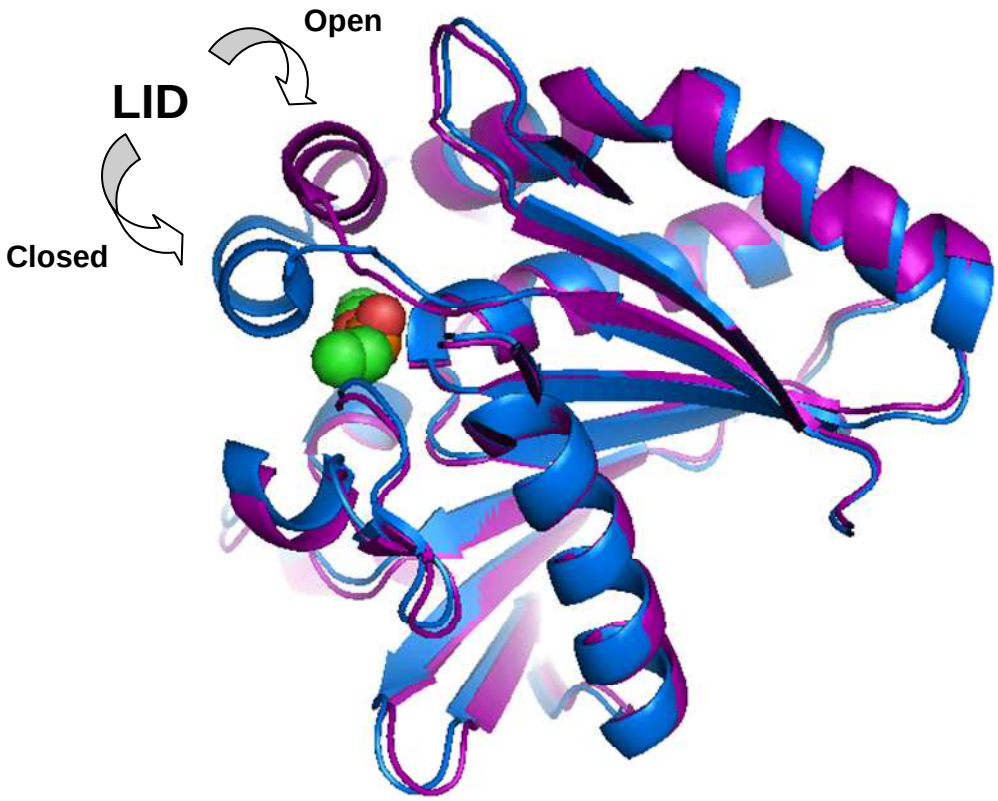


Figure 4. Rhizomucor miehei lipase. In purple its open conformation with diethyl phosphonate, PDB 4TGL, (Derewenda et al., 1992a) and in blue its closed conformation, PDB: 3TGL, (Brzozowski et al., 1992).

4.5 Substrate binding site

The active site of lipases is located in the inside of a pocket on the top of the central β sheet of the protein structure. The surface of the pocket's border mainly consists in hydrophobic residues in order to interact with the hydrophobic substrate. The active sites of lipases differ in their shape, size, deepness of the pocket and physicochemical characteristics of their amino acids (Pleiss et al., 1998). Pleiss et al., 1998 classified lipases in three groups according to the geometry of their binding site (*Figure 5*). The first group has a hydrophobic, crevice-like binding site located near the surface of the protein. Lipases from *Rhizomucor* and *Rhizopus* display such a crevice-like binding site. The second group has a funnel-like binding site. This group includes lipases from *C. antarctica*, *Burkholderia sp.* and *P. cepacia,*

as well as mammalian pancreas. The last group has a tunnel-like binding site and comprises lipases from *C. rugosa*.

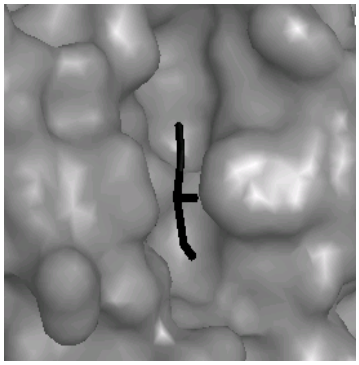
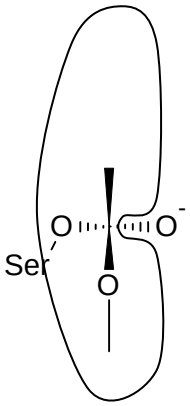
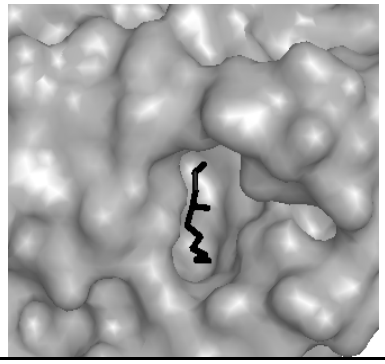
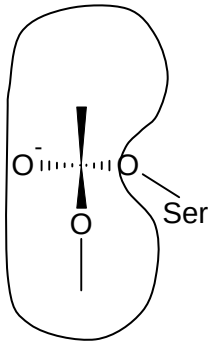
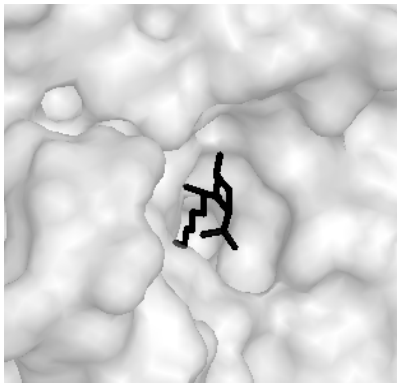
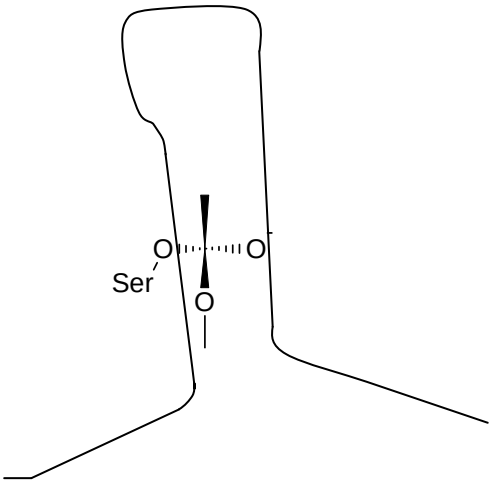
Source	Top View	
***Rhizomucor miehei* lipase**		
***Candida antarctica* Lipase B**		
***Candida rugosa* lipase**		

Figure 5. Shape of the three types of binding site of lipases as identified by (Pleiss et al., 1998).

4.6 Catalytic mechanism

The catalytic mechanism of lipases is shown in *Figure 6*. The mechanism starts by an acylation. This step consists in the transfer of a proton between the aspartate, the histidine and the serine residues of the lipase, causing the activation of the hydroxyl group of the catalytic serine. As a consequence, the hydroxyl residue of the serine, with subsequently increased nucleophilicity, attacks the carbonyl group of the substrate. The first tetrahedral intermediate is formed with a negative charge on the oxygen of the carbonyl group. The oxyanion hole stabilizes the charge distribution and reduces the state energy of the tetrahedral intermediate by forming at least two hydrogen bonds. The deacylation step then takes place, where a nucleophile attacks the enzyme, releasing the product and regenerating the enzyme. This nucleophile can be either water in the case of hydrolysis or an alcohol in the case of alcoholysis.

5. Selectivity

Lipase selectivity is related to its preference to perform given reactions. Three types of selectivity can be distinguished: type-selectivity, regioselectivity, and enantioselectivity. The basis of these types of selectivity is discussed below.

5.1 Type-selectivity

Type-selectivity is associated to the preference for a given substrate, for example tri, di or monoglycerides. For instance, a monoacylglycerol lipase isolated from human erythrocytes was shown to hydrolyze only mono-oleoylglycerol, compared to the corresponding di and tri-glycerides (Sommadelpero et al., 1995). This selectivity also refers to the preference of lipases towards short, medium or long chain fatty acids and to the degree of unsaturation and potential substitutions of the substrate. The preference of a lipase for acyl groups of different sizes is directly influenced by the shape of its binding site (cf. 4.5), and the nature of the amino acids composing this binding site. Indeed, the very homologous *C. rugosa* lipase isoforms differ in chain length specificity due to slight modifications of the amino acids in their tunnel-shaped binding site (Lopez et al., 2004). In addition lipases can show chemo-selectivity which is the specificity of lipases toward a specific chemical group.

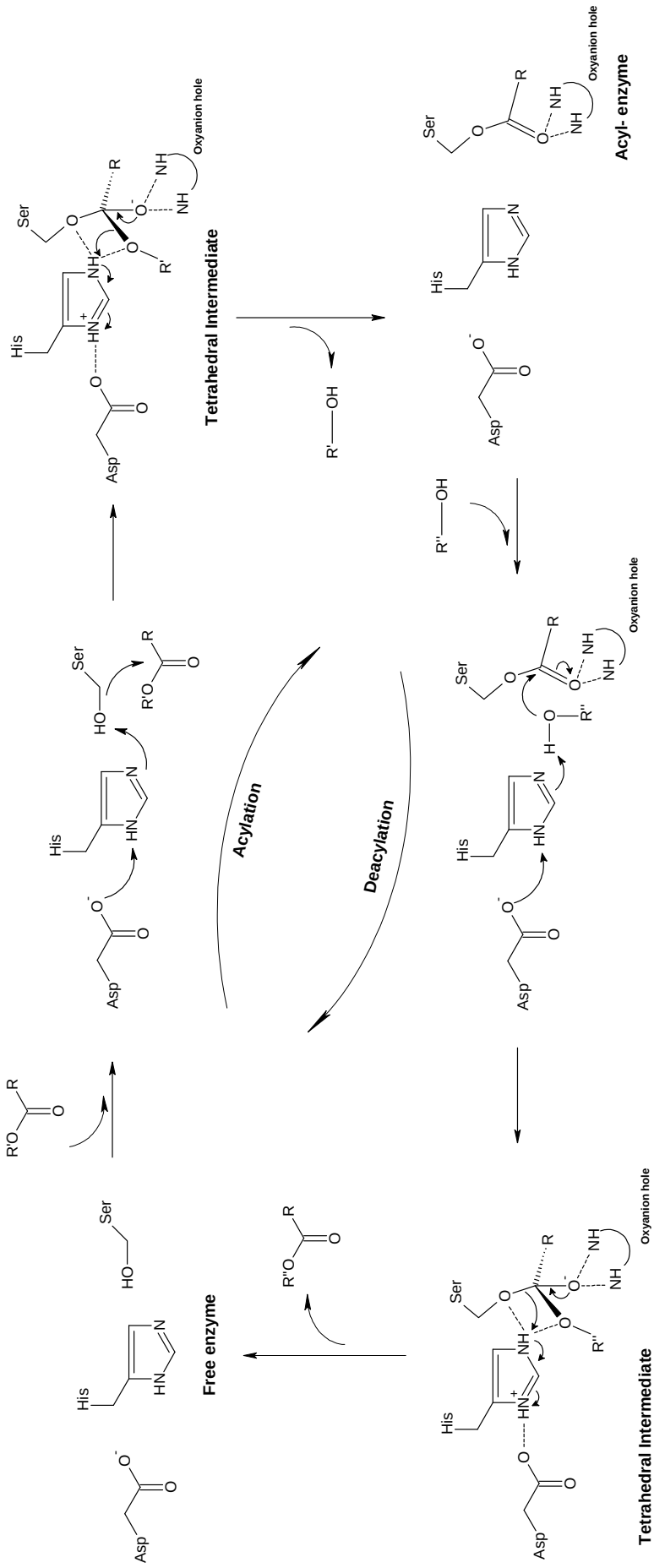


Figure. 6. Catalytic mechanism of lipases.

5.2 Regioselectivity

Regioselectivity is defined as the preferential attack of lipases toward a given ester bond in the glycerol backbone of triglycerides, *i.e.* primary or secondary ester bond. Regioselectivity can be *sn-1(3)*, or *sn-2* (*Figure 7*). Lang et al. crystallized the lipase from *B. cepacia* with triglyceride analogues and could unambiguously detect four binding pockets for the triglycerides (Lang et al., 1998). The binding pockets include the oxyanion hole and three pockets that accommodate the *sn-1*, *sn-2* and *sn-3* fatty acid chains. The size and hydrophobicity of these different pockets will control the regioselectivity of lipases.

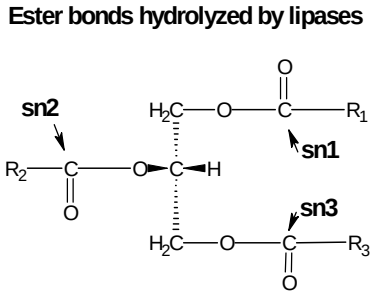


Figure 7. Identification of the ester bonds potentially hydrolyzed by lipases in a triacylglycerol molecule.

Most microbial lipases hydrolyze the *sn-1(3)* positions of triglycerides and only few are capable of hydrolyzing the *sn-2* position. Lipases with *sn-1(3)* specificity are produced by *R. arrizhus, Aspergillus níger, Y. lipolytica, R. miehei, R. delemar* and *T. lanuginosus.* Lipases with *sn-2* specificity are unusual, and include those from *Staphylococcus* (Horchani et al., 2010) and lipase C from *Geotrichum sp* FO401B (Ota et al., 2000). Finally, some lipases are non specific lipases that act at randomly on the triglycerides. Examples of non regio specific lipases are those from *S. aureus* (Vadehra and Harmon, 1967), *S. hyicus* (Vanoort et al., 1989), *Corynebacterium acnes* (Hassing, 1971), *C. viscosum* (Sugiura and Isobe, 1975) and *C. antarctica*.

5.3 Enantioselectivity

A chiral molecule is a molecule with an asymmetric center, which can adopt two enantiomeric forms, *R* and *S*. Enantiomers *R* and *S* are non-superimposable mirror images of each other (*Figure 8*), whose chemical properties, such as melting point, solubility and reactivity, are very similar. However they often have different biological properties. As a matter of fact, a given enantiomer might show therapeutic activity, while the other might be

inactive or even toxic (Soykova Pachnerova, 1963). Enantioselectivity refers to the preference of lipases towards a particular enantiomer of a chiral molecule, in a chemical reaction implying a racemate mixture (mixture of both enantiomers). Enantioselectivity is thus of great interest in the pharmaceutical industry.

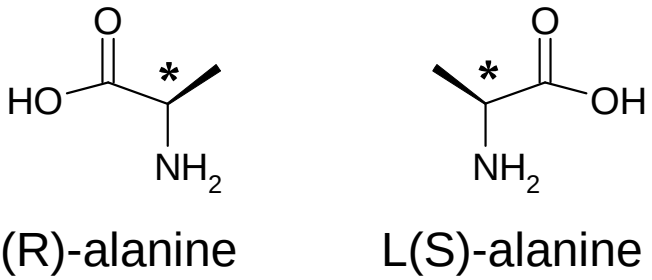


Figure 8. Representation of a chiral alanine in its two possible enantiomeric forms R and S. The chiral center is represented by an asterisk.

An empirical rule based on the relative size of the substituents at the stereocenter was proposed for the reaction of lipases with secondary alcohols (Kazlauskas et al., 1991). This rule was also relevant to predict enantioselectivity of lipases for hydrolysis and transesterification reactions, the substrate being an ester or an alcohol, respectively. Unfortunately, rules predicting the enantioselectivity of lipases towards primary alcohols are far less reliable, even though natural substrates of lipases, *i.e.* esters of long chain fatty acid with glycerol, fall in this category. The X-ray resolution of substrate-enzyme complexes structures, additionally to molecular modelling of the tetrahedral intermediate or docking of the substrate in the active site, helped deepen the comprehension of lipase enantioselectivity (Bordes et al., 2009). By comparing the microbial lipases from *C. antarctica* (lipase B)*, R. oryzae*, *R. miehei* and *C. rugosa* and their interaction with a trioctanoin substrate, Pleiss and collaborators highlighted the importance of both the topology of the binding site (and of a particular His-gap motif implying the catalytic histidine), and the structure of the substrate (flexible/rigid character of the sn-2 substituent) to explain lipase stereopreference (*i.e. sn-1* versus *sn-3* ester bond) (Pleiss et al., 2000b). Besides the interaction in the active site and the nature of the substrate, stereospecificity was also shown to be dependent on physicochemical factors such as temperature and solvent. Finally, it happens that enantioselectivity can be explained neither by structural nor by energetic analysis. By use of a novel *in silico* approach based on efficient path-planning algorithms, Guieysse et al. explained the enantioselectivy of *B. cepacia* lipase towards (*R,S*)-

bromophenylacetic acid ethyl ester by the relative accessibility of the enantiomers to the catalytic center (Guieysse et al., 2008).

6. Applications

Enzymes are key components in a large number of industrial fields. Actually, their worldwide market was estimated in US $4.7 billions in 2008 (CBDM.T®, 2008), and was anticipated to reach US $7 billions in 2013 with an average annual increase of 6.3% per year (Freedonia, 2009). The sole US enzyme demand is expected to reach US $2.8 billion in 2014, with a market distribution of 29% in pharmaceuticals, 18% in biofuels, 14% in food and beverage associated processes, 11% in research and biotechnology and the rest in industrial fields such as animal feed, pulp and paper processes, cosmetics and cleaning products (Freedonia, 2010). In this context, lipases are the third largest group of commercialized enzymes, after proteases and carbohydrases, and represent one billion dollar per year (Hasan et al., 2006).

Lipases are of great importance in the industry due to their stability in organic solvents, their wide variety of substrates, their selectivity and their ability to catalyze reactions without addition of expensive cofactors. Moreover, they are also easily produced and active at ambient conditions.

Therefore, lipases are used in many different industrial areas such as:

- **Food industry**, including production of dairy products, such as cheese, modification of fats and oils (e.g. manufacture of butter and margarine, new cooking oils), production of baby food and structured lipids with unique properties (e.g. cocoa butter equivalent, human milk substitute, high or reduced calorie fats, poly unsaturated fatty acids PUFA enriched oils). They are used as emulsifiers in the improvement of baked products and pasta and as additives in animal feeding (Pignede et al., 2000a; Houde et al., 2004; Aloulou et al., 2007b). Finally, they are also used to modify flavours and produce fragrance compounds.
- **Detergents and cleaning agents**, as additives since they are active and stable at high temperatures and alkaline pH. They are also essential in the production of soap, dish washing products, dry cleaning solvents and contact lens cleaning (Pandey et al., 1999; Hasan et al., 2006).

- **Fine chemicals**. In the pharmaceutical industry for the production of pure enantiomers through resolution of racemic mixtures (e.g. chiral molecules such as prostaglandins, cephalosporines, non-steroid anti-inflammatory drugs, hydantoins and penicillins). Chiral molecules are also used as herbicides in the agrochemical industry (Jaeger and Eggert, 2002). In the perfumes and cosmetic industry, they are employed to produce surfactants and scents, and as emollients in personal care products.
- **Medical applications**, an alternative application of lipases is as diagnostic tools, since their presence and level can indicate an infection or disease, and as new drugs for treatment of digestive aids and high cholesterol levels (Hasan et al., 2006).
- **Pulp and paper industry**, in pitch control, for removal of triglycerides and waxes. Moreover, their presence increases whiteness and reduces the pollution in waste waters.
- **Lipase bioremediation and environmental processes** such as treatment of residual waters rich in oil, degradation of organic debris and sewage treatment from a wide range of industries (Hasan et al., 2006). They are also used to degrade petroleum hydrocarbons in oils spills.
- **Energy industry**, production of lubricants, biodiesel an biokerosene from renewable sources by transesterification of vegetable/animal oils (Jaeger and Eggert, 2002). They are also used to produce additives that decrease the viscosity of biodiesel.
- **Further applications** include production of biopolymers such as polyphenols, polysaccharides and polyesters (Jaeger and Eggert, 2002), lipase-mediated lipophilization, production of biosensors and modification of phenolic acids and antioxidants. Textile industry also uses lipases for enzymatic wash and jeans treatment (Hasan et al., 2006).

Table 2 summarizes some of the applications of lipases.

The application of lipases in industrial processes requires, in most cases, an over expression of the gene of interest in order to obtain larger quantities of the desired lipase. The production method and further purification of the desired enzyme will depend on the quantity and purity needed for a given application. Lipases are mainly produced by submerged fermentation (Sharma et al., 2001), however they can also be produced by solid state fermentation (Chisti and Flickinger, 2009). The purification methods used in the industry should be rapid, efficient, inexpensive and high yielding. Purification methods often involve a first concentration step, also known as pre-purification, by precipitation, ultra-filtration or

organic solvent extraction (Gupta et al., 2004). These techniques generate lipases employed in the cleaning agents industry. However pharmaceutical applications will require highly purified enzymes. Further enzyme purification is mainly achieved by hydrophobic or affinity chromatography, immunopurification, reversed micellar system and membrane processes (Gupta et al., 2004). Some commercial lipases available in the market are listed in *Table 3*.

Table 3. Commercially available lipases, (Jaeger and Reetz, 1998; Houde et al., 2004; Vakhlu and Kour, 2006; Aimee Mireille Alloue et al., 2008).

Type	Source	Form	Producing company
Fungal	*Candida rugosa*	Powder	Atlus Biologics, Amano, Biocatalysts, Boehringer Mannheim, Meito Sangyo, Fluka, Genzyme, Sigma
	Candida antarctica A/B	Immobilized	Boehringer Mannheim, Novo Nordisk, Sigma
	Thermomyces lanuginosus	Immobilized	Novo Nordisk, Boehringer Mannheim
	Rhizomucor miehei	Immobilized/Liquid	Novo Nordisk, Biocatalysts, Amano
	Yarrowia lipolytica	Powder	Amano, Artechno S.A.
	Geotricumcandidum	Liquid	Boehringer Mannheim, Novo Nordisk
Bacterial	*Burkholderia cepacia*	Powder	Amano, Fluka, Boehringer Mannheim
	Pseudomonas alcaligenes	Powder	Gist-Brocades, Genencor International
	Pseudomonas mendocina	Powder	Genencor International
	Chromobacterium viscosum	Liquid	Asahi, Biocatalysts
Animal	Pig pancreatic lipase	Granulated	Solvay pharma

*Table 2. Industrial applications of lipases and some patented processes, *lipases are used in all food industry applications (Pandey et al., 1999; Sharma et al., 2001; Houde et al., 2004; Hasan et al., 2006).*

Industry	Lipase	Action	Product or application	Patent	Reference
Food industry* Dairy	*Aspergillus niger* and *oryzae* *Candida rugosa, utilis* and *antarctica*	Cheese flavoring and ripening Hydrolysis of milk fat Lipolysis and modification of butterfat and cream (Schrag et al., 1997)	Flavoring agents (acetoacetate, beta-keto acids, methyl ketones, flavour esters and lactones) Fragrance agents in cheese, milk and butter	WO 2009068098 US 2004033571 PT 102638 ES 2167205 ES 2149689 US 6162623 US 3973042 WO 2009106575	(Haering et al., 2010b) (Irimescu et al., 2005) (Regalo Da Fonseca et al., 2002) (Martinez Rodriguez et al., 2002) (Aracil Mira et al., 2000) (Grote et al., 2000) (Kosikowski and Jolly, 1976) (Efimova et al., 2009)
Fats and oils	*Penicillium roquefortii* and *camembertii*	Transesterification	Butter substitutes (cocoa butter) Glycerides for butter and margarine SL rich in PUFA Low caloric triglycerols	EP 0191217 KR 20090031740 CA 1318624 US 2006141592 JP 2004283043	(Yamaguchi et al., 1986) (Uehara et al., 2009) (Ergan et al., 1993) (Sumida and K., 2006) (Abe and Arai, 2004)
	Porcine pancreas *Pseudomonas* sp.	Hydrolysis	Concentrate or purified FA Diglycerols for cooking oils	WO 03040091 US 2002197687 CA 1050908	(Chrostensen et al., 2003) (Brunner et al., 2002) (Komatsu, 1979)
Baked products and confectionery	*Rhizomucor miehei* and *javanicus*	Flavor improvement Control non-enzymatic browning Quality improvement	Extend shelf-life Increase loaf volume Improve crumb structure Mayonnaise and dressings Emulsifiers	US 2003180418 CA 1050908 WO 2007096201 RO 121070 EP 1586240	(Rey et al., 2004) (Komatsu, 1979) (Laan Van Der and Schooneveld-Bergmans, 2007) (Teodorescu et al., 2006) (Lejeune-Luquet et al., 2005)
Others	*Rhizopus oryzae* *Thermomyces lanuginosus*	Transesterification Improve aroma, flavour and fermentation Synthesis	Sausage manufacture, ripening Alcoholic beverages (e.g. apple win) Sugar esters		

Industry	Lipase	Action	Product or application	Patent	Reference
Detergents	*Acinetobacter* sp. *A. oryzae* *Candida* sp. *Chromobacterium* sp. *Pseudomonas mendocina* and *alcaligenes* *T. lanuginosus*	Hydrolysis	Fats removal (decomposition of lipids) Soap production Dish washing, dry cleaning solvents, liquid leather cleaner, contact lens cleaning	US 6017866 WO 9708281 WO 9600292 US 5763383 EP 0385401	(Aehle et al., 2000) (Nitsh et al., 1997) (Frenken et al., 1996) (Hashida et al., 1998) (Pierce et al., 1990)
Fine chemicals Pharmaceutics	*Achromobacter* sp. *Alcaligenes* sp. *Arthrobacter* sp. *Aspergillus* sp *Bacillus subtilis* *Burkholderia cepacia* *C. antarctica* and *rugosa* *Chromobacterium viscosum* *Pseudomonas stutzeri, fluorescens* and *cepacia* Pig pancreatic lipase *Rhizopus delemar* *R. miehei* *Saccharomyces cerevisae* *Streptomyces* sp.	Enantioselectivity Synthesis Transesterification Hydrolysis	Resolution of racemic mixtures Building blocks for pharmaceuticals, agrochemicals and pesticides Digestive aids	WO 2006136159 US 2007105201 WO2005092370 US 2006003428 US 2005153404 EP 1061132 US 5645832 US 5380659 WO 9118623 CN 101191137 WO 2007078176 WO 2007035066 ES 2292341 EP 1223223	(Svendsen et al., 2006) (Bertolini et al., 2007) (Shlieout et al., 2005) (Tsai, 2006) (Bosch et al., 2005) (Gatfield and Hilmer, 2000) (Braatz et al., 1997) (Holla and Keller, 1995) (Huge-Jensen, 1994) (Hui et al., 2008) (Hwang and Chung, 2007b) (Hwang and Chung, 2007a) (Ramirez Fajardo et al., 2008) (Gatfield et al., 2002)
Cosmetics and perfumes	*R. miehei* *C. rugosa* and *antarctica* B	Synthesis of additive	Emollient in creams and bath oils Anti obese creams Emulsifiers Moisturizing	DE 102007039736	(Vosmann et al., 2009)
Pulp and paper industry	*C. rugosa* *Pseudomonas* sp.	Hydrolysis	Pitch control Wastepaper deinking Increase paper whiteness Reduce waste water pollution	US 2010269989 WO 2007035481 WO 2006029404 US 2003124710	(Wang et al., 2010) (Wang et al., 2007) (Wang et al., 2006b) (Borch et al., 2003)

Industry	Lipase	Action	Product or application	Patent	Reference
Bioremediation					
Waste treatment	*Acinetobacter calcoaceticus* *C. rugosa*. *Pseudomonas cepacia* and *aeruginosa* *Yarrowia lipolytica* *R. oryzae*	Hydrolysis	Remove fat layer Degreasing (e.g. holding tanks) Degradation of organic debris Clearing of drains Sewage treatment plants Water reconditioning	DE 19834359 EP 1707540 DE 10261349 FR 2846984	(Festet et al., 2000) (Meier and Marquis, 2006) (Sommer, 2004) (Valentin, 2004)
Oil biodegradation	*Acinetobacter* sp. *Mycobacterium* sp. *Rhodococcus* sp.	Biodegradation of petroleum hydrocarbons	Degradation of oil spills (e.g. *n*-alkanes, aromatic hydrocarbons and polycyclic aromatic hydrocarbons)		
Energy	*A. niger* *C. rugosa* and *antarctica* *T. lanuginosus* *R. javanicus* *P. camembertii* and *cepacia* *R. oryzae* and *niveus*	Transesterification	Biodiesel Reduction of biodiesel viscosity Lubricants	CN 1687313 CN 101381614 CN 101260417 CN 101250424 CN 101240201 JP 2006272326	(Liu et al., 2005) (Wei et al., 2009) (Wei et al., 2008) (Wei and Dehua, 2008) (Jiaxin et al., 2008) (Sato et al., 2006)
Others					
Polymers		Synthesis	Biodegradable polyesters Aromatic polyesters Lubricants	US 2010048927	(Haering et al., 2010a)
Textiles		Removal of lubricants Stone and enzymatic washing Bio polishing	Jeans, yarns, fabrics, rugs		
Medical application	*A. niger* *C. rugosa* *Galleria mellonella* *Serratia marcescens*	Marked enzymes Drug targets Sources of drugs	Diagnostic tools Treatment of gastrointestinal disturbances Treatment malignal tumors Cholesterols lowering drug	WO 2004018660 US 5075231 US 2010216212 WO 2008079685 CN 101518646	(Albang et al., 2004) (Moreu et al., 1991) (Morita et al., 2010) (Svendsen et al., 2010) (Qinghui and Jianying, 2009)
Leather degreasing	*Rhizopus nodosus* *C. rugosa*	Hydrolysis of fats	Waster water treatment Water proof leather		

Part II: Lip2 from *Yarrowia lipolytica*

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Lip2 from *Yarrowia lipolytica*

The non-conventional yeast of *Yarrowia lipolytica* is considered as a potential host for the production of proteins due to its high secretion levels (Guieysse et al., 2004; Fickers et al., 2011). This yeast produces one extracellular lipase (Lip2) and two cell bound lipases (Lip7 and Lip8) (Pignede et al., 2000a), which have been characterized (Pignede et al., 2000a; Fickers et al., 2005b). The sequence analysis of the lipases from *Y. lipolytica* showed 76% identity between Lip7 and Lip8, 33.9% between Lip2 and Lip7 and 35.2% between Lip2 and Lip8 (Fickers et al., 2005b). Lip2 is responsible for all the extracellular lipase activity of *Y. lipolytica* (Pignede et al., 2000a) and is easily adaptable for production and secretion of the lipase. Lip2 has been studied and characterized by several research teams (Pignede et al., 2000a; Aloulou et al., 2007b; Yu et al., 2007a; Yu et al., 2007b; Bordes et al., 2010).

1. Characteristics

The mature Lip2 from *Y. lipolytica* is a glycosylated protein of 38 kDa and 301 amino acids encoded by the Lip2 gene (Pignede et al., 2000a; Yu et al., 2007b). The Lip2 gene encodes 334 amino acids, from which the first 33 represent a signal peptide or *prepro* (Yu et al., 2007a). The *prepro* has 13 amino acids, followed by four X-Ala or X-Pro dipeptides (substrates of diamino peptidases), a 12 amino acids pro region and a Lys-Arg dipeptide (substrate of the endopeptidase encoded by the gene XPR6 in *Y. lipolytica*) (Pignede et al., 2000a). Four different isoforms of Lip2, with different glycosylation patterns and molecular weights between 36.874 and 38.485 kDa, have been identified (Aloulou et al., 2007b).

1.1 Structure

Lip2 from *Y. lipolytica* has homology with the fungal lipases from *R. miehei*, PDB: 3TGL (Brzozowski et al., 1992) and 4TGL (Derewenda et al., 1992a), sequence identity 29%, sequence homology 46 %, gap 16 %; *R. niveus,* PDB: 1LGY (Kohno et al., 1996), sequence identity 33%, sequence homology 47 %, gap 17% and *T. lanuginosus*, PDB: 1GT6 (Yapoudjian et al., 2002), sequence identity 31 %, sequence homology 47%, gap 14% (Bordes et al., 2009).

Lip2 is an extracellular lipase, which is a type of protein that in general have several disulphide bonds which are crucial for their structure and stabilization of their tertiary structure and essential for protein structure and function (Bordes et al., 2009; Bordes et al., 2010). The crystal structure of Lip2 (1.7 Å resolution) (*Figure 9*) shows that this lipase has a

typical α/β-hydrolase fold and four disulfide bridges (Cys30-Cys299, Cys43-Cys47, Cys120-Cys123, Cys265-Cys273), having only one free Cys residue (Cys244) (Bordes et al., 2010). The crystal structure also showed confirmed the two glycosylation sites at N113 and N134 (Jolivet et al., 2007; Bordes et al., 2010).

The catalytic triad of Lip2 is formed by the Ser162, located in the nucleophilic elbow after the β5 sheet, the ASP230 and the His289 found after the β7 and β8 sheets, respectively. Lip2 catalytic Ser shows a typical GxSxG lipase signature which in this case is GHSLG. The residues involved in the oxyanion hole are the Leu163, positioned next to the catalytic Ser162, and a rather hydrophilic residue the Thr88, located in a loop after the β3 sheet next to a Gly residue (Bordes et al., 2009). This oxyanion hole is of the *G**X*** type which usually hydrolyzes substrates with medium and long carbon chain length (Pleiss et al., 2000b). Asp 97 is the anchor residue, which interacts through a hydrogen bond with the side chain of the hydrophilic residue of the oxyanion hole in the open form of the lipase.

As explained in the previous section another important structural element of lipases is the lid. The lid is a mobile element composed of one or more α helices, which uncovers the active site in the presence of a lipid-water interface, generating a conformational change that enables the access of the substrate to the active site. From the homology analysis with *R. miehei*, *R. niveus* and *T. lanuginosus* in Lip2 the lid is formed by the residues between Thr88 and Leu105 (Bordes et al., 2009).

1.2 Catalytic properties

Optimal temperature and pH for Lip2 have been studied by several authors (Pignede et al., 2000a; Aloulou et al., 2007b; Yu et al., 2007a; Yu et al., 2007b). Lip2 from *Y. lipolytica* is active in a pH range of 4 to 8, having its optimal pH between 6 and 8.0, depending on the substrates and experimental conditions (Yu et al., 2007b; Fickers et al., 2011). This enzyme is stable between pH of 3.5 to 9.0 (Fickers et al., 2011) but it suffers irreversible inactivation at pH of 3.0 and 8.5 (Aloulou et al., 2007b).

Lip2 is active at low temperatures (5°C) and is rapidly deactivated over 50°C, with an optimal temperature between 30°C and 40°C (Destain et al., 1997; Aloulou et al., 2007b; Yu et al., 2007b). Immobilized Lip2 has been used in bulk polymerization at high temperatures, up to 150°C (Sandoval et al., 2010). A Lip2 variant with improved thermostability was recently isolated by error prone PCR (Bordes et al., 2011). In this variant the free cysteine 244 was

replaced by an alanine and had a half life time 127 fold higher at 60°C when compared with the wild type (1.5 min to 3h).

Lip2 activity is also affected by the presence of solvents and metal ions. Lip2 preserved 90% of its activity after contact for 30 min at 30°C with 10% acetone, methanol, ethanol, isopropanol and DMSO (Yu et al., 2007b). However, it was deactivated in the presence of acetonitrile in the same conditions. After exposure with 20% organic solvent no activity was detected in acetone, ethanol and isopropanol. Nevertheless, it conserved 60% of its activity in methanol and 95% in DMSO. Lip2 activity increases in the presence of Ca^{2+} and Mg^{2+} and is inhibited by Zn^{2+}, Ni^{2+} and Cu^{2+} (Yu et al., 2007b).

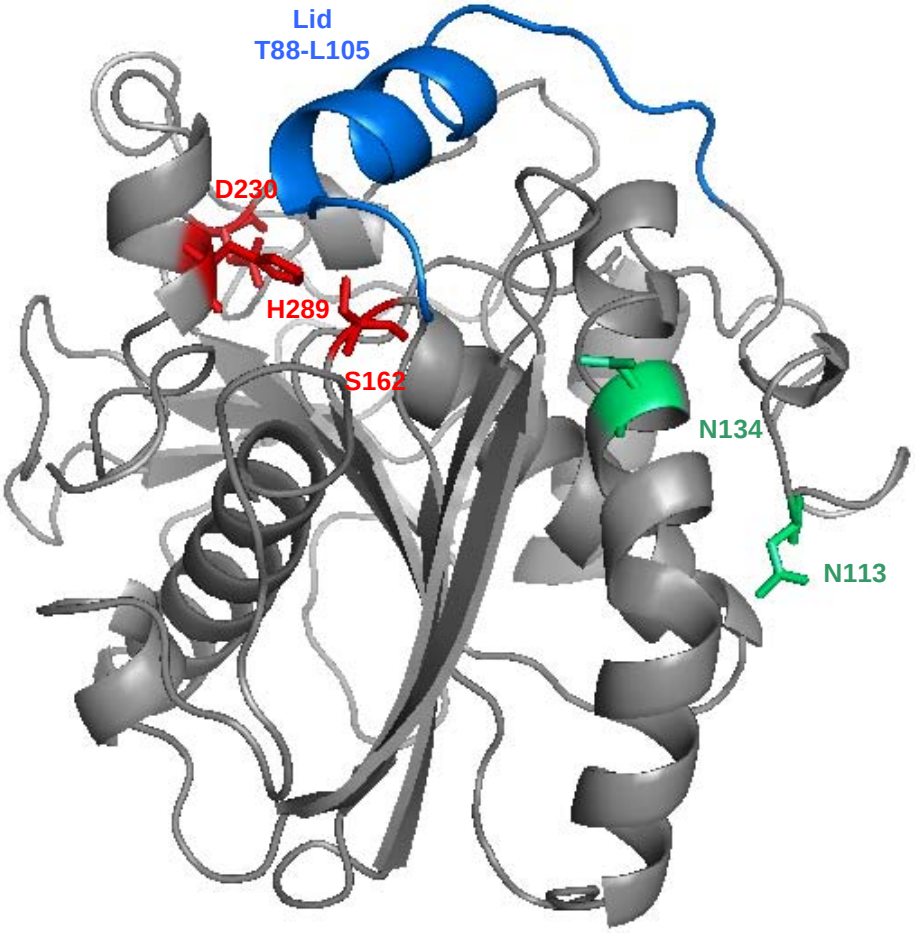

Figure 9. Ribbon representation of the structure of Lip2 from Y. lipolytica, PDB 3O0D (Bordes et al., 2010). The lid (T88–L105) is shown in blue, the catalytic triad (S162, D230, and H289) in red sticks and the glycosylation residues in green sticks (N113 and N134).

1.3 Substrate specificity

The substrate specificity of Lip2 was studied for different triglycerides and fatty acid methyl esters with different chain length. Lip2 shows higher activity toward triglycerides than hydrophilic esters, such as fatty acids methyl esters (Yu et al., 2007b). For triglycerides, Lip2 was found highly active versus tricaprylin, olive oil and triolein (Aloulou et al., 2007b; Yu et al., 2007a). For fatty acid methyl esters, Lip2 showed activity towards C12-C16 methyl esters, with a higher preference towards methyl myristate (Yu et al., 2007b). Lip2 stereoselectivity was studied following the hydrolysis of triolein and the release of partial glycerides (Aloulou et al., 2007b). Monoglycerides concentration continuously increased until 66% hydrolysis, phase at which triglycerides and diglycerides had almost disappeared. This profile is characteristic of *sn-1,3* lipases since they produce 2-monoglycerides and can not hydrolyse the ester bond at the *sn-2* position. Lip2 stereoselectivity toward chemically alike but sterically non equivalent ester groups, showed a slight stereopreference for the hydrolysis of the ester bond at the *sn-3* position compared to the *sn-1* position of the triglyceride (Aloulou et al., 2007b). However, the apparent stereopreference changed according to the hydrolysis degree and the diglycerides excess slightly reversed after 25% of lipolysis.

2. Cloning and production

Overexpression of Lip2 has been studied due the wide range of applications this lipase could have. The Lip2 gene was cloned and expressed in *Y. lipolytica* using the JMP3 integrative multi-copy vector under the control of the POX2 promoter (Pignede et al., 2000b; Nicaud et al., 2002). The POX2 promoter allows high lipase production and is inducible by oleic acid or methyl oleate (Fickers et al., 2005a). The multi-copy strain JMY184 produced up to 1500 U/ml in a flask culture, while the lipase produced by the wild type had an activity of 50 U/ml (Pignede et al., 2000a; Pignede et al., 2000b). Another strategy was the chemical mutagenesis, using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, to isolated overproducing mutants from the *Y. lipolytica* strain CBS6303 (Destain et al., 1997; Fickers et al., 2003). The second generation mutant LgX64.81 had a lipase activity over 1100 U/ml (Destain et al., 1997). The LgX64.81 mutant was later improved by amplification of the Lip2 gene, producing the JMY1105 strain (Fickers et al., 2005c). This strain produced a lipase with an activity of 26450 U/ml in a batch culture, using olive oil and tryptone as carbon and nitrogen sources. Feeding tryptone and olive oil at the end of the exponential growth phase led to a lipase production of 158246 U/ml after 80h in a 20 liters fed batch fermentor.

Lip2 production, from a non-genetically modified strain, is of interest from a biotechnological point of view and has been optimized in batch and fed batch bioreactors. The overproducing mutant LgX64.81, grown in a 20 litres fermentor, showed that feeding with a complete medium grave a two fold increase in lipase production (2000 U/ml) while glucose and olive oil addition gave a three fold increase (Fickers et al., 2009). This same mutant, LgX64.81, produced 2145 U/ml in a 32h batch culture with a medium supplemented with 10 g/l of tryptone (Turki et al., 2010). Production was optimized by a stepwise feeding strategy, with methyl oleate and tryptone, and by decoupling cell growth and lipase production phases, leading to a production of 10000 U/ml after 80h. Lip2 has also been successfully produced in a large sale fermentor (2000lt), using a mixture of whey powder, corn steep liquor, glucose and olive oil as medium (Fickers et al., 2006). After 53h fermentation an activity of 1100 U/ml was obtained.

Fed-batch production in a mineral medium was also attempted (Leblond et al., 2009). The synthetic medium provided sources for *Y. lipolytica* growth and protein expression using oleic acid as inducer of the promoter POX2. Growth phase was carried out using glucose as sole carbon source, reaching 60g/L of biomass after 15h at 28°C and pH 6. For the protein expression phase the carbon source was switched to oleic acid in a fed batch mode. After other 50h fermentation the biomass reached a concentration of 100g/L with 60000 U/ml of lipase activity in triolein and 380 U/ml of *p*-NPB activity (*Figure 10*).

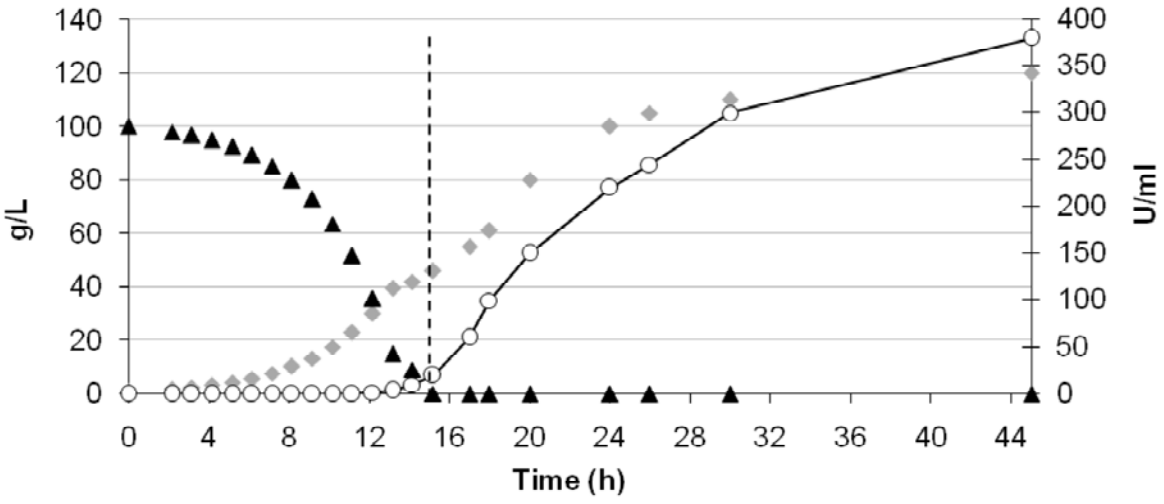

Figure 10. Production of Lip2 from Y. lipolytica in mineral medium. Batch system for biomass production using glucose as carbon source; fed-batch for protein expression using oleic acid as carbon source, indicated with a dash line. ▲ Glucose concentration (g/L) ◆ Biomass (g/L) ○ p-NPB activity (U/ml).

The Lip2 gene has also been heterologously produced in *Pichia pastoris* (Aloulou et al., 2007a; Yu et al., 2007a). Lip2 was expressed in the methylotrophic yeast *P. pastoris* X-33 and secreted using the secretion signal peptide (α-factor) from *S. cerevisiae* and under the control of the methanol inducible promoter AOX1 (Yu et al., 2007a). After fed batch culture, a lipase activity of 12500 U/ml was reached.

2.1 Improving Lip2 using Y. lipolytica as an expression system

Y. lipolytica is a good host for gene expression and secretion; it has been successfully used to produce plasmids and proteins from several organisms (Nicaud et al., 2002; Madzak et al., 2004). This system shows high secretion and is efficient in post-translational modifications (Barth and Gaillardin, 1996). In addition, strains deleted for extracellular proteases and lipases allow high protein purity in the supernatant (Pignede et al., 2000a; Nicaud et al., 2002; Fickers et al., 2005b).

Y. lipolytica was used to develop a high-throughput screening protocol using Lip2 as expressed enzyme (Bordes et al., 2007; Cambon et al., 2010) and was later used for directed evolution of this enzyme (Bordes et al., 2009). The Lip2 gene was carried by the expression cassette contained in plasmid JMP8 (*Figure 11*), flanked by two zeta regions and composed of URA3 marker (ura3d1), POX2 promoter and Lip2 gene. The expression cassette (*Figure 12A*) can be recovered by NotI digestion and used directly in *Y. lipolytica* transformation. This research resulted in the construction of the stain JMY1212, which contains a zeta docking platform that allows integrations at a specific site avoiding the random insertion observed in strain JMY1165 (*Figure 12B -12C*). With strain JMY1165 a coefficient variance of 36.3% for the full process (transformation, picking, expression and activity test) was obtained. The new strain JMY1212 allows high transformation frequency and lower coefficient variance of 18.9% for transformation, growth and expression of protein (Bordes et al., 2007).

Strain JMY1212 is the first expression system that allows direct comparison of activities between the enzymes or variants directly from the supernatants (Cambon et al., 2010). The zeta docking platform forced the integration of the expression cassette at this locus. Analysis of 102 transformants expressing Lip2 from *Y. lipolytica* showed that only one transformant had abnormal activity (57% increase) (*Figure 13*). The other variants had a normal distribution, with a coefficient variation of 9.1% where the interval mean ± two standard deviations represents 95% of the transformants. This strain was used to compare the activities of Lip2 mono mutants library in position 232, crucial for enantiomer discrimination.

The 95% confidence intervals around the mean enabled variant activities to be statistically compared. The high reproducibility in the expression levels avoids protein purification and quantification steps for false positives and avoids real positives from being discarded.

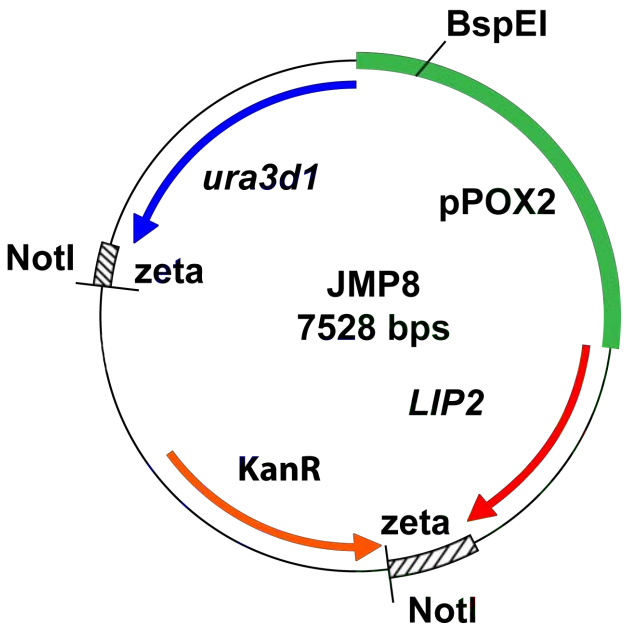

Figure 11. Schematic diagram of JMP8 expression vector. Vector contains the ura3d1 marker for selection of Ura+ transformants in Y. lipolytica, the kanamycin gene (KanR) for selection in E. coli and Lip2 gene expressed under the control of the POX2 promoter.

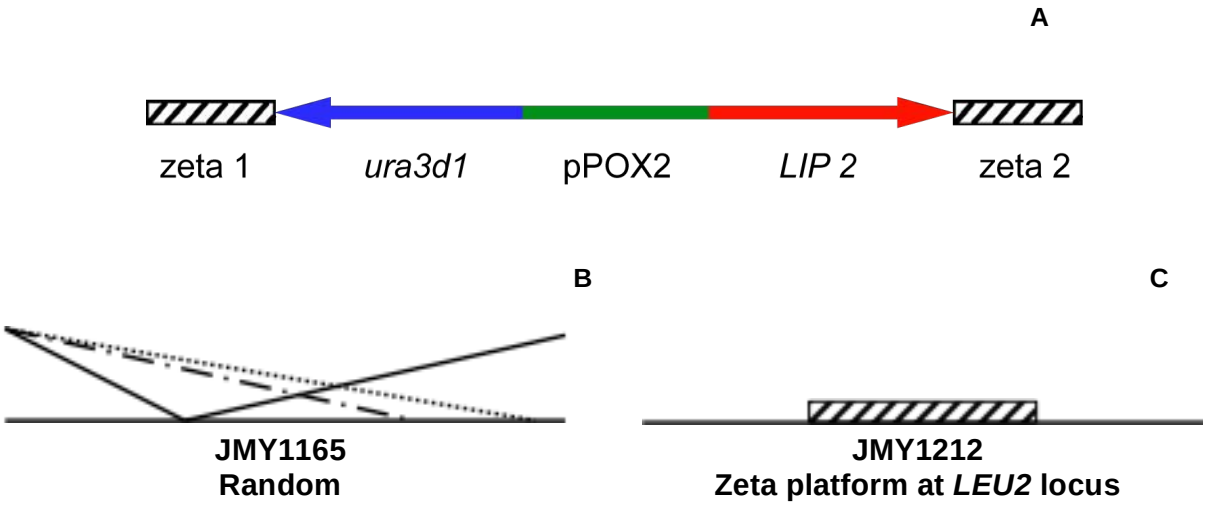

Figure 12. (A) Expression cassette flanked by the zeta region, liberated from the plasmid upon NotI digestion (Pignede et al., 2000b). Expression cassette (B) Random insertion in strain JMY1165 (C) Unique and targeted integration at the zeta platform in strain JMY1212.

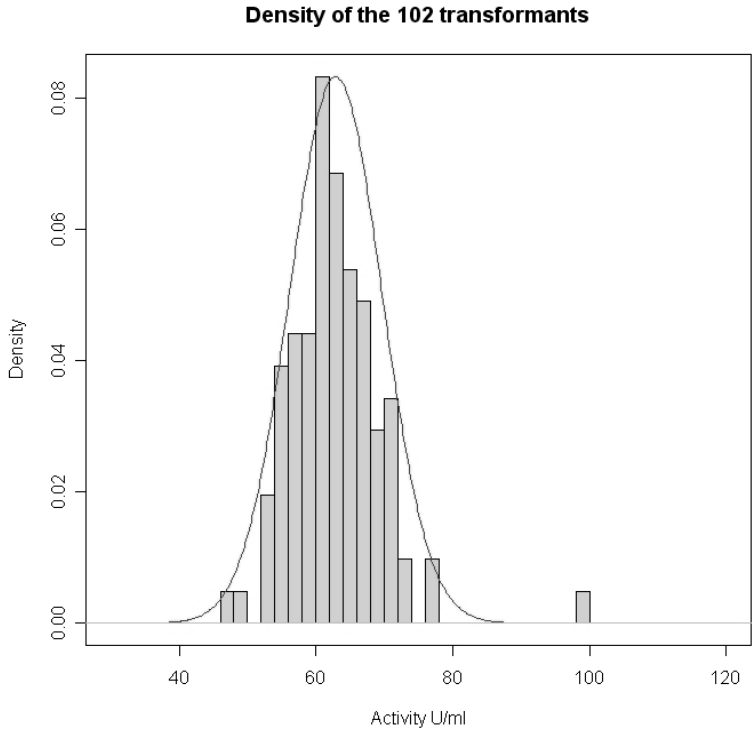

Figure 13. Comparison of the experimental activity distribution (represented as a histogram) with the theoretical normal distribution with a mean of 62.9 U/ml and a standard deviation of 6.7 U/ml (represented as a line) for 102 transformants.

Using this platform the enantioselectivity of Lip2, for the resolution of 2-bromo-arylacetic acid esters, an important class of chemical intermediates in the pharmaceutical industry, was improved (Bordes et al., 2009). Using site directed mutagenesis to the substrate binding site the enantioselectivity was modified. Five amino acid residues (T88, V94, V97, V232 and V2385) from the active site and in direct contact with the substrate were selected, since they could potentially be involved in the enzyme selectivity. Position V232 was found essential for the discrimination of enantiomers, variants V232A had enhanced enantioselectivity and variant V232L had selectivity inversion. Position V232 was saturated and the screening of this library identified variant V232S with a highly increased E value (>200) and an eight-fold increase in activity.

Thermostability of Lip2 has also been improved using the high efficient *Y. lipolytica* expression system (Bordes et al., 2011). This lipase has low thermostability at temperatures higher than 40°C, however it is a promising candidates for many industrial applications. Using error prone PCR, the screening of the library identified a thermostable variant where the free cysteine 244 mutated for an alanine. Saturation of position 244 showed that thermal denaturation is caused by the presence of a cysteine in this position.

3. Applications

Some of the most important applications of Lip2 are summarized in *Table 4*. This lipase can be use in several areas such as bioremediation, fine chemistry, food and pharmaceutical industries. In addition, due to its homology to the lipases from *R. miehei* and *T. lanuginosus* it could be used for the purification of polyunsaturated fatty acids of the Omega-3 family, such as docosahexaenoic and eicosapentaenoic acid, and for the production of structural lipids (1,3 specificity), which are two of the objectives of this research work.

Table 4. Industrial applications of Lip2 from Yarrowia lipolytica.

Industry	Product or application	Reference
Bioremediation	Treatment for olive mill and oil industry waste waters.	(Lanciotti et al., 2005) (Scioli and Vollaro, 1997) (Wu et al., 2009)
	Treatment of palm oil mill effluent.	(Oswal et al., 2002)
	Treatment of seafood wastes.	(Yano et al., 2008)
	Bioremediation of crude oil contamination.	(Zinjarde and Pant, 2002)
Fine chemistry	Polyester synthesis: ring-opening polymerization reaction of ε-caprolactone.	(Barrera-Rivera et al., 2008)
	Optically pure amines: resolving agents, chiral adjuvant, and chiral synthetic building blocks, e.g. (±)α-phenylethyl amine.	(Wen et al., 2008)
	Production secondary metabolites: ciric ans isocitric, γ and δ lactones, and dicarboxylic acids.	(Thevenieau et al., 2009)
Food industry	Synthesis of MAG. Maturation of cheeses. Dry fermented sausages.	(Esmelindro et al., 2008) (Suzzi et al., 2001) (Wyder et al., 1999) (Gardini et al., 2001)
Pharmaceutical industry	Substitution therapy for exocrine pancreatic insufficiency.	(Leblond and Mouz, 2007)
	Resolution of racemic mixtures: e.g. 2-halogeno-carboxylic acids, intermediates in the synthesis pathways of drugs and ibuprofen.	(Guieysse et al., 2004) (Cancino et al., 2008) (Bordes et al., 2009) (Liu et al., 2009)

Part III: Omega-3 polyunsaturated fatty acids

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Omega-3 polyunsaturated fatty acids

1. Fatty acids

Oils and fats are derived from fatty acids and are used to store energy. They represent the most important and abundant compounds in foods (Badui et al., 1993). Fatty acids (FA) are carboxylic acids with hydrocarbon tails of 4 to 36 carbons and with a terminal carboxyl group (-COOH). The fatty acids can be saturated or unsaturated. Saturated fatty acids have between four and twenty four carbons (*Table 5*) and their basic formula is:

$$CH_3\text{-}(CH_2)_n\text{-}COOH$$

Some examples are the butyric acid, found in milk fat and the lauric acid, which abounds in coconut and palmitic oils. The foods rich in saturated fats are milk and meat, and their derivatives.

Unsaturated fatty acids have double bonds in the chain, between one and six, and they can be monounsaturated or polyunsaturated. The instaurations can have *cis* or *trans* configuration. Unsaturated fatty acids produced naturally have a *cis* configuration in the double bonds. However *trans* fatty acids are found in dairy products and meats, as well as in vegetal oils as a consequence of the hydrogenation process. A high consumption of *trans* fatty acids increases the levels of low density lipoproteins (LDL) and reduces the concentration of high density lipoproteins (HDL) (Lehninger et al., 2005). Lipoproteins transport triacylglycerols and cholesterol esters through the blood. There are four main groups, the chylomicrons, which are the biggest and with lowest density, the very low density lipoproteins (VLDL), the low density lipoproteins (LDL) and the high density lipoproteins (HDL). Each one has different amounts of triacylglycerols, cholesterol, phospholipids and proteins (Gunstone et al., 1994 ; Lehninger et al., 2005).

Unsaturated fatty acids are found in vegetal and fish oils (*Table 6*). The oleic acid is found in all vegetal oils, mainly in olive oil. The polyunsaturated fatty acids (PUFA) of the Omega-6 family, which is the linoleic acid, are found in corn, safflower, soy bean and sunflower oils (Badui et al., 1993). Fish is rich in polyunsaturated fatty acids of the Omega-3 family. The two fatty acids that represent the largest proportion in fish are the eicosapentaenoic, EPA, and docosahexaenoic, DHA (Botanical-Online, 2011).

Table 5. Saturated fatty acids.

Common name	Scientific name	Abbreviation	Formula	Sources
Butyric acid	Butanoic acid	C 4:0	$CH_3(CH_2)_2COOH$	Ruminant milk
Caproic acid	Hexanoic acid	C 6:0	$CH_3(CH_2)_4COOH$	Ruminant milk
Caprylic acid	Octanoic acid	C 8:0	$CH_3(CH_2)_6COOH$	Ruminant milk and coconut oil
Capric acid	Decanoic acid	C 10:0	$CH_3(CH_2)_8COOH$	Ruminant milk and coconut oil
Lauric acid	Dodecanoic acid	C 12:0	$CH_3(CH_2)_{10}COOH$	Coconut oil and palm oil
Myristic acid	Tetradecanoic acid	C 14:0	$CH_3(CH_2)_{12}COOH$	Coconut, palm and other vegetable oils
Pentadecanoic acid	Pentadecanoic acid	C 15:0	$CH_3(CH_2)_{13}COOH$	Uncommon in all tissues
Palmitic acid	Hexadecanoic acid	C 16:0	$CH_3(CH_2)_{14}COOH$	Common in all fats
Stearic acid	Octadecanoic acid	C 18:0	$CH_3(CH_2)_{16}COOH$	Animal fats and cacao
Arachidic acid	Eicosanoic acid	C 20:0	$CH_3(CH_2)_{18}COOH$	Pig lard and peanut oil
Behenic acid	Docosanoic acid	C 22:0	$CH_3(CH_2)_{20}COOH$	Rare in all tissues
Lignoceric acid	Tetracosanoic acid	C 24:0	$CH_3(CH_2)_{22}COOH$	Rare in all tissues

The smallest lipids that fatty acids can form are the triacylglycerols (TAG). Triacylglycerols consist of three fatty acids, each one with an ester bond to a glycerol molecule (*Figure 14*). The glycerol has each one of the three hydroxyl groups esterified to the fatty acids, which can be saturated or unsaturated. Triacylglycerols can have the same or different fatty acids in the three positions. These compounds are essential for the formation of more complex lipids which are stored as fats and oils and lipases are required in order to hydrolyze them and release the fatty acids (Lehninger et al., 2005).

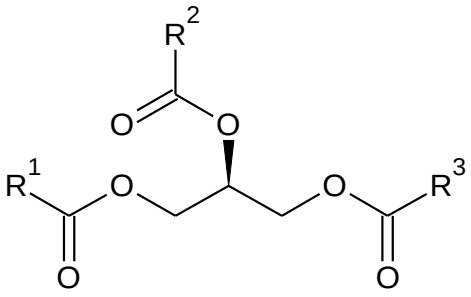


Figure 14. Triacylglycerol

Lipids represent a wide variety of chemical compounds which are mainly characterized by their insolubility in water. However they are soluble in certain organic solvents such as chloroform, hexane, benzene and ethanol (Spiller, 1996). They have several biological functions that can be classified in four major groups: storage, structural, signalling and transport. Most of the organisms use them to store energy as oils and fats since they release more energy than sugars during their oxidation process. Mammals accumulate lipids as fats in several adipose tissue sites while fishes accumulate them as oils. In plants, lipids are stored as protective oils with characteristic scents and flavors. One of their most important structural functions is their presence as phospholipids and sterols in the biological membrane. Lipids form a double film in the membranes, known as bilipid layer, which acts as a barrier to polar molecules and ions. There are five general types of membranes: glycerophospholipids, galactolipids, sulfolipids, sphingolipids and sterols (Lehninger et al., 2005).

Other functions include acting as enzymes co-factors, electron carriers, chaperons, emulsifiers, hormones and intracellular messengers. These lipids have an active role in the metabolic traffic as metabolites and messengers. They can act as signals, hormones that travel in the blood from one tissue to another or as intracellular messengers that generate an external response, since they can act like binding sites on the membrane. Another function is as eicosanoids, derivates from the fatty acids involved, among others, in the reproductive functions and the blood pressure regulation. Three types of eicosanoids are present prostaglandins, thromboxanes and leukotrienes. The prostaglandins affect the blood flow in specific organs and their response affect hormones like epinephrine and glucagon. The thromboxanes are involved in the production of clots and the leukotrienes are strong biological signs. In electron transference reactions they operate as enzymatic cofactors. Lipids can also act as molecules capable of visible light absorption (Lehninger et al., 2005). Other functions include thermal isolation and they provide mechanical protection.

Table 6. Unsaturated fatty acids.

Common name	Scientific name	Abbreviation	Formula	Sources
Caproleic acid	9-decenoic acid	C10:1 n-1	$C_9H_{17}COOH$	Ruminant milk
Lauroleic acid	2-dodecenoic acid	C12:1 n-3	$C_{11}H_{21}COOH$	Cow milk
Myristoleic acid	9-tetradecenoic acid	C14:1 n-5	$C_{13}H_{25}COOH$	Fish oils
Palmitoleic acid	9-hexadecenoic acid	C16:1 n-7	$C_{15}H_{29}COOH$	Macadamia nut and fish oils
Oleic acid	9-octadenoic acid	C18:1 n-9	$C_{17}H_{33}COOH$	Vegetable oils
Vaccenic acid	11-octadecenoic acid	C18:1 n-7	$C_{17}H_{33}COOH$	Ruminant fat
Gadoleic acid	9-eicosenoic acid	C20:1 n-11	$C_{19}H_{37}COOH$	Fish oils
Cetoleic acid	11-docosenoic acid	C22:1 n-11	$C_{21}H_{41}COOH$	Fish oils
Erucic acid	13-docosenoic acid	C22:1 n-9	$C_{21}H_{41}COOH$	Colza oil
Linoleic acid	9,12-octadecadienoic acid	C18:2 n-6	$C_{17}H_{31}COOH$	Vegetable oils
Linolenic acid	9,12,15-octadecatrienoic acid	C18: 3 n-3	$C_{17}H_{29}COOH$	Soy bean and other vegetal oils
Gamma linolenic acid	6,9,12-octadecatrienoic acid	C18:3 n-6	$C_{17}H_{29}COOH$	Onagra and borage oil
Stearidonic acid	6,9,12,15-octadecatetraenoic acid	C18:4 n-3	$C_{17}H_{27}COOH$	Fish oils and onagra and borage seeds
Araquidonic acid	5,8,11,14-eicosatetraenoic acid	C20:4 n-6	$C_{19}H_{31}COOH$	Fish oils
Eicosapentaenoic acid	5,8,11,14,17-eicosapentaenoic acid	C20:5 n-3	$C_{19}H_{29}COOH$	Fish oils
Docosapentaenoic acid	7,10,13,16,19-docosapentaenoic acid	C22:5 n-3	$C_{21}H_{33}COOH$	Fish oils
Docosahexaenoic acid	4,7,10,13,16,19-docosahexaenoic acid	C22:6 n-3	$C_{21}H_{31}COOH$	Fish oils

1.1 Essential fatty acids

The essential fatty acids (EFAs) are those indispensable for human health but can not be synthesized in the organism. The essential fatty acids are the linoleic acid, Omega -6 and the linolenic acid, Omega-3 (University of Maryland Medical Center, 2011). Both of them have important roles in brain functions, as well as in growth, normal development of the organism and synthesis of prostaglandins. In general they stimulate skin and hair growth, they regulate the metabolism, maintain bone health and preserve reproductive capability (Botanical-Online, 2011; University of Maryland Medical Center, 2011).

1.1.1 Omega-6

The most important fatty acid in the Omega-6 family is the gamma linolenic acid (GLA) which is a polyunsaturated fatty acid (*Figure 11*). In processed foods the linolenic acid is saturated with hydrogen in order to increase its stability, unfortunately this process produces *trans* fatty acids. The seeds oils from black currant, borage and evening primrose are rich in linolenic acid, as well as walnut, avocado, sunflower, sesame and wheat oils (Botanical-Online, 2011; University of Maryland Medical Center, 2011). Other fatty acids of the Omega-6 family are the arachidonic acid (*Figure 15*), predecessor in the synthesis of prostaglandins, and the homo-gamma-linoleic acid (*Table 7*) (Botanical-Online, 2011; University of Maryland Medical Center, 2011). The arachidonic acid is found in meat and egg.

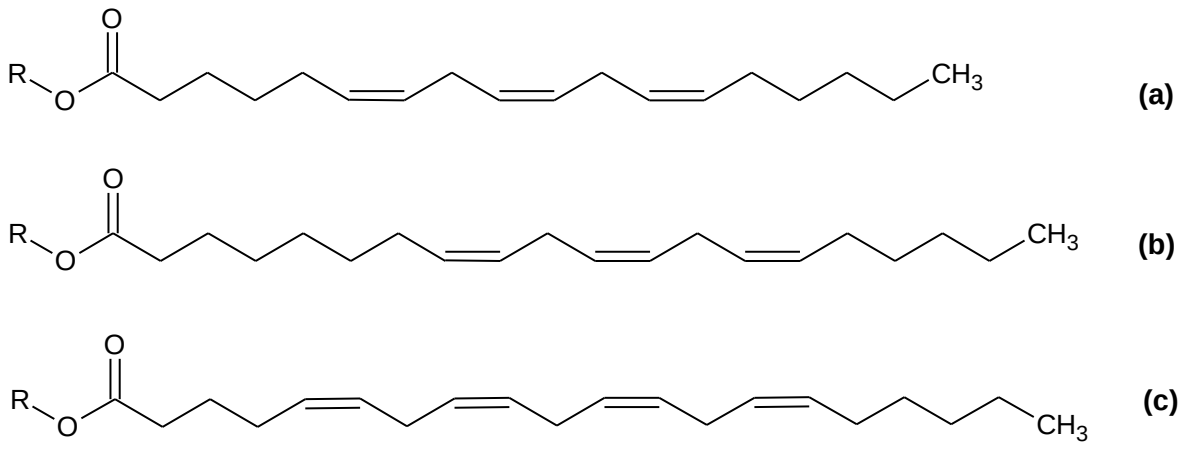
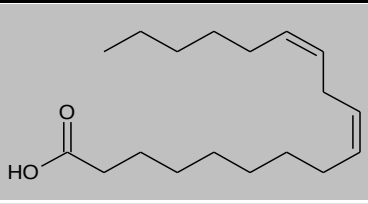
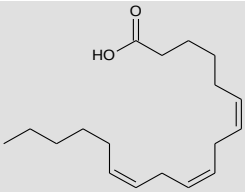
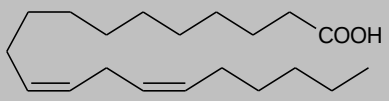
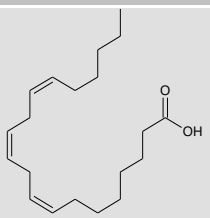
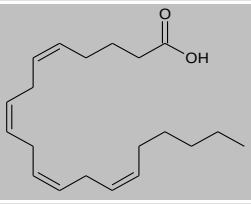
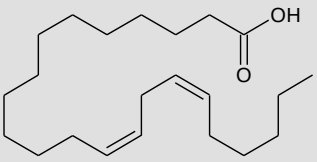
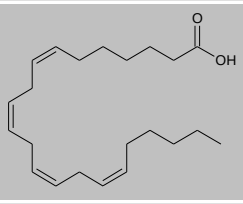
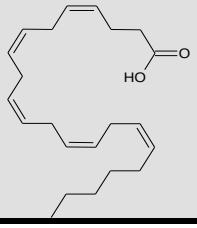


Figure 15. Structure of (a) gamma linolenic acid (b) homo-gamma-linolenic acid and (c) arachidonic acid (Lipomics Technologies, 2009).

Table 7. Fatty acids of the Omeg-6 family (Lipomics Technologies, 2009)

Common name	Abbreviation	Structure	Scientific name
Linoleic acid	18:2 (n−6)		9,12-octadecadienoic acid
Gamma linolenic acid	18:3 (n−6)		6,9,12-octadecatrienoic acid
Eicosadienoic acid	20:2 (n−6)		11,14-eicosadienoic acid
Homo-γ-linolenic acid	20:3 (n−6)		8,11,14-eicosatrienoic acid
Arachidonic acid	20:4 (n−6)		5,8,11,14-eicosatetraenoic acid
Docosadienoic acid	22:2 (n−6)		13,16-docosadienoic acid
Adrenic acid	22:4 (n−6)		7,10,13,16-docosatetraenoic acid
Docosapentaenoic acid	22:5 (n−6)		4,7,10,13,16-docosapentaenoic acid

Some functions of the Omega-6 in the organism are the formation of cell membranes and the production of hormones, maintaining a good functioning of the immune system, the development of the retina and appropriate functioning of neurons and chemical transmissions. However there are other benefits that these compounds proportionate to the organism. Some of the benefits to the circulatory system include the reduction in the amount of cholesterol, the prevention in the formation of clods in the arteries and a reduction of the blood pressure (University of Maryland Medical Center, 2011). It also protects the organism against heart attacks, apoplexies and angina, among others (Botanical-Online, 2011).

Fatty acids of the Omega-6 family have been used in the treatment of nervous anorexia, in order to avoid metabolic complications related with the deficiency of these polyunsaturated fatty acids. They also have anti-inflammatory properties, which make them appropriate in the treatment of articulation diseases such as rheumatoid arthritis. Linoleic acid also helps with the production of prostaglandins which makes it useful in the treatment of premenstrual syndrome (University of Maryland Medical Center, 2011). Another application of these fatty acids is in the diabetic treatment because it helps to maintain the insulin levels and its consumption prevents diseases of the nervous system. In Mexico according with the IMSS (Mexican Institute of Social Security) the diabetes is the first cause of death in the country with 21, 388 deaths in the country in 2007 (El Universal, 2008).

The deficiency of the essential fatty acids can induce the loss of bone density and osteoporosis, reason why their appropriate consumption is necessary. Pharmaceutical supplements of GLA and EPA keep and increase the bone density, they improve the calcium absorption and reduce the calcium loss by urine (University of Maryland Medical Center, 2011). This fatty acid could be used in the alcoholism treatment by reducing the anxiety and the hepatic damage. It is also given to allergic people who can have low blood levels of this fatty (University of Maryland Medical Center, 2011).

The gamma linoleic acid can be used externally for skin diseases such as eczemas (University of Maryland Medical Center, 2011). Some clinical researches have shown that the linoleic acid is capable of reducing the amount of acne and psoriasis on the skin by reducing the amount of facial oil. Some properties of the linoleic acid are anti-inflammatory, anti-esclerotic, anti-hemorragic and hepatoprotector among others (Botanical-Online, 2011).

1.1.2 Omega-3

Omega-3 is a family of polyunsaturated fatty acids. The most important fatty acids of the Omega-3 family are the α-linolenic acid, ALA, the eicosapentaenoic acid, EPA and the docosahexaenoic acid, DHA (*Figure 16*). The body is capable of converting the ingested ALA to EPA and DHA, which are more readily available in the body. Unfortunately the amount of ALA that can be converted is less than 10% of the amount ingested (*Figure 17*) (Caballero et al., 2006).

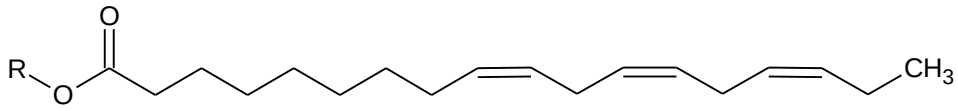
ALA can be found in vegetable seeds such as linseed, canola and nuts among others. EPA is found in blue fish oil and breast milk. Blue fish oil and some microscopic microalgaes are also rich in DHA. The main sources of Omega-3 are blue fish, salmon, tuna, halibut, herring, mackerel, anchovies and sardines, which are rich in EPA and DHA, the fish oil and the vegetable oils of linseed, canola and nuts. Other important sources are lettuce, soy, spinaches, strawberries, cucumbers, Brussels sprouts, pineapples, almonds and nuts (Botanical-Online, 2011). *Table 8* shows other fatty acids of the Omega-3 family.

The functions in the organism of the Omega-3 fatty acids are similar those of the Omega-6 (Botanical-Online, 2011). They have a crucial role in normal growth and development, in addition to healthy brain functions, like memory and performance (University of Maryland Medical Center, 2011). DHA is an important structural component of the gray matter of the brain, eye retina and hearth tissue (Ward and Singh, 2005). These fatty acids reduce inflammation and reduce risk factors of diseases such as arthritis, cancer and heart diseases, myocardial infarction or bronchial asthma (Rubio-Rodriguez et al., 2009). The deficiency of Omega-3 can provoke fatigue, dry skin, heart problems, poor circulation, depression and memory loss, among others.

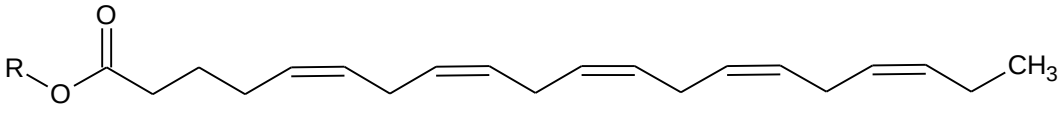
Omega-6 family		**Omega-3 family**
Linoleic acid (18:2)		α-linolenic acid (18:3)
⬇	Δ^6-desaturase	⬇
γ-Linolenic acid (18:3)		Octadecatraenoic acid (18:4)
⬇	elongase	⬇
Dihomo-γ-linolenic acid (20:3)		Eicosatetraeonic acid (20:4)
⬇	Δ^5-desaturase	⬇
Araquidonic acid (20:4)		Eicosapentaenoic acid (20:5)
⬇	elongase	⬇
Adrenic acid (22:4)		Docosapentaenoic acid (22:5)
⬇	Δ^4-desaturase	⬇
Docosapentaenoic acid (22:5)		Docosahexaenoic acid (22:6)

Figure 17. Metabolism of fatty acids from the Omega-6 and Omega-3 family, (Carvalho et al., 2003).

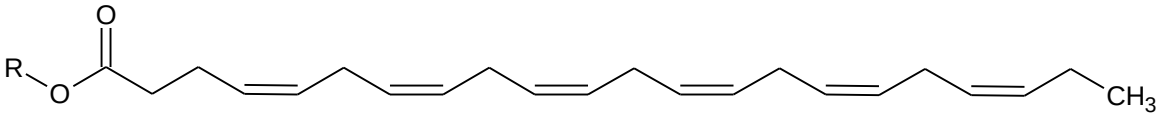
I (a)



(b)



(c)



II

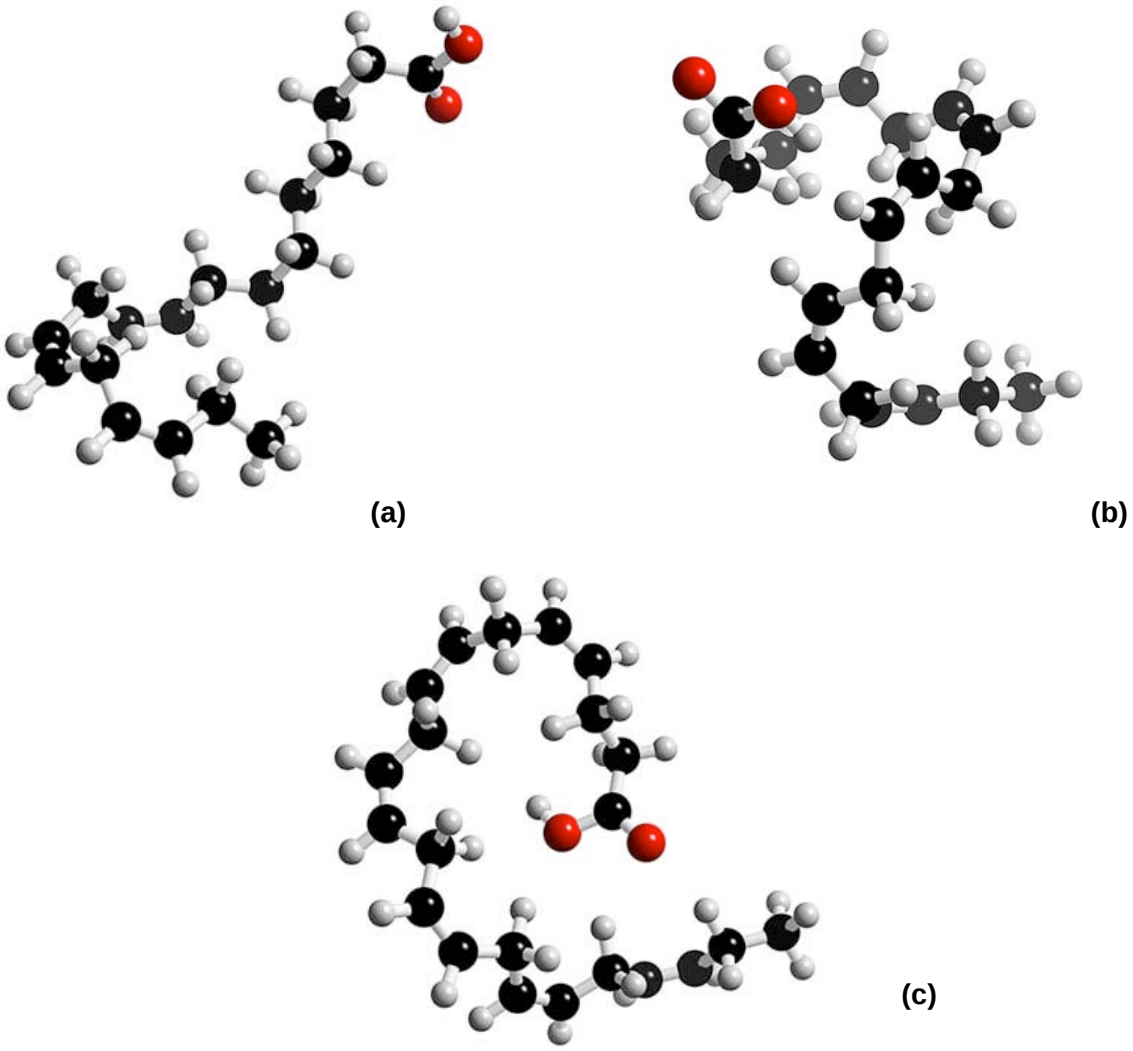
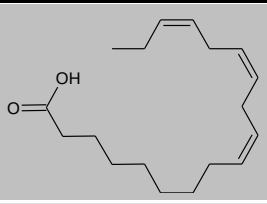
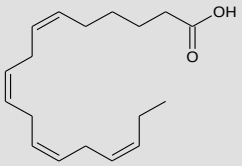
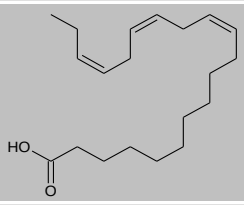
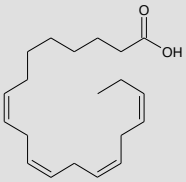
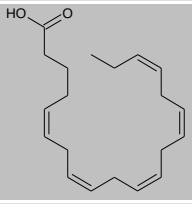
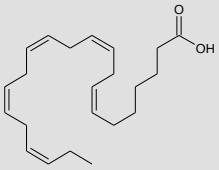
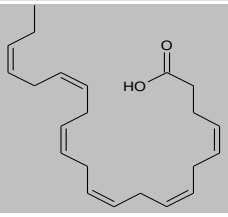
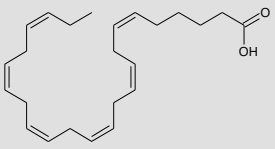


Figure 16. I Linear structure II Three dimensional structure of (a) ALA (b) EPA y (c) DHA.

They also have a cardio protector effect, which is a property of interest considering that the cardiovascular diseases represent the first cause of death in many countries around the world, which make them an important health problem (WHO, 2009). Each year the average of hearth disease deaths is of 102.9 deaths per 100,000 people around the world.

Table 8. Fatty acids of the Omeg-3 family (Lipomics Technologies, 2009).

Common name	Abbreviation	Formula	Scientific name
α-linolenic acid ALA	18:3 (n−3)		*cis*-9,12,15-octadecatrienoic acid
Stearidonic acid STD	18:4 (n−3)		*cis*-6,9,12,15-octadecatetraenoic acid
Eicosatrienoic acid ETA	20:3 (n−3)		*cis*-11,14,17-eicosatrienoic acid
Eicosatetraenoic acid	20:4 (n−3)		*cis*-8,11,14,17-eicosatetraenoic acid
Eicosapentaenoic acid EPA	20:5 (n−3)		*cis*-5,8,11,14,17-eicosapentaenoic acid
Docosapentaenoic acid DPA	22:5 (n−3)		*cis*-7,10,13,16,19-docosapentaenoic acid
Docosahexaenoic acid DHA	22:6 (n−3)		*cis*-4,7,10,13,16,19-docosahexaenoic acid
Tetracosahexaenoic acid	24:6 (n−3)		*cis*-6,9,12,15,18,21-tetracosahexaenoic acid

Omega-3 induces the production of eicosanoids, compounds that make the blood less viscous and reduce the formation of clods in the blood vessels. Some of their benefits include the diminishment of the blood pressure, the reduction of the cholesterol and triacylglycerol levels and they also prevent arrhythmia (Botanical-Online, 2011). Besides they aid the dilatation of the blood vessels increasing the irrigations of different organs. The Omega-3 acid ethyl esters have been prescribed in the treatment of very high triacylglycerols in blood (Sadovky and Kris-Etherton, 2009). In addition they have the ability of reducing the growth of cancer cells and they prevent breast, prostate and colon cancer. The Omega-3 is basic in the development vision and nervous system. It can also be used in the treatment of Attention deficit/hyperactivity disorder (ADHD) (University of Maryland Medical Center, 2011).

Omega-3 have anti-inflammatory properties which are used in the treatment of arthritis rheumatoid, psoriasis and lupus, as well as inflammatory intestinal diseases. Their ingestion helps maintaining a metal equilibrium and reduces the risk of depression. They ensure skin health and prevent diseases like prioriasis. The consumption of Omega-3 is essential during pregnancy to ensure a healthy development of the fetus brain (Botanical-Online, 2011) and it has been shown that they reduce premature pregnancies (Olsen and Secher, 2002). Clinical studies show that Omega-3 fatty acids increase calcium levels in the body and improve strength and reduce osteoporosis (University of Maryland Medical Center, 2011). The ethyl ester form of the Omega-3 has also been used in medical treatments. The ethyl ester of EPA (E-EPA) has been used in the treatment of arteriosclerosis obliterans (Shimada et al., 1997d).

The appropriate functioning of the organism requires an optimal relationship in the consumption of Omega-3 and Omega-6, which is a ratio of 4:1, Omega-6 to Omega-3. However in most diets the consumption of Omega-6 is higher than the optimal, reaching up between 11 and 30 times more Omega-6 than Omega-3. The appropriate intake of Omega-3 is of 1.6 g/day for men and 1.1 g/day for women, (*Table 9*) (IOM, 2005). An excess in the consumption of Omega-6 can contribute to the development of long term diseases such as cancer, asthma, arthritis, depression and hearth diseases (University of Maryland Medical Center, 2011).

Table 9 Amount of EPA and DHA in fish (Kris-Etherton et al., 2002; Caballero et al., 2006)

Fish	DHA + EPA content g/3-oz serving fish	Fish	DHA + EPA content g/3-oz serving fish
Sardines	0.98-1.7	**Sole**	0.42
Wild trout	0.84	**Oyster**	0.37-1.17
Fresh Tuna	0.24-1.28	**Salmon**	0.68-1.83
Halibut	0.4-1.0	**Shrimp**	0.27
Cod	0.13-0.24	**Clam**	0.24
Mackerel	0.34-1.57	**Lobster**	0.07-0.41
Herring	1.71-1.81	**Alaska crab**	0.35
Halibut	0.4-1.0	**Commercial products** *Cod liver oil* *Omega-3 concentrates* *Omacor*	 0.19 0.5 0.85

2. Concentrates of Omega-3

The necessity to have an appropriate consumption of polyunsaturated fatty acids has increased the interest of researchers and industries to produce Omega-3 concentrates from marine oils. Marine oils can be concentrated as triacylglycerols, as free fatty acids (FFA) or their esters (Shahidi and Wanasundara, 1998) and are the most important source of Omega-3 polyunsaturated fatty acids (ω-3 PUFA). These oils are the most common raw material used to prepare ω-3 PUFA concentrates, from which the production of concentrates with high percentages of EPA and DHA are the most important. Some alternative sources of ω-3 PUFA are single cell oils (Ward and Singh, 2005). The marine protists and dinoflagellates species of *Thraustochytrium, Schizochytrium* and *Crypthecodinium* are good sources of DHA and the microalgaes like *Phaeodactylum* and *Monodus* are rich in EPA. Other alternative source that has been studied is the species belonging to the fungal genus *Mortierella*, which mainly produce ARA and EPA (Dyal and Narine, 2005).

Different methods are used for concentrating the ω-3 PUFA, which include adsorption chromatography, molecular or fractional distillation, low temperature crystallization, urea complexation, supercritical fluid extraction, and enzymatic splitting (*Figure 18*). A brief explanation of these techniques will be described in the following paragraphs.

Adsorption chromatography is a method in which the fatty acids are separated according to their carbon number or unsaturation degree (Shahidi and Wanasundara, 1998). Some chromatography techniques that have been used are high performance liquid chromatography (Tokiwa et al., 1981; Beebe et al., 1988; Perrut, 1988), silver resin chromatography (Adlof and Emiken, 1985) and column chromatography on silver nitrate impregnated silica gel (Teshima et al., 1978). More recent attempts have used selective extraction to enrich polyunsaturated fatty acid methyl esters from fish oil with π-complexing sorbents (Li et al., 2009). This technique can increase the amount of polyunsaturated fatty acid methyl esters from 18 to 80%.

The distillation method is capable of a partial separation of fatty acid esters in a mixture. This method is based on the different boiling points and molecular weights of the fatty acids under low pressure. The most common method is the fractional distillation of fatty acid methyl esters at reduced pressure (Shahidi and Wanasundara, 1998). The low temperature crystallization method explodes the different solubilities of triacylglycerols, fatty acids, esters and other lipids in organic solvents. The PUFA are soluble at low temperatures while long

chain saturated fatty acids crystallize. The urea complexation method can be used to separate the fatty acids, or their esters, since their presence changes the structure and diameter of the crystallized urea. Using this method the saturated fatty acids of a mixture are crystallized with urea and filtrated, leaving a liquid fraction rich in ω-3 PUFA (Shahidi et al., 1994). The supercritical fluid extraction technique uses gases that have solvent properties when are taken above their critical value. The separation of PUFA with this technique is based on the different molecular size of the molecules involved (Mishra et al., 1993; Riha and Brunner, 2000). Riha and Brunner used supercritical carbon dioxide fractioning to separate fish oil fatty acid ethyl esters. Supercritical fluid chromatography can concentrate the DHA and EPA ethyl esters (Alkio et al., 2000). This technique can produce DHA ester concentrates with 95%wt purity using CO_2 as the mobile phase at 65°C and 145 bar and octadecyl silane-type reversed- phase silica as the stationary phase.

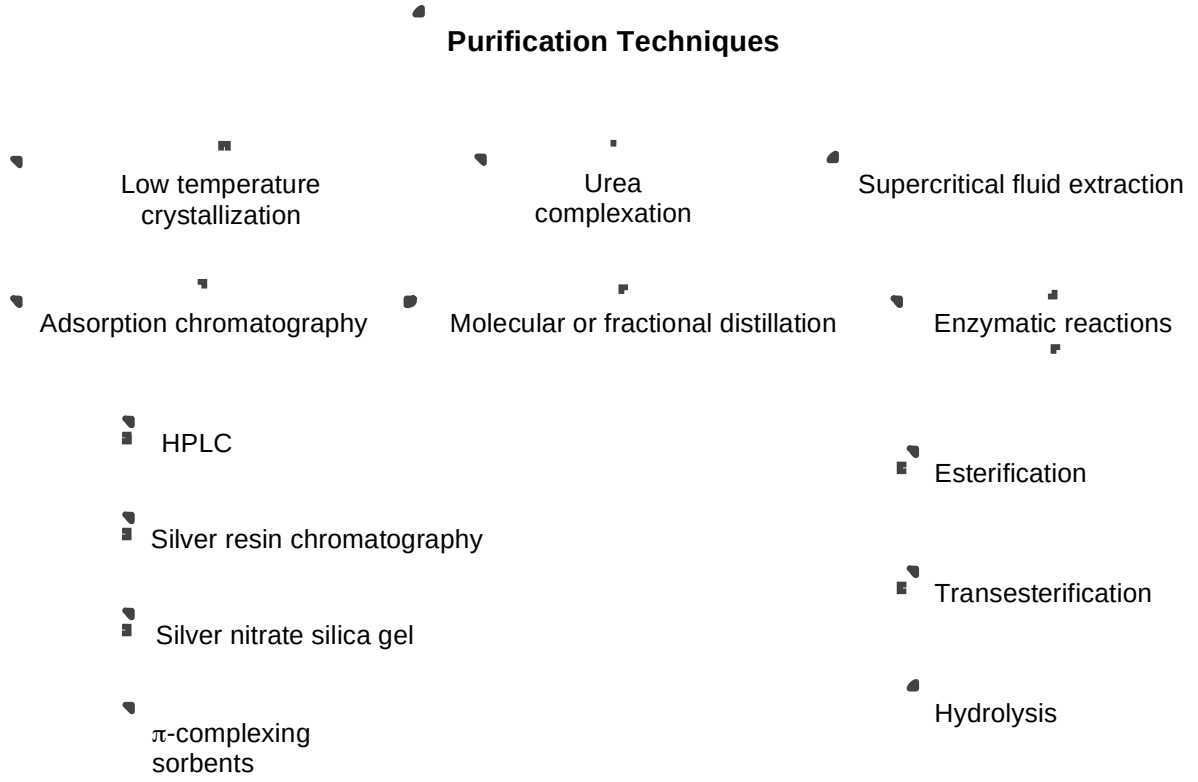
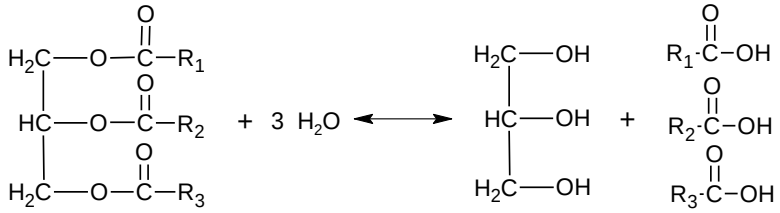


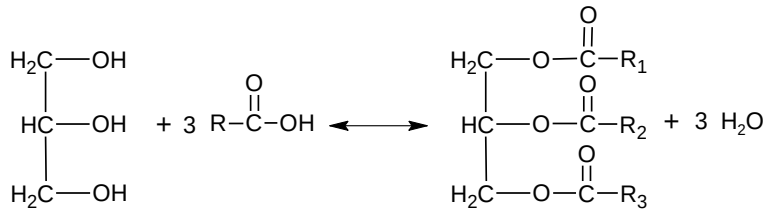
Figure 18. Techniques used for concentrating the ω-3 PUFA.

Another method of interest is the enzymatic splitting. The purification and concentration of ω-3 PUFA can be achieved by esterification, transesterification or hydrolysis (*Figure 19*) (Shahidi and Wanasundara, 1998; Carvalho et al., 2003). Lipases have been used to produce concentrates of eicosapentaenoic acid and docosahexaenoic acid from fish oil.

Hydrolysis

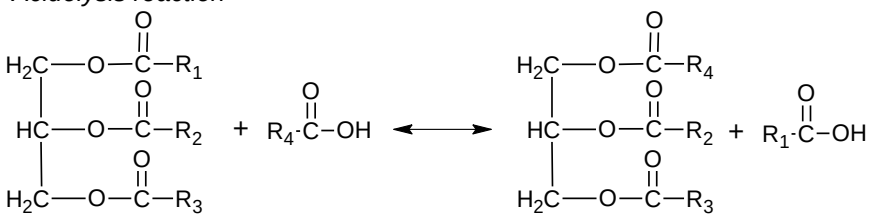


Esterification reaction



Transesterification reactions

Acidolysis reaction



Alcoholysis reaction

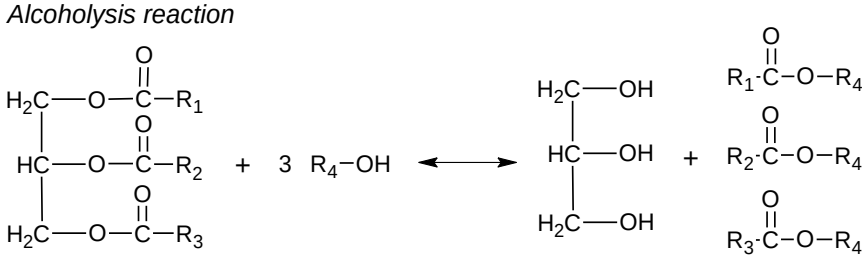


Figure 19. Reactions catalyzed by lipases for ω-3 PUFA purification.

2.1 Esterification

The lipases from *Chromobacterium viscosum* and *Candida cylindracea* were used for the selective esterification reaction between glycerol and individual FFA, including EPA and DHA (Osada et al., 1992). The esterification with *C. viscosum* lipases had a reaction yield between 89-95%for EPA and DHA, while *C. cylindracea* had a reaction yield of 71-75% for EPA and of 63% for DHA. Similar experiments were carried out between ω-3 PUFA concentrates, containing 23.8% EPA and 53.1% DHA, and glycerol, in order to produce acylglycerols (He and Shahidi, 1997). These researchers tested the commercial lipases from

C. viscosum, Rhizomucor miehei, Pseudomonas sp., *Candida rugosa, Rhizopus niveus, Aspergillus niger* and *Rhizopus oryzae.* The degree of synthesis was of 68.5%, 44.1%, 46.0%, 13.9%, 0%, 0% and 39.8% respectively. Under optimal reaction conditions (0.4 g of ω-3 PUFA concentrates, 4 g of glycerol, 40 mg of lipase, 0.5 g of molecular sieve and 1ml of isooctane), with *C. viscosum* lipase the maximal yield was of 94.3%, with a relative content of TAG of 37.4%, 43.1% for diglycerols and 13.8% of monoglycerols.

This reaction has also been studied by Medina et al. (Medina et al., 1999). The synthesis of triacylglycerols by enzymatic esterification of PUFA with glycerol was catalyzed by *Candida antarctica* lipase (Novozym 435). The reaction was performed with 100mg of enzyme, 9ml of hexane, at 50ºC and a molar ratio of 1.2:3 glycerol to PUFA. The triacylglyceride yield was of 93.5% using cod liver oil PUFA concentrate, generating a product with 25.7% EPA and 44.7% DHA. Similar experiments were developed by Lie and Molin (Lie and Molin, 1992). The lipases from *R. miehei* and *C. viscosum* incorporated free PUFA into glycerol to a concentration of 75% and 80%, respectively. Both lipases showed a slight preference for EPA over DHA. Using the commercial lipase from *C. viscosum* immobilized, Tanaka et al. were able to produce a triglyceride with 46.2% DHA (Tanaka et al., 1994).

The esterification reaction has also been used to separate the EPA and DHA in fish oil (Halldorsson et al., 2003). The reaction used FFA with glycerol and was catalyzed by *R. miehei* lipase (Lipozyme RM IM) at 40ºC with a lipase amount of 10%. Under these conditions most of the FFA and the EPA were converted to acylglycerols and the DHA was concentrated in the residual FFA. Using FFA from tuna oil with an initial proportion of 5% EPA and 25% DHA the esterification converted 90% to acylglycerols in 48h. The FFA fraction had a DHA concentration of 78% and only 3% of EPA, and 79% of the DHA was recovered. The EPA recovered in the acylglycerol fraction represented 91% (Halldorsson et al., 2003).

Concentrates of ω-3 PUFA can also be produced as monoacylglycerols (MAG) (Pawongrat et al., 2007). MAG rich in EPA and DHA were produced by glycerolysis of tuna oil FFA, catalyzed with Lipase AK, from *Pseudomonas fluorescens* (Amano). The conditions of the reaction were 10% w/v of tuna oil in ter-butyl methyl ether, molar ratio of 3:1 glycerol to tuna oil, water with 4%wt in glycerol and 45ºC. A 24h reaction yielded 24.6% of MAG with 56%wt content of ω-3 PUFA.

An alternative source of ω-3 PUFA is the effluents of the sardine canning industry which have up to 10% of EPA and 10% DHA (Schmitt-Rozieres et al., 2000). The recovery of the fatty acids required a pre-treatment of the effluents that included removing solid particles, proteins and peptides. The obtained oil was hydrolyzed and the EPA and DHA enriched from the free fatty acid fraction by enzymatic esterification with butanol. The enzymes tested were *R. miehei* (Lipozyme IM60) and immobilized *C. rugosa* lipase on Amberlite IRC50 cation-exchange resin. *R. miehei* enriched up to 80% DHA but did not increase the concentration of EPA (12%). The *C. rugosa* lipase enriched EPA to 30% and DHA to 41.0%. FFA produced as by-products in the seafood industry, are rich in DHA (46%wt) and were used to produced a TAG rich in DHA using *R. miehei* lipase (Lipozyme RM IM) (Nagao et al., 2011). The TAG produced had high concentrations of DHA which was distributed 51.7%mol in the *sn-1,3* positions and 17.3%mol in the *sn-2* position.

Thanks to its specificity, a great amount of research has focus on the application of the enzyme of *C. rugosa* to purify the PUFA content (Jonzo et al., 2000). Jonzo et al. used two isoforms A (Lip1 isoform) and B (mixture of Lip2 and Lip3 isoforms) of the *C. rugosa* lipase (Lipase My) that were purified and immobilized in Duolite A 568. The selective esterification was performed between FFA from sardine oils and cholesterol. Both isoforms had preference toward saturated and monosaturated fatty acids. The esterification with Lip A produced an unesterified FFA fraction enriched four times in DHA which increase its content from 7.4 to 32%, with a recovery of 95%. The unesterifed FFA fraction was enriched 3.4 times when LipB was used, increasing the percentage from 7.42 to 25.3% with 93.8 recovery. The selectivity toward EPA was less and its concentration change from 10.59% to 5.21% and 12.11% with Lip A and Lip B respectively (*Figure 20*).

2.2 Transesterification

The transesterification reaction with lipases can also be used to produce concentrates of ω-3 PUFA. The alcoholysis reaction of fish oil with ethanol released the saturated and monosaturated fatty acids as ethyl esters concentrating the polyunsaturated fatty acids in the acylglycerol mixture. The *Pseudomonas* species lipases show the highest activity toward the saturated and monounsaturated fatty acids in the fish oil, and a lower specificity toward EPA and DHA. The initial triacylglycerol substrate concentration was of 15.9% EPA and 9.8% DHA, obtaining a final concentration of 46% EPA + DHA, after a reaction of 24h at 20°C, with 90% recovery for EPA and 80%for DHA (Haraldsson et al., 1997). The *Pseudomonas* species lipases are some of the few lipases that favour towards DHA as a substrate over EPA.

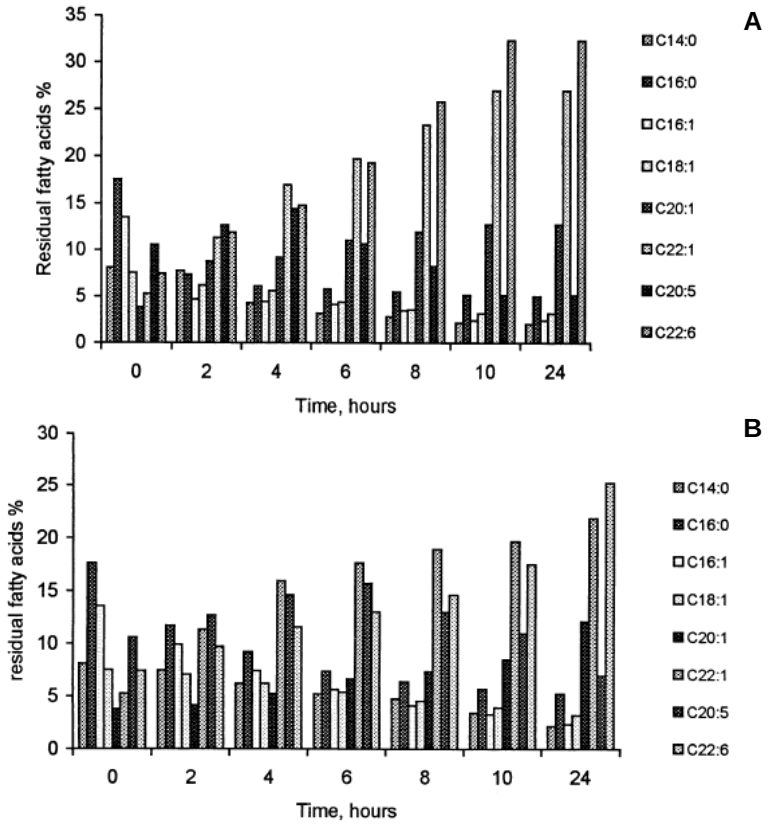


Figure 20. Esterification of fatty acids from sardine oil and cholesterol with immobilized Lip A (A) or Lip B (B) at 40°C in cyclohexane for 24 h (Jonzo et al., 2000).

The commercial lipase from *R. miehei* (Lipozyme OM-60, Novozymes) was used for the acidolysis reaction to enrich the ω-3 PUFA from menhaden oil under supercritical carbon dioxide conditions, using a concentrate of ω-3 PUFA (Lin et al., 2006). After transesterification, saponification and urea inclusion, 80.1%wt of ω-3 PUFA was concentrated with a concentration of 29.4%wt EPA and 41.8%wt DHA. The optimal pressure and temperature were 103.4 bars and 50°C.

The purification of ethyl esters of fish oils represents another approach to produce concentrates of ω-3 PUFA. The ethyl docosahexaenoate (E-DHA) was purified by alcoholysis of fatty acid ethyl esters using the immobilized lipase from *R. delemar* (Ta-lipase, 120000 U/g; Tanabe Seiyaku) (Shimada et al., 1997d). The original tuna oil had a 23%mol content of E-DHA. The alcoholysis reaction was carried out at 30°C, with a molar ratio of 1:3 E-tuna to lauryl alcohol and 4%v of lipase. After 50h of reaction the E-DHA content was increased from 23 to 52%mol with a 90% recovery. Using ethyl esters mixtures with high contents of E-DHA, 45%mol and 60%mol, after a 50h reaction the content of this ester increased to 72%mol and 83% mol respectively, with more than 90% recovery.

Optimization of the purification of ethyl esters of DHA was conducted by the same authors (Shimada et al., 1998). The selective alcoholysis of ethyl esters from tuna oil, with *R. delemar* lipase (Ta-lipase), efficiently enriched the E-DHA. As mentioned in the previous paragraph, the alcoholysis can increased the content of E-DHA in the unreacted ethyl ester fraction from 23 to 49%mol with 90% recovery. Unfortunately the concentration of ethyl eicosapentaenoate (E-EPA) also increased. The concentration of E-EPA was reduced using the lipase from *R. miehei* (Lipozyme IM). The alcoholysis reaction conditions were 30ºC, with a molar ratio of 1:3 ethyl ester to lauryl alcohol and 4%wt of *R. miehei* lipase. This reaction effectively increases the E-DHA content from 45 to 74%mol and reduced the concentration of E-EPA from 12 to 6.2%mol. Using a higher molar ratio of ethyl esters/lauryl alcohol increased the E-DHA content from 60 to 93% and decreased the E-EPA content from 8.6 to 2.9%.

This reaction has been used to efficiently separate EPA and DHA acid in fish oil (Haraldsson and Kristinsson, 1998). The ethanolysis reaction was carried out with *R. miehei,* (Lipozyme IM) at 20ºC with stoichiometric amount of ethanol (*Figure 21*). The original amount of PUFA in the tuna oil was 6% EPA and 23% DHA and after 24h transesterification 65% was converted into ethyl esters. The residual glyceride fraction had 49% DHA and 6% EPA with 90% DHA recovery in the glyceride mixture and 60% EPA recovery in the ethyl ester fraction.

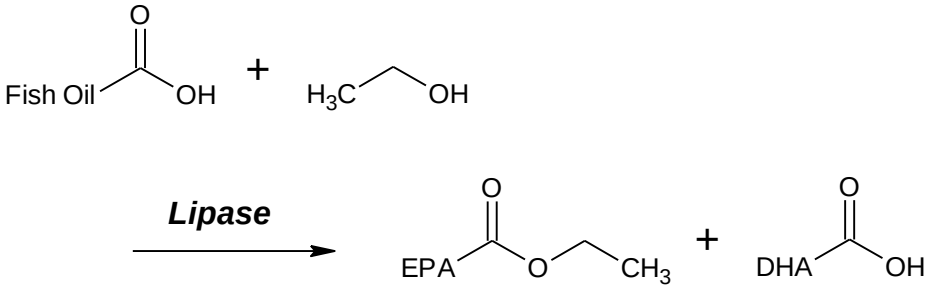


Figure 21. Ethanolysis reaction between PUFA of tuna oil and R. miehei lipase, (Haraldsson and Kristinsson, 1998).

The preparation of highly purified concentrates of eicosapentaenoic acid and docosahexaenoic acid were produced by a three step process: lipase catalyzed alcoholysis with *Pseudomonas* sp. lipase (PSL), combined with short distillation separation and ethanolysis with *C. antarctica* lipase (CAL) (*Figure 22*) (Breivik et al., 1997). The transesterification reaction conditions were room temperature with a stoichiometric amount of ethanol. This reaction concentrated the EPA and DHA from 14.9% and 9.8% to 40.1% of

EPA+DHA. After distillation the concentration increased to 47.3% EPA+DHA. Finally the ethanolysis with CAL showed complete conversion to DHA and EPA ethyl esters.

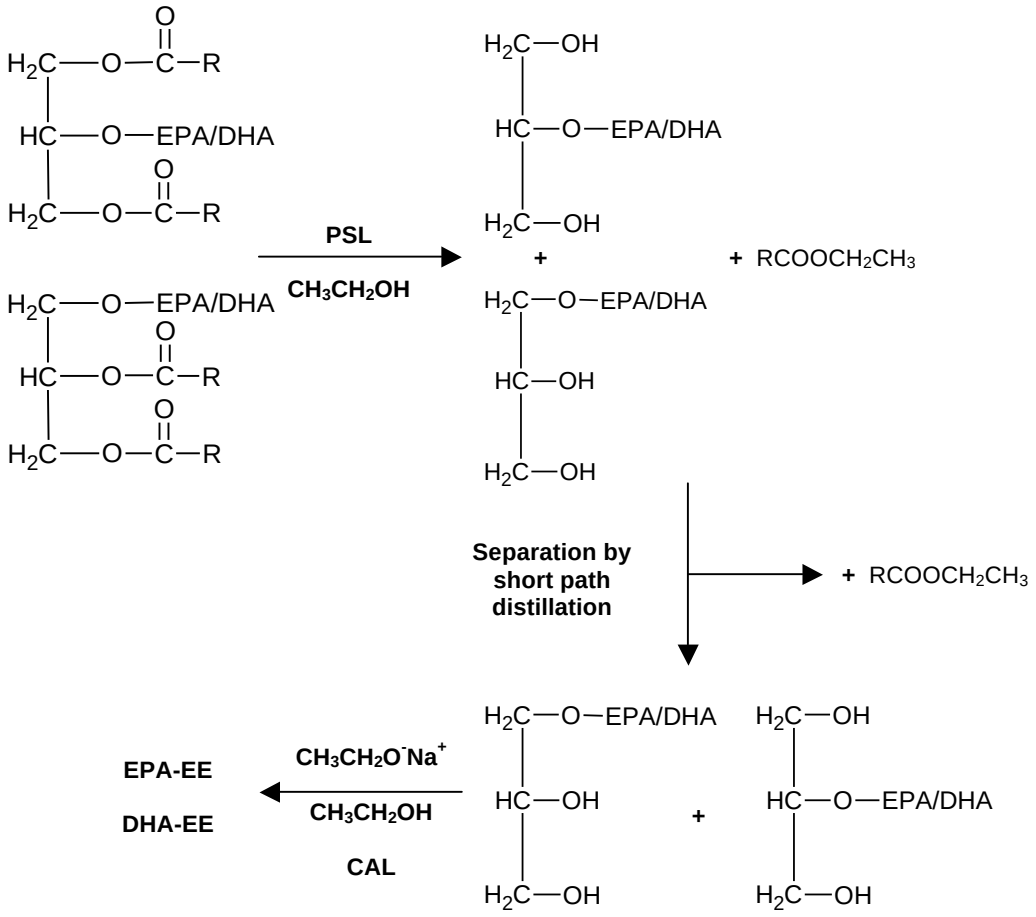


Figure 22. Preparation of highly purified concentrates of EPA and DHA. PSL is Pseudomonas sp. lipase and CAL is C. antarctica lipase (Breivik et al., 1997).

2.3 Hydrolysis

Lipases present a mechanism of resistance toward long chain PUFA. The presence of *cis* bonds in the fatty acid chain of PUFAs allows them to bend and causes a steric effect between the terminal methyl group and the ester bond (Bottino et al., 1967). As a result, the lipases cannot reach the ester linkage between these fatty acids and the glycerol. The saturated and monounsaturated fatty acids do not present barriers to the lipases so they are easily removed from fish oils by lipase hydrolysis to produce Omega-3 concentrates rich in EPA and DHA (Shahidi and Wanasundara, 1998). Some advantages of the lipase-assisted hydrolysis include that it is performed under mild pH and temperature conditions, it avoids the oxidation of the *cis* bonds in the PUFA, it requires less energy as a production system

and it increases the product selectivity (Sun et al., 2002). Some examples of lipase assisted purification of fish oils are presented in *Table 10*.

Table 10. Purification of fish oils by lipase catalyzed hydrolysis.

Substrates	Lipase	Purity (%)	References
Cod Liver Oil 22% ω-3 PUFA	*T. lanuginosus* *C. cylindracea* *A. niger*	40% ω-3 50% ω-3 50% ω-3	(Hoshino and Yamane, 1990)
Fish oil 25% DHA	*C. cylindracea*	53% DHA	(Tanaka et al., 1992).
Tuna Oil 32% EPA+DHA	*G. candidum*	48.7% EPA+DHA	(Shimada et al., 1994).
Anchovy Oil 27% PUFA	*R. miehei*	39.6% PUFA	(Ustun et al., 1997)
Chilean Fish Oil 30.3% ω-3 PUFA	*C. rugosa* *G. candidum*	48.9.% ω-3 46.0% ω-3	(McNeill et al., 1996)
Sea Blubber Oil 20.2% ω-3 PUFA	*C. cylindracea*	45% ω-3 54.3% ω-3	(Wanasundara and Shahidi, 1998b) (Wanasundara and Shahidi, 1998a)
Menhaden Oil 30% ω-3 PUFA	*R. oryzae* *C. cylindracea*	44.6% ω-3 54.5% ω-3	(Wanasundara and Shahidi, 1998b) (Wanasundara and Shahidi, 1998a)
Sardine Oil 48.3% ω-3 PUFA 26.9% EPA 13.6% DHA	*C. rugosa* *C. cylindracea*	33.7% EPA 29.9% DHA 63.8% ω-3 31.9% EPA 26.5% DHA	(Okada and Morrissey, 2007)
Marine fish oil	Pancreatic phospholipase A2	24% EPA 40% DHA	(Tocher et al., 1986)
Marine fish oil 2.9% EPA 22.5% DHA	*Penicillium abeanum*	3% EPA 47.3% DHA	(Sugihara et al., 1996)
	C. cylindracea	4.3% EPA 42.8% DHA	
	G. candidum	3.7% EPA 36% DHA	
Cod oil 12.2% EPA 6.9% DHA	*P. fluorescens* AK-lipase HU-lipase	16.8% EPA 44.6% DHA 43.1% EPA 7% DHA	(Kojima et al., 2006)
Salmon oil 30.1% ω-3 PUFA	*A. niger*	45% ω-3	(Carvalho et al., 2009)
Oil from Nile perch viscera	*T. lanuginosus*	38% DHA	(Mbatia et al., 2010)

The lipases from *Thermomyces lanuginosus* (Novozymes)*, C. cylindracea* (Meito Sangyo)*, A. niger* (Amano)*, Rhizopus delemar* (Amano)*, Geotrichum candidum* (Amano) and porcine pancreas (crude) (Sigma Chemical Co.) were tested to concentrate the ω-3 PUFA of cod liver oil, CLO, and refine sardine oil, RSO, as triacylglycerols (Hoshino and Yamane, 1990). The initial amount of ω-3 PUFA in CLO was of 22% and it was increased to 50% with the lipase of *C. cylindracea* and *A. niger,* and up to 40%, 38%, 35% and 32% with the lipases of

T. lanuginosus, R. delemar, G. candidum and porcine pancreas respectively. The hydrolysis with *C. cylindracea* lipase increased the amount of DHA while keeping the amount EPA almost constant. On the other hand, the lipase from *A. niger* concentrated DHA and partially concentrated EPA. After extended hydrolysis these two enzymes hydrolyzed the EPA. The other four enzymes concentrated the DHA with a small increment in the amount of EPA. The hydrolysis of RSO with an initial concentration of ω-3 PUFA of 28% presented a hydrolysis, with *C. cylindracea* and *A. niger,* similar to the one obtained with CLO, reaching up to 50% the amount of ω-3 PUFA.

The hydrolysis has been used to concentrate the amount of DHA in a fish oil glyceride mixture of triglyceride, diglyceride and monoglyceride (Tanaka et al., 1992). The lipases used were *C. cylindracea* (Meito Sangyo)*, A. niger* (Amano)*, Pseudomonas* sp. (Amano), *R. delemar* (Lyberg and Adlercreutz)*, Rhizopus javanicus* (Amano) and *C. viscosum* (Asahi Chemical)*.* The original fish oil glyceride mixture had 13.3% EPA and 5.9% DHA. After the hydrolysis, the free fatty acids were removed from the mixture and the glyceride mixture analyzed. After a 70% hydrolysis of the reaction mixture with the lipase of *C. cylindracea,* the amount of DHA increased to 30% while the amount of EPA decreased to 70% less than the original mixture. The other lipases did not showed changes in the concentration of DHA and EPA. The hydrolysis of a tuna oil mixture rich in DHA (25.1%) with *C. cylindracea* lipase increased the content of DHA to 53.1%.

The ability of the lipase from *C. cylindracea* to discriminate between different fatty acids of marine oils and wax esters was studied by (Lie and Lambertsen, 1986). The triacylglycerols in fish oils were hydrolyzed faster than the esters and the enzyme showed preference in the hydrolysis of the C14 to C18 saturated and monounsaturated fatty acids. The long chain monoenes (20:1 and 22:1) and the polyunsaturated fatty acids C18:4, EPA and DHA were resistant to the hydrolysis in both, the triacylglycerols and the wax esters.

The enzyme from *G. candidum,* was used for concentrating the EPA and DHA in the glyceride fraction of tuna oil (Shimada et al., 1994). The hydrolysis was carried out at 30°C for 16h and after a 33.5% hydrolysis the resulting glycerides increased its concentration of DHA and EPA from 32.1% to 48.7%. A second hydrolysis was performed and produced glycerides with 57.5% of DHA and EPA with a recovery of 81.5% of the initial DHA end EPA. This hydrolysis product had an 85.5% of triacylglycerols.

Comparable experiments were carried out to produced an enriched glyceride mixture of PUFA from anchovy oil with 27% of PUFA (7.6% EPA, 12.7% DHA) (Ustun et al., 1997). The enzyme used was *R. miehei* (Lipozyme) lipase that is not specific toward PUFA. After a 3 h hydrolysis at 35°C with a pH of 4.0 the amount of PUFA in the glyceride mixture was of 39.6% with only 2% lost as free fatty acids.

The ability of different enzymes to selectively enrich the amount of ω-3 PUFA was later studied by (McNeill et al., 1996). The commercial enzymes screened were *C. rugosa* (Meito Sangyo), *G. candidum* (Amano), *R. niveus* (Amano), *R. miehei* (Novozymes), *T. lanuginosus* (Novozymes) and *C. viscosum* (Shizuoka, Japan), and the oil used was Chilean fish oil. The hydrolysis with *C. rugosa* or *G. candidum* lipases showed the highest increment in the DHA and EPA concentration in the acylglycerols. *C. rugosa* lipase increased the concentration of total ω-3 PUFA from 30.3 to 48.9% and the lipase from *G. candidum* to 46%. The lipase from *C. rugosa* has strong discrimination toward DHA but moderate discrimination against EPA.

Other marine oils that have been enriched in their ω-3 PUFA content by hydrolysis are the sea blubber oil (SBO) and the menhaden oil (MHO) (Wanasundara and Shahidi, 1998b). The lipases tested were *A. niger* (AN) (Amano), *R. miehei* (RM) (Novo Nordisk), *R. oryzae* (RO) (Amano), *R. niveus* (RN) (Amano), *C. cylindracea* (CC) (Amano), *C. viscosum* (CV) (Asahi Chemicals), *G. candidum* (GC) (Amano) and *Pseudomonas* sp (PS) (Amano). The total ω-3 PUFA content after hydrolysis in the mixture of monoglycerides, diglycerides, and triacylglycerols, is shown in the following figure (*Figure 23*).

All lipases concentrated the ω-3 PUFA content from both oils. The CC lipase significantly increased the total ω-3 PUFA content of EPA and DHA in SBO from 20.2 to 45%, but extended hydrolysis reduced the amount of EPA. This lipase reached a maximum ω-3 PUFA concentration in SBO of 9.75% EPA, 8.61% DPA and 24.0% DHA and of 18.5% EPA, 3.62% DPA and 17.3% DHA in MHO. The RO lipase was able of concentrate the DHA of both oils, but the amount of EPA decreased from 6.4 to 4.3% in SBO and from 13.2 to 12.5% in MHO. In the MHO the total ω-3 PUFA content increased from 30% to 44.6, 44.1 and 41.7% by RO, CC and GC lipase respectively, where the corresponding DHA content increased from 10.1 to 23.5, 17.3 and 14.8% for these three lipases. In the SBO the RO, GC, MM, PS, CV, RN and AN lipases reached 33.2, 30.6, 29.3, 26.1, 25.5, 25.3, and 24.6% respectively. After further research the optimal conditions were obtained for the hydrolysis with CC lipase (Wanasundara and Shahidi, 1998a). A maximum of 54.3% total ω–3 PUFA was obtained from SBO with an enzyme concentration of 308 U/g oil, 40h reaction at 37°C. From MHO a

maximum of 54.5% total ω-3 PUFA was obtained with an enzyme concentration of 340 U/g oil.

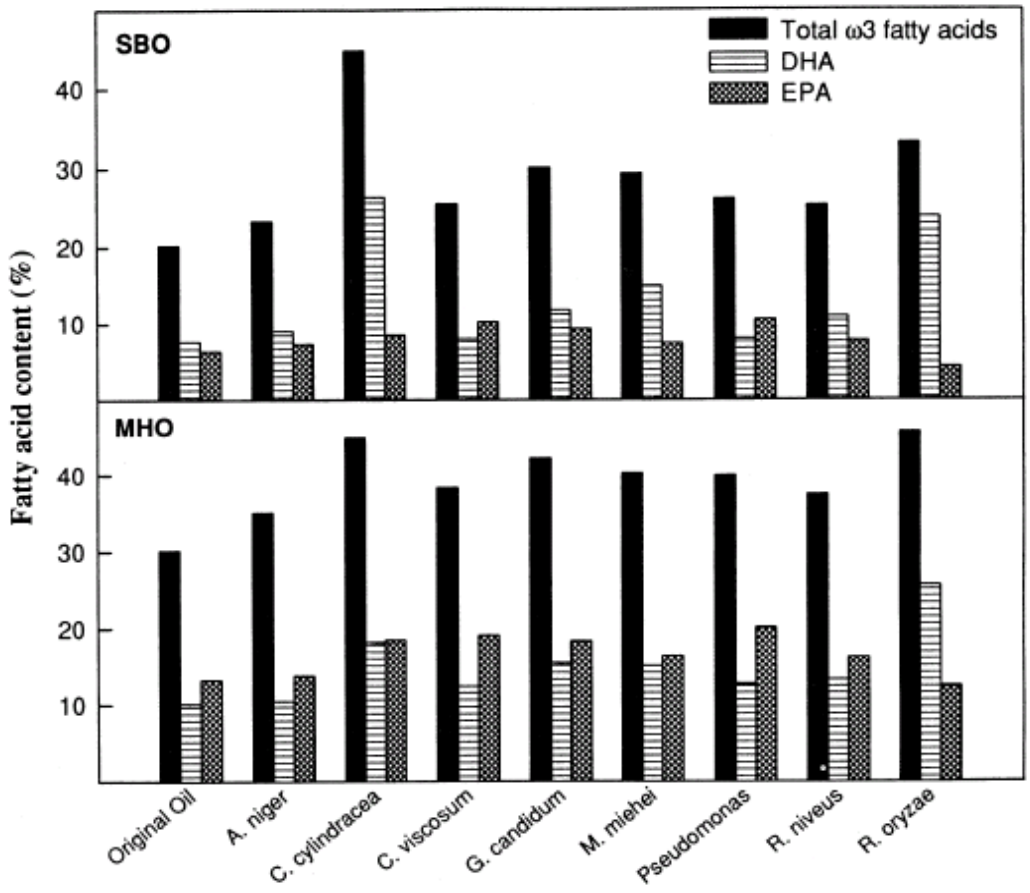


Figure 23. Fatty acid content of SBO and MHO after hydrolysis (Shahidi and Wanasundara, 1998)

Further research regarding the concentration of sardine oil (*Sardinops sagax*) produced a ω-3 PUFA concentrate (Okada and Morrissey, 2007). The commercially available microbial lipases used were *C. rugosa* (CR, Sigma–Aldrich), *C. cylindracea* (CC, Fluka Chemie AG), *Mucor javanicus* (MJ, Aldrich) and *A. niger* (AN, Aldrich) and the PUFA fraction in the crude oil was of 48.29%. The sardine oil was rapidly hydrolyzed and the highest hydrolysis was obtained with CR (78.4%) and CC (69.33%) enzymes, in agreement with previous researches (*Figure 24*). This research revealed that the EPA concentration depends on the enzyme and enzyme concentration while DHA is affected by enzyme and reaction time. Using CR the EPA increased from 26.87 to 33.74% in 1.5h and then remained constant, while the DHA content increased from 13.63% to 23.12% in the same time but kept increasing and reached 29.94% (*Figure 25*). The highest PUFA concentration was found with CR after 6h hydrolysis reaching 63.86%. In the same reaction time using CC the concentration of EPA increased to 31.91% and the DHA concentration reached 22.65%, 26.16% and 26.54% in 1.5, 6 and 9 hours.

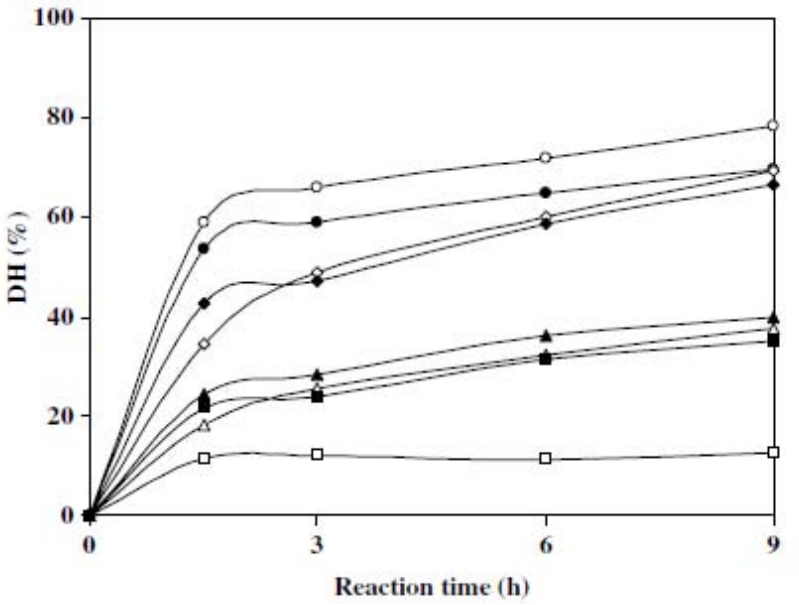


Figure 24. Degree of hydrolysis (%) of hydrolyzed sardine oil by lipases at 37°C. ○ CR 250U; ● CR 500U; ◇ CC 250U; ◆CC 500U; △ MJ 250U; ▲ MJ 500U; □ AN 250U; ■ AN 500U, (Okada and Morrissey, 2007)

Supplementary research regarding the concentration of DHA in the glyceride fraction by hydrolysis of tuna oil, using *C. rugosa* lipase was performed by Japanese researchers (Yan et al., 2002). The hydrolysis conditions were 40°C with phosphate buffer. After 28h reaction the concentration of DHA increased almost three times, reaching a concentration in the acylglycerol fraction of 56%.

Other lipases that have been used to enriched marine fish oil in ω-3 PUFA are the pancreatic phospholipase A2 (Tocher et al., 1986) and the *Penicillium abeanum* lipase (Sugihara et al., 1996). The method of Tocher et al. is based on the specificity of the enzyme to the ester bond of the *sn-2* position which is rich in EPA and DHA. The free fatty acids oil concentrate obtained had a concentration of 24% EPA and 40% DHA. The lipase of *P. abeanum* hydrolyzes the *sn-1*and *sn-3* position nine times faster than the *sn-2* position and has lower activity to the ester bonds of the PUFA. After hydrolysis with *P. abeanum* the tuna oil increased its concentration of EPA from 2.9 to 3.0% and from 22.5 to 47.3% with 67% recovery of DHA. In comparison with other enzymes under the same reaction conditions *C. cylindracea* produced a concentrate with 4.3% EPA and 42.8% DHA and *G. candidum* one with 3.7% EPA and 36.0 DHA, with 86% and 94% of DHA recovery respectively.

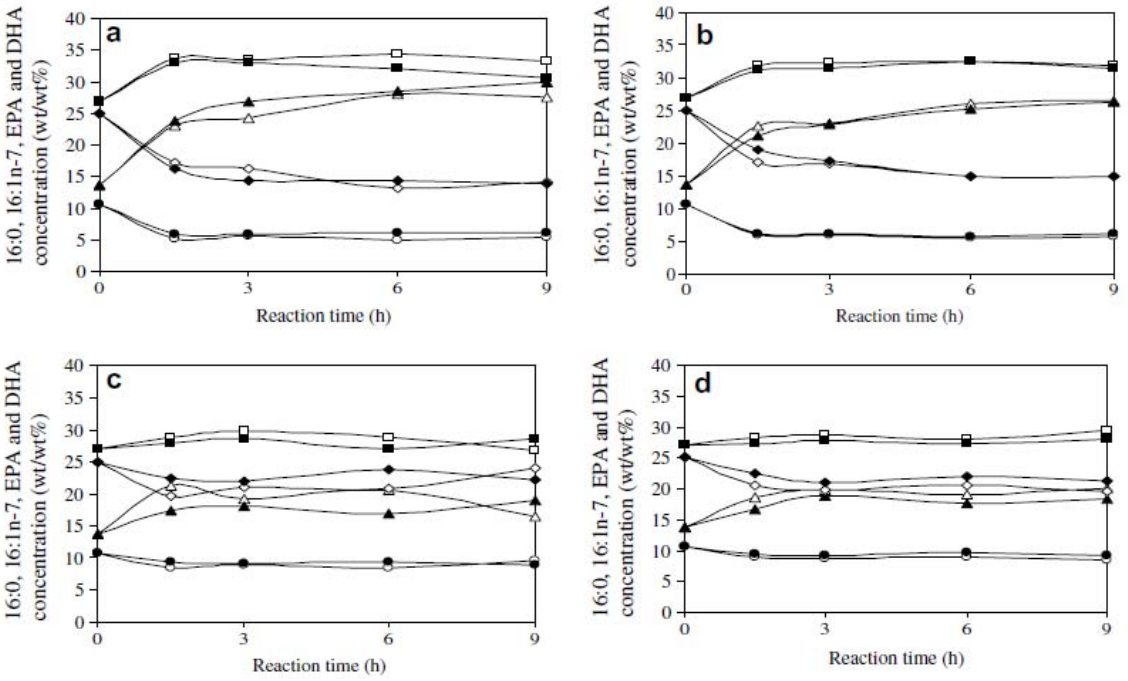


Figure 25. Changes in 16:0, 16:1n - 7, EPA, and DHA concentration (wt/wt%) in final w-3 PUFA concentrate with lipases from (a) CR, (b) CC, (c) MJ and (d) AN during hydrolysis at 37 °C. ◇ 16:0 with 250 U; ◆ 16:0 with CR 500 U; ○ 16:1n-7 with 250 U; ● 16:1n- 7 with 500 U; □ EPA with 250 U; ■ EPA with 500 U; △ DHA with 250 U; ▲ DHA with 500 U (Okada and Morrissey, 2007).

The specificity of the commercially available non immobilized lipases from *C. rugosa, R. miehei* (Amano), *T. lanuginosus, P. fluorescens* (Amano) *and Pseudomonas cepacia* (Amano) towards EPA and DHA in the hydrolysis of fish oils, squid oil and methyl esters was studied by (Lyberg and Adlercreutz, 2008). All the lipases were able to discriminate against EPA and DHA, being less hydrolyzed as methyl esters. The lipase from *C. rugosa* showed the highest discrimination toward methyl docosahexaenoate followed by the lipases from *T. lanuginosus* and *R. miehei.* However in the fish and squid oils the highest discrimination against DHA was achieved by the *T. lanuginosus* and *R. miehei* lipases. Concerning EPA the highest discrimination was observed by *P. fluorescens* in all three systems (Haraldsson et al., 1997; Lyberg and Adlercreutz, 2008). Regarding regioselectivity, all lipases showed a *sn-1*, *sn-3* specificity, except *C. rugosa*. Applying these enzymes to enrich the glyceride fraction of fish oil showed enrichment by *T. lanuginosus* and *R. miehei* in the early stages; however the highest overall enrichment was achieved by *C. rugosa* but with elevated losses. The concentration of EPA was observed with the lipases from *P. cepacia* and *P. fluorescens.*

The hydrolysis specificity of two lipases produced by *P. fluorescens* (AK-lipase and HU-lipase) toward C20 fatty acids with a Δ5 unsaturated double bond was studied by (Kojima et al., 2006). The HU-lipase had no specificity regarding the Δ5 unsaturated bond but showed low reactivity for DHA. The Ak-lipase was less reactive toward C20 fatty acids with this Δ5 unsaturated bond. The lipase catalyzed hydrolysis of cod oil (12.2% EPA, 6.9% DHA) followed by urea adduction produced FFA with 43.1% EPA and 7% DHA with HU-lipase. Under the same reaction conditions the hydrolysis of cuttlefish oil with AK-lipase increase the amount of EPA from 14.2to 16.8% and that of DHA from 16.3 to 44.6% in the hydrolyzed FFA fraction.

The concentration of the content of ω-3 PUFA (30.1%) in the residual acylglycerol fraction of salmon oil was attempted using native lipases from *A. niger, R. javanicus* and *Penicillium solitum* (Carvalho et al., 2009). All the lipases had 1,3-specificity, which preserved the PUFA in the *sn-2* position during hydrolysis. The most efficient enzyme was the lipase from *A. niger* which after a 60% hydrolysis, increased the content of DHA from 14.4 to 34% with a final total ω-3 PUFA content of 45% after 24h reaction at 45ºC. The hydrolysis with the other lipases was only of 20% and 3% respectively.

More recently the release of ω-3 PUFA from sardine oil by hydrolysis, using commercial lipases immobilized in different supports was studied (Fernandez-Lorente et al., 2011a; Fernandez-Lorente et al., 2011b). The lipases from *C. antarctica* lipase B, *T. lanuginosus* and *R. miehei* were immobilized in the porous support octyl-Sepharose (Fernandez-Lorente et al., 2011b). *C. antarctica* lipase B showed the highest selectivity towards PUFA versus oleic and palmitic acid, while *T. lanuginosus* and *R. miehei* lipases showed higher selectivity toward EPA versus DHA. The lipases from *C. antarctica* lipase B, *T. lanuginosus* and *R. miehei*, *C. rugosa*, *R. oryzae*, *P. fluorescens* and *Y. lipolytica* were immobilized in octyl Sepharose CL-4B and CNBr-Sepharose (Fernandez-Lorente et al., 2011a). The enzymes immobilized in octyl-sepharose were more active and had higher selectivity toward EPA. Immobilized *Y. lipolytica* lipase was the most selective while the *P. fluorescens* immobilized lipase was the most active but not selective. All the lipases hydrolyzed EPA faster than DHA and can be used to release a mixture of ω-3 PUFA or pure DHA by a first selective release of EPA.

Another source of fish oil and ω-3 PUFA are the viscera from the fish processing industry, which are generally considered waste. An attempt to increase the concentration of EPA and DHA by hydrolysis, using Atlantic salmon (*Salmo salar* L.) viscera as a source of fish oil, was

performed by (Sun et al., 2002). The commercially available lipases from Amano tested were derived from *A. niger*, *P. fluorescens*, *C. rugosa*, *R. oryzae*, *M. javanicus,* and *P. cepacia.* The concentration of EPA and DHA was obtained by hydrolysis and further isolation of the acylglycerols. The reaction conditions were 35°C for 20h. The highest hydrolysis, 70%, was achieved with the *C. rugosa* lipase after 80h. The concentration of EPA and DHA in the acylglycerol fraction was increased by the lipases from *P. cepacia* and *C. rugosa*. The concentration of EPA+DHA decreased with *C. rugosa* after 12h hydrolysis.

Alternative, oil was extracted from Nile perch viscera, and EPA and DHA were enriched in the glyceride fraction (Mbatia et al., 2010). Enzymatic hydrolysis was carried out with lipases from *C. rugosa*, *T. lanuginosus* and *P. cepacia*. The *sn-2* position of this oil was rich in palmitic acid, representing 51%, whit only 16% of EPA. DHA was equally distributed in the three position of the TAG. The highest enrichment of EPA and DHA was obtained with *C. rugosa* lipase, since it is a non-regioselective enzyme that effectively hydrolyzed the palmitic acid in the sn-2 position. This lipase increased EPA from 3% to 6%mol and DHA from 9% to 23%mol with recoveries of 42% and 55% respectively. *T. lanuginosus* lipase was unable to enrich EPA but increase DHA up to 38% mol with a recovery of 39%.

The optimization of the hydrolysis system using emulsions has also been studied (Byun et al., 2007; Koike et al., 2007). In the research of Byun et al., sardine oils were hydrolyzed in a water emulsion system by six commercially available lipases, lipases from porcine pancreas, *C. rugosa, C. cylindracea, R. niveus, M. miehei* and *Pseudomonas sp*. The optimal emulsion system found had a water-oil ratio of 40%(w/v), pH of 80.1, 40°C and gelatin as emulsifier. The sardine oil hydrolysis in the emulsion system was 50% higher than in the non-emulsion system. The degree of hydrolysis after 24h was higher with the *Pseudomonas sp*. lipase. The profile of the fatty acids in the acylglycerol fraction after hydrolysis in the emulsion system is shown in the following table (*Table 11*).

Table 11. Fatty acids of acylglycerols fractions of sardine oil hydrolyzed by various lipases, SFA: saturated fatty acids, MUFA: monounsaturated fatty acids and PUFA: polyunsaturated fatty acids (Byun et al., 2007)

Fatty acids	*Pseudomonas sp*	*Candida cylindracea*	Porcine pancreas	*Candida rugosa*	Sardine oil
SFA	46.5	48.4	42.8	50.2	60.2
MUFA	32.5	33.6	30.9	33.7	19.8
PUFA	21.0	18.0	26.3	16.1	20.0
Total	100.0	100.0	100.0	100.0	100.0

Koike et al. also increased the amount of DHA in the acylglycerol fraction by performing the hydrolysis reaction in a water oil microemulsion using soybean lecithin as emulsifier (Koike et al., 2007) (*Figure 26*). The lipase chosen was the lipase from *C. rugosa* (Fluka) because of its steric hindrance with the ester bonded DHA. The hydrolytic specificity of this enzyme is toward saturated and mono saturated fatty acids. The optimal conditions were found at a lecithin concentration close to the critical micelle concentration in organic solvent. This technique increased the DHA concentration in the triacylglycerols to 97%.

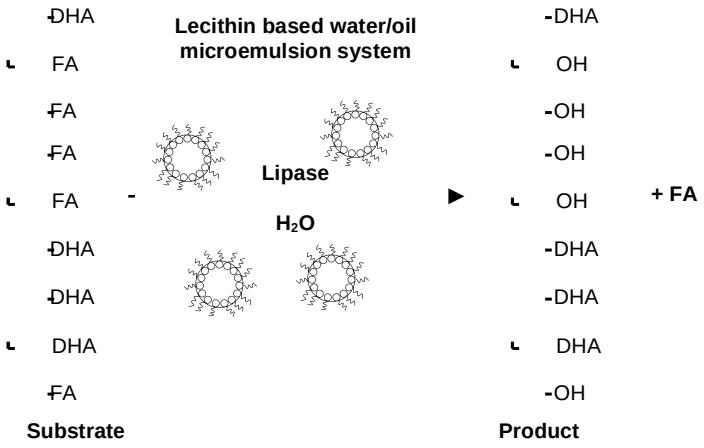


Figure 26. Microemulsion system, FA represent the different fatty acids present in fish oil (Koike et al., 2007).

2.4 Combined techniques

The combination of concentration methods has also been studied. A two step method, hydrolysis and selective esterification, offers the possibility of increasing the concentration of DHA (Moore and McNeill, 1996; Shimada et al., 1997a; Shimada et al., 1997b). Moore and McNeill used the lipase from *C. rugosa* (Amano) to hydrolyze Chilean fish oil and produce a glyceride fraction enriched in DHA (14.2 to 39.5%) and almost depleted in EPA (10.1 to 7.5%). The esterification step was carried out with *R. miehei* lipase (Novo).

Shimada et al. (1997b) chemically hydrolyzed tuna oils with a NaOH-ethanol solution to release the DHA, creating a FFA mixture with 23.2% DHA. The second step was the selective esterification of the FFA mixture with lauryl alcohol catalyzed by *R. delemar lipase (*Meito Sangyo). After a 20h reaction at 30°C, 72% of the FFA mixture was esterified and DHA was purified to 73% with 84% recovered in the unesterified fraction. In order to further increase the percentage of DHA in the unesterified fraction, this fraction was extracted and esterified again with *R. delemar* and *C. rugosa* lipase. After a second esterification with *C.*

rugosa DHA was purified to 83% with an 81% recovery of the initial but only 15% esterification. Using *R. delemar* lipase the second esterification reached 30% and DHA was purified to 89% recovering 71% of the initial content.

Optimization of the method was developed using enzymatic hydrolysis (Shimada et al., 1997a). The tuna oil (22.9%wt DHA) was hydrolyzed with *Pseudomonas* sp. lipase (Lipase AK, Amano), which has strong activity toward DHA. This lipase has preference toward DHA ester over the EPA ester, resulting in a FFA mixture rich in DHA (24.2%). Under the reaction conditions of 2.5 g of oils, 2.5 g of water and 5000U of lipase at 40ºC, after 48h 83%of the tuna oils DHA was recovered as FFA. The enzyme used for esterification was the lipase from *R. delemar* (Ta-lipase; Tanabe Seiyaku) because it esterifies selectively the other fatty acids present over DHA. The selective esterification was conducted at 30ºC, with a mole ratio of 1:2 FFA to lauryl alcohol, catalyzed with *R. delemar* lipase and with a reaction time of 20h. After esterification the concentration of DHA in the unesterified fraction increased from 24 to 72%wt, with 68.5% recovery. Once again a second esterification was performed under the same conditions and the DHA content increased to 91% with 60.3% recovery.

Some research has focused on the combination of chemical and enzymatic techniques in order to produce better PUFA concentrates (Gamez-Meza et al., 2003). Gamez-Meza et al. used the enzymatic hydrolysis to release the EPA and DHA of fish oil as free fatty acids (FFA). The FFA would be further concentrated using urea complexation. The hydrolysis of sardine oil was accomplished with commercial lipases from *Pseudomonas*, three immobilized (PS-CI, PS-CII and PS-DI) and two soluble lipases one from *P. fluorescens* and the other from *P. cepacia* (AK-20 and PS-30). The immobilized enzymes had higher EPA and DHA hydrolysis over the soluble enzymes (*Figure 27*). The highest degree of hydrolysis was obtained after 24h, with the PS-CI enzyme, releasing 81.5% of EPA and 72.3% of DHA, from the original content in the oil. The urea complexation reduced the content of saturated FFA (14:0, 16:0, 18:0, and 20:0) and monounsaturated FFA (16:1 and 18:1). Using the hydrolyzed mixture from PD-CI, urea complexation enriched the EPA from 14.5 to 46.2% and the DHA from 12.5 to 40.3%.

Another combination of purification techniques is hydrolysis, filtration and re-esterification (Linder et al., 2002). The hydrolysis was carried out with a specific *sn*-1 *sn*-3 hydrolytic lipase from *Aspergillus oryzae* (Novozyme SP 398). After a 40% hydrolysis in 24h the acylglycerol and fatty acid fractions were filtrated to separate the saturated fatty acids. After filtration the content of PUFA increased from 39.2 to 43.3%mol. The FFA were re-esterified with the 1,3-specific *R. miehei* (Lipozyme IM). The 90% re-esterification took 48h, producing

a mixture with 22.1% monoglycerides, 28.7% diglycerides and 43.4% triacylglycerols without modifying the PUFA content.

2.5 Patents

Some of the patents that exist to produce PUFA concentrates are presented in the following paragraph. A Japanese patent concentrates EPA and DHA using different lipases like *C. cylindracea, A. niger* and *R. miehei*. By selective hydrolysis the ester concentration of EPA reached 25% and 17%for DHA (Noguchi and Hibino, 1984). Other patent describes the process for concentrating and separating PUFA esters (Makoto and Hideki, 1992). The method separates EPA ester by a three step process. First a solution of fatty acid esters in a nonpolar solvent is placed in contact with zeolite so the EPA ester is adsorbed. Then the impurities are desorbed from the zeolite and finally the EPA ester is desorbed using a polar solvent. Another patent describes a process for making a mixture of PUFA esters (Luthria, 2002). The process is based on transesterification of oil from *Schizochytrium* sp. and alcohol in a base media to produce fatty acid esters. The next step is urea complexation to produce a urea fraction with saturated fatty acid esters and a liquid fraction with PUFA esters. This process can generate a methyl ester mixture with 23.4%wt DPA and 65.2%wt DHA

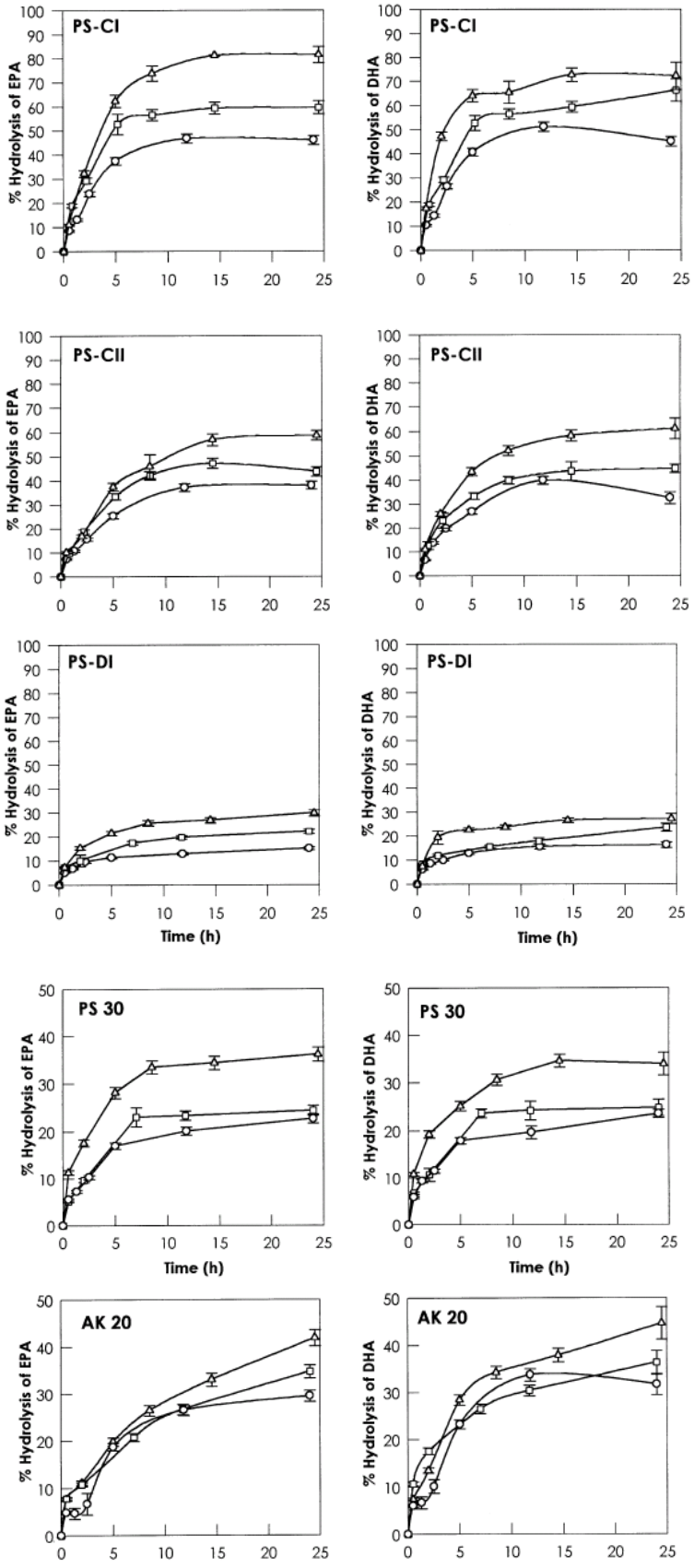


Figure 27. Enzymatic hydrolysis of EPA and DHA of sardine oil by different Pseudomonas lipases at 40°C and pH 7.0. Amount of enzyme (% w/w oil): ○ 0.25; □, 0.50; △, 0.75 (Gamez-Meza et al., 2003).

3. Conclusions

Lipases are able to discriminate between fatty acids in function of their chain length and saturation degree in three types of reactions: hydrolysis, trans-esterification, and esterification. These enzymes react more efficiently with the bulk of saturated and mono-unsaturated fatty acids than with the more resistant PUFAs. Indeed, the 5 and 6 double bonds, in EPA and DHA respectively, enhance steric hindrance in the active site of the lipases.

Lipases present different discrimination depending of the reaction used for ω-3 purification. Reactions can be classified in their order of efficiency: hydrolysis of triacylglycerides, esterification of free fatty acids and the most efficient one, hydrolysis of fatty acid ethyl esters. Therefore, the hydrolysis of fish oil or fish oils ethyl esters with lipases represent one of the most viable techniques for the purification of DHA since it can be carried out under mild conditions and the high specificity of the lipases does not generate undesirable by-products.

Several lipases have been used to concentrate ω-3 PUFAs, being *T. lanuginosus*, *C. rugosa* and *R. miehei* the most efficient ones. However, these lipases are incapable of producing concentrates with purities high enough for pharmaceutical applications. Discovering more specific enzymes for PUFAs purification is still a great challenge. In this thesis, the potentialities of the lipase Lip2 from *Yarrowia lipolytica* are investigated, in comparison with the lipases identified as efficient from *T. lanuginosus* and *C. rugosa*. However is possible that no lipases will be sufficiently active and selective to fulfil industrial requests, DHA purity higher than 85% with high yields of DHA recovery. Therefore, selectivity improvements of the lipases can be achieved using enzyme engineering tools.

Part IV: Structured Lipids

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Structured Lipids

1. Introduction

The production of fats and oils in 2011 was over 176 million tons (REA Holdings PLC, 2012). However many oils are not appropriate for human consumption, so they require specific modifications. Structured lipids are also known as functional lipids and can be produced by new chemical or technological techniques such as genetic engineering and enzymatic reactions. Structured lipids (SL) can be defined as triacylglycerols (TAG) that have been chemically or enzymatically modified in order to have specific fatty acids in the different positions of the glycerol (Iwasaki et al., 1999; Iwasaki and Yamane, 2000). The SL have modified properties to meet specific nutrition requirements and functional applications for food and pharmaceutical industries (Xu, 2000). Some desired nutritional benefits include the composition of the fatty acids, the concentration of essential fatty acids and the composition of the triacylglycerols (Gunstone, 2002).

The TAG composition modifies the way they are metabolized in the organism (digestion and absorption) and it changes its physical characteristics, like melting point and crystallization pattern. The dietary TAG cannot be absorbed as so and needs to be converted into more soluble products, so they are digested by the *sn-1,3* regiospecific gastric lipase. This lipase has preference for short and medium chain TAG, over long chain TAG (Reis et al., 2009).

The production of SL searches the development of modified lipids without the negative health effects exhibited by hydrogenated fats, where a decrease in the amount of unsaturated and essential fatty acids and the formation of *trans* isomers is observed (Wilkes, 2006). There are several methods that can be used to produce SL and they can be classified as technological or biological. The technological methods include mixing, distillation, urea fractionation, fractionation, hydrogenation, chemical and enzymatic interesterification. The biological methods are domestication of wild crops, modification of oils from a conventional approach, production of oils using genetic engineering techniques in oilseed crops and production of single cell oils (Willis and Marangoni, 1999).

The most interesting technological method is transesterification. Chemical and enzymatic transesterification are the best choice for post production modification of vegetable oils (Willis and Marangoni, 1999). Chemical transesterification can be defined as the shuffling of the fatty acids moieties within and among the TAG until they reach thermodynamic equilibrium (Marangoni and Rousseau, 1995). This method was first used in order to

improve the crystallization properties of lards. It has also been used in the margarine industry, to change the melting point profile of solid fats, to improve the compatibility of triacylglycerols and to change the emulsifying properties and crystallization behaviour (Wilkes, 2006). This method does not modify the original composition of the unsaturated fatty acids and avoids the production of *trans* isomers. The most common catalyst is an alkali metal. The catalyst can be destroyed by acid, water or peroxide, so all the impurities must be removed. The five main steps of chemical interesterification are: pre-treatment, reaction with the catalyst, deactivation, bleaching and deodorization of the interesterified fats. Chemical interesterification has low cost production and is easily scaled up. However, the disadvantages include random acyl transfers and changes of the original position of the fatty acids in the *sn*-2 position of the TAG (Willis and Marangoni, 1999; Gunstone, 2002; Gunstone, 2003). In vegetal oils the polyunsaturated fatty acid are mainly esterified in the *sn-2* position where they can provide nutritional benefits.

Chemical interesterification has been used to produce mixtures of butterfat and corn oil that can be used as butter analogues richer in PUFA but with similar organoleptic properties to butter (Rodrigues and Gioielli, 2003). A fat stock blend was produced by chemical interesterification of palm stearin and olive oil (da Silva et al., 2010) . It has also been used to enrich tuna oil with ω-3 PUFA, in order to avoid overconsumption of fish oils, which are also rich in cholesterol and saturated fatty acids (Klinkesorn et al., 2004). Chemical interesterification between ω-3 methyl esters and tuna oil, using sodium methoxide as catalyst, produced highest incorporation of EPA and DHA after 5 hours reaction at 80°C. The percentage of EPA in the triglyceride increased almost 70% and almost 50% for DHA.

2. Enzymatic production of structured lipids

Enzymatic interesterification has several advantages over the chemical process, since it can be developed under mild conditions, like low temperature and atmospheric pressure, the process can be performed in continuous mode, the products are free of impurities and the catalyst, the enzyme, can be reused (Xu, 2000; Neklyudov and Ivankin, 2002; de Castro et al., 2004). In addition the enzyme shows high stability in organic solvents, it does not require the presence of co-factors, is highly specific and can be improved by genetic engineering (Xu, 2000; de Castro et al., 2004). However, enzymatic interesterification has shown several problems in industrial implementation including the scale-up of the process and the cost of the enzyme, especially for the production of low added-value commodity fats for food industry and when biocatalysts with low operational stability are used.

By designing a SL with a precise chemical structure, the nutritional and pharmaceutical properties can be controlled. Since the synthesis of SL requires specific modifications, chemical interesterification is inadequate due to the random products it generates. The application of enzymatic interesterification, with specific lipases, promises the desired products due to the lipases fatty acid selectivity and regiospecificity (Willis and Marangoni, 1999). In most cases, the production of SL is achieved using immobilized enzymes since the immobilization process increases the stability and life of the enzyme, it is cost efficient and is easily removed from the reaction medium (Holm and Cowan, 2008). Immobilized lipases are an essential tool for the modification of lipids and they reduce the environmental impact of the process (Holm and Cowan, 2008). Nonetheless the amount and variety of commercial immobilized lipases is limited. Currently, new lipases are being obtained and new supports and immobilization methods are being used for the production of structured lipids.

Table 12. Lipases for the production of structured lipids. (L) long-chain fatty acids, (S) short-chain fatty acids and (M) medium-chain fatty acids. (Xu, 2000)

Lipase source	Fatty acid specificity	Regio specificity (sn)
Aspergillus niger	S, M, L	1, 3 >> 2
Candida lipolytica	S, M, L	1, 3 > 2
Humicola lanuginosa	S, M, L	1, 3 >> 2
Mucor javanicus	M, L >> S	1, 3 > 2
Rhizomucor miehei	S > M, L	1 > 3 >> 2
Pancreatic	S > M, L	1, 3
Pre-gastric	S, M >> L	1, 3
Penicillium roquefortii	S, M >> L	1, 3
Rhizopus delemar	M, L >> S	1, 3 >> 2
Rhizopus javanicus	M, L > S	1, 3 > 2
Rhizopus japonicus	S, M, L	1, 3 > 2
Rhizopus niveus	M, L > S	1, 3 > 2
Rhizopus oryzae	M, L > S	1, 3 >>> 2
Pseudomonas fluofescens	M, L > S	1, 3 > 2
Pseudomonas sp	S, M, L	1, 3 > 2
Rhizopus arrhizus	S, M > L	1, 3

The *sn*-1,3-specific lipases are the most important tool for the production of SL since these enzymes react on the *sn-1* and *sn-3* bonds without modifying the groups in the *sn-2* position. Different lipases can be used for SL production, however in recent years most research has been focus in microbial lipases and recombinant or mutant lipases (Xu, 2000). The *sn*-1,3-specific lipases from *A. niger, M. javanicus, R. miehei, Rhizopus arrhizus, R. delemar* and *R. niveus* are useful catalysts for interesterification (Gunstone, 2001). *Table 12* shows some lipases used for SL production.

In addition to the lipases, the production of SL requires vegetables oils or animal fats and oils as raw material. The selection of the appropriate oils is essential for the design of the SL. *Table 13* shows some examples of oils and fats rich in a specific fatty acid in the *sn*-2 position and those rich in a specific triacylglycerol (TAG). However the current production of oilseeds has decreased since the use of cultivable land is in competition between the production of grains and oilseeds. In addition, the market demand of grains and oil seeds has increased since they are also used as raw material in the production of biofuels (López Pérez, 2008). Therefore, the interest of producing SL from industrial residues and non-edible oils has increased.

Table 13. Oils rich in a specific fatty acid in the *sn*-2 position and those rich in a specific TAG. (P) Palmitic acid, (O) Oleic acid and (St) Stearic acid (Xu, 2000).

Fatty acid abundant in the *sn*-2 position	**Oils**	**Individual TAG**	**Oils rich in individual TAG**
Short-chain	Artificial oils: tributyrin, tricaproin, etc.	Tributyrin	Artificial tributyrin
Medium-chain	Artificial oils: medium-chain triacylglycerols	Medium chain TAG	Artificial medium-chain triacylglycerols
Lauric	Coconut oil	Tripalmitin	Palm stearin, urushi wax
Palmitic	Human milk fat, palm stearin,lard, urushi wax	POP	Palm oil mid fraction Chinese vegetable tallow
Stearic	Fully hydrogenated soybean oil, canola oil, etc.	Triolein	High oleic sunflower oil and canola oil, olive oil, teaseed oil
Oleic	High oleic sunflower oil, teaseed oil, olive oil, high oleic canola oil, palm oil mid-fraction, cocoa butter, Chinese vegetable tallow	StOSt	Sal fats, mango fat, kokum fat, shea oil
Linoleic	Safflower oil, sunflower oil, corn oil, soybean oil, cottonseed oil	Tristearin	Fully hydrogenated soybean oil and canola oil
Linolenic	Linseed oil, perilla oil	Trilinolein	Safflower oil, sunflower oil
EPA and DHA	Fish oils, microbial oils rich in long-chain polyunsaturated fatty acids	Trilinolenin	Linseed oil

The main types of SL can be classified, according to the FA present and their distribution in the glycerol backbone as: AAA, ABA, AAB and ABC types (*Figure 28*) (Iwasaki and Yamane, 2000). From these SL, type AAA can be synthesized chemically or enzymatically between glycerol and FA. However, the other types of SL require a regiospecific lipase. ABA type lipids can be synthesized with *sn*-1,3 lipases by catalyzing the reaction between two TAG or between one TAG and FFA or their ethyl ester. This type can also be produced from the acylation of glycerol and FFA with a *sn*-1,3 lipases in order to produced 1,3-diacyl-*sn*-glycerol and finalizing the reaction with a chemical acylation of the *sn*-2 position. ABB type is produced by the monosubstitution of the *sn*-1 or the *sn*-3 position of TAG with FFA or their ethyl esters. ABB and ABC types can be obtained with lipases that show higher *sn*-1 or *sn*-3 position stereo preference.

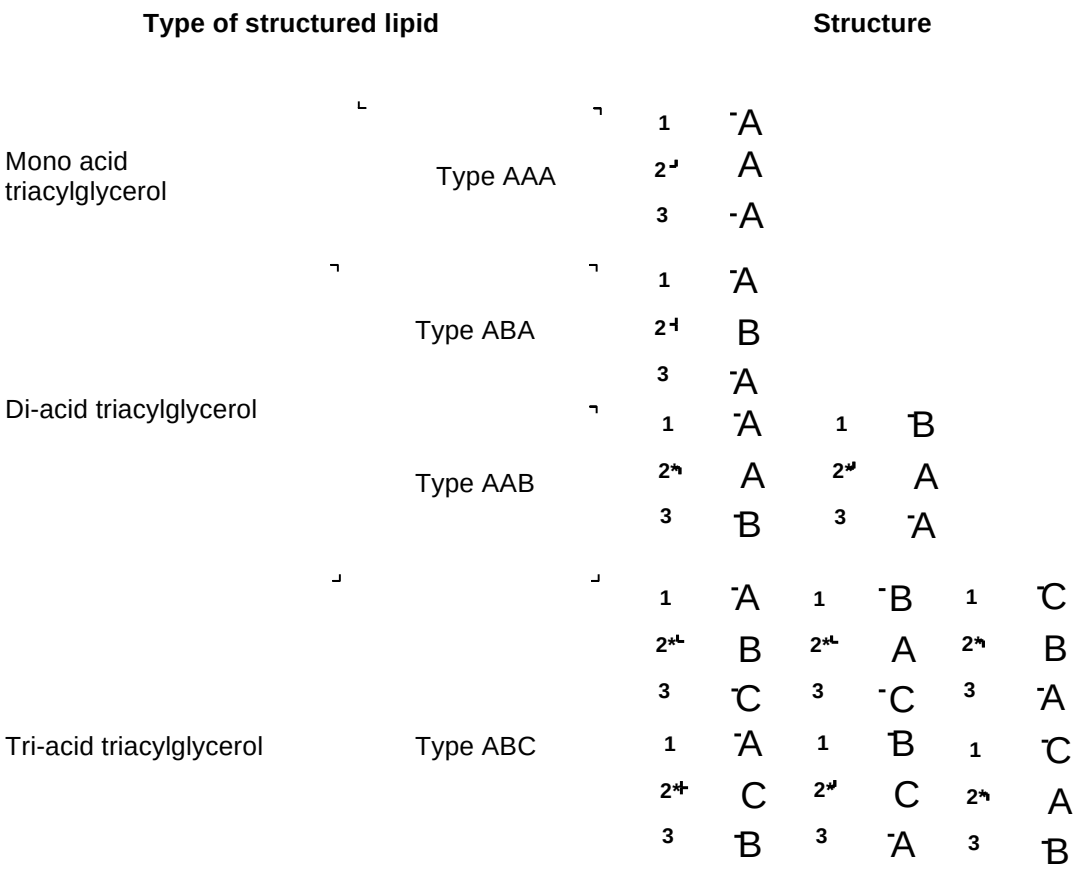

*Figure 28. Classification of structured lipids. A, B and C represent any fatty acid but they are not identical. Types AAB and ABC have chiral centers indicated by * (Iwasaki and Yamane, 2000).*

2.1 Cocoa butter equivalent and modified butter fats.

One successful application of enzymatic interesterification is the modification of the intermediate fraction of palm oil, to produce a cocoa butter equivalent, CBE. The process exchanges the palmitic acid in the *sn-1* and *sn-3* position for stearic acid, without changing the oleic acid in the *sn-2* position (Gunstone, 2001). CBE can also be produced by interesterification of tea seed oil with methyl palmitate and methyl stearate using immobilized pancreatic lipase as catalyst and reaching similar characteristics to those of cocoa butter (Wang et al., 2006a). Immobilized pancreatic lipase was also used to catalyze the production of CBE from the acidolysis reaction between refined olive pomace oil with palmitic and stearic acid (Ciftci et al., 2009). Using a high enzyme load of 40%, a molar ratio of 1:2:6 oil to palmitic and stearic acid at 45°C, maximum conversion was obtained after 3h. The product obtained showed no drastic differences from cocoa butter.

CBE can also be obtained by the incorporation of palmitic and stearic acid into triolein using Lipozyme RM IM (Ciftci et al., 2008). Under optimal conditions (10h, 45°C, enzyme load 20% and molar ratio 1:3:3 triolein to palmitic and stearic acid), the main TAG obtained were 1,3-dipalmitoyl-2-oleoyl--glycerol (POP), 1(3)-palmitoyl-3(1)-stearoyl-2-oleoyl-glycerol (POS) and 1,3-distearoyl-2-oleoyl-glycerol (SOS) with a percentage of 15.2%, 30.4% and 15.2%, respectively.

Butter fat can be modified by interesterification with oleic acid using the immobilized lipase from *M. circinelloides* (Balcao et al., 1998a). The amount of total saturated TAG decreased by 27% and the percentage of oleic acid in the TAG increased 27%, by reducing the presence of lauric, myristic and palmitic acids. Further research was carried out with the immobilized commercial enzyme from *M. javanicus* reducing the amount of lauric, myristic and palmitic acid in butter fat (Balcao et al., 1998b).

2.2 Modified oils

A substitute of margarine fat was produced by interesterification of two products of the palm oil industry, palm stearin and palm kern olein (Zainal and Yusoff, 1999). The interesterification reaction was catalyzed with the immobilized lipase of *R. miehei* at 60°, with a reaction time of 5h. The thermal characteristics of the product were comparable to those of the commercial margarines and it contained less than 0.5% of *trans* fats.

Oils can also bee modified in their fatty acid composition to change their original characteristics. One example is the enzyme catalyzed acidolysis between sunflower oil and a mixture of palmitic and stearic acid (Carrin and Crapiste, 2008). This reaction was carried out in a batch reactor using Lipozyme RM IM as catalyst, with a temperature between 50-60°C and a reaction time of 24-48h. The product had different melting profiles from those of the sunflower oil. Lipozyme RM IM was also used in a packed bed reactor to catalyse the interesterification of palm kernel oil with soybean oil obtaining an interesterification percentage of 19.6% (Nelson Moreno and Aide Perea, 2008).

Corn and canola oils were enriched with conjugated linolenic acid from bitter ground seed oil fatty acids (Elibal et al., 2011). Using a 10% enzyme load of the immobilized lipase from *T. lanuginosus* (Lipozyme TL IM) after 3h reaction, the optimal conditions for corn oil were 53.5°C and a ratio of 5.9:1 fatty acids to oil, which gave an incorporation of 41.4%. With the same enzyme load and reaction time, optimal conditions for canola oil were 54.2°C and a molar ratio of 6.8:1 with an incorporation of 37%.

2.3 Human milk fat substitutes

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, 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.

Human milk fat substitutes (HMFS) can be produced by interesterification of tripalmitin with oleic acid or methyl oleate. This SL has palmitic acid in the *sn*-2 position and oleic acid in the *sn*-1,3 positions, OPO , making it similar to human milk fat. OPO was synthesized using Lip1 from *C. rugosa* at 45°C, obtaining an incorporation of oleic acid of 37.7% with methyl oleate as acyl donor and of 26.3% with oleic acid (Srivastava et al., 2006). The same researches also produced OPO using Lipozyme RM IM as biocatalyst, reaching an incorporation of 49.4% at 65°C with methyl oleate as acyl donor. OPO was also produced using a lipase from *Bacillus stearothermophilus* which showed good thermo stability and conversion of 50% in 48h (Guncheva et al., 2008). Lipase DF from *R. oryzae* can catalyse the production of OPO in short reaction times, obtaining an oleic acid incorporation of 50.4% in 1h (Esteban et al., 2011). The production of HMFS from tripalmitin and oleic acid, in solvent-free media, catalysed by commercial immobilized lipases (Lipozyme TL IM, Lipozyme RM IM and Novozym 435), *Candida parapsilosis* lipase/acyltransferase (Tecelão et al., 2010), *Carica*

papaya latex (Tecelão et al., 2012b) and a heterologous *R. oryzae* lipase (Tecelão et al., 2012a) was also reported.

Chen et al. (2004) produced OPO from palm oil using a three-step method (Chen et al., 2004). The first step consisted of a low temperature fractionation of palm oil fatty acids to produce two acid fractions, one rich in palmitic acid (87.8%) and the second one rich in oleic acid (96%). The palmitic acid was transformed to ethyl palmitate by selective enzymatic esterification with ethanol, obtaining a concentration of 98.3%. In the second step, tripalmitin was obtained from the esterification reaction between ethyl palmitate and glycerol using Novozym 435. The final step was the production of OPO from tripalmitin and oleic acid using the lipase IM 60 from *R. miehei*. The product had 74%mol OPO with an incorporation of oleic acid of 66% and the *sn-2* position had 90.7% mol of palmitic acid. OPO was also efficiently produced with a two step process (Schmid et al., 1998). The first step consisted in the alcoholysis of tripalmitin with ethanol, using *R. miehei* commercial immobilized lipase to produce *sn*-2-monopalmitin with a purity of 95%. The second step consisted in the esterification with oleic acid for the synthesis of OPO with a yield of 72%.

Lard can be modified into human milk fat substitute by acidolysis with soybean oil fatty acids using Lipozyme RM IM (Yang et al., 2003). The optimal reaction conditions found were temperature 61°C, water content 3.5%, lard to fatty acids molar ratio 1:2.4, enzyme load 13.7% and reaction time 1h, obtaining a product similar to human milk fat. The same reaction was studied using a packed bed reactor with Lipozyme RM IM and producing a human milk fat substitute on a kg scale (Nielsen et al., 2006). However, the human milk fat substitute had lower oxidative stability than commercial products.

Human milk fat substitute rich in γ–linolenic acid was obtained from enzymatic interesterification of tripalmitin with hazelnut fatty acid and γ–linolenic acid in hexane, using the lipases Lipozyme RM IM and TL IM (Sahin et al., 2005). Incorporation percentages of 10% for γ–linolenic and 45% for oleic acid were obtained with both enzymes after 24h reaction at 55°C with molar ratios of 1:14.8 and1:14, total fatty acid to tripalmitin, for RM IM and TL IM respectively. Another human milk fat analogue rich in stearidonic acid was produced by acidolysis between the FFA and tripalmitin using Lipozyme TL IM (SilRoy and Ghosh, 2011). The enrichment of butter oil milk fat with conjugated linoleic acid was carried out with immobilized lipases from *C. antarctica* and *M. miehei* (Garcia et al., 2001). They were capable of reaching 80-90% interesterification of conjugated linoleic acid in a packed bed reactor with *C. antarctica*. Enrichment of human fat substitute with medium chain fatty

acids was also studied (Ilyasoglu et al., 2011). Reaction between tripalmitin and MCFA was catalyzed by Lipozyme RM IM and, under optimal conditions, a SL with 12.8% caprylic acid, 10.6% capric acid and 30% palmitic acid was obtained.

Human milk fat substitutes enriched with palmitic and DHA acids at the *sn-2* position and oleic acid at the *sn-1* and *sn-3* positions were also produced using a four-step process (Robles et al., 2011). First, tuna oil rich in palmitic acid and DHA was obtained, followed by a purification of these TAG. The third step consisted in the incorporation of oleic acid in the *sn-1* and *sn-3* positions and keeping the palmitic acid and DHA in the *sn-2* position, using the *sn*-1,3 selective lipase from *R. oryzae*. The final step was the purification of the SL. Another attempt to produce human milk fat substitute rich in ω-3 PUFA, from tripalmitin and a concentrate of in ω-3 PUFA rich in DHA, using commercial lipases and the lipase/acyltransferase of *Candida parapsilosis,* showed that the highest incorporation was obtained with Novozym 435 (21.6%) and Lipozyme TM IM (20%) while *Candida parapsilosis* lipase only gave 8.5% (Tecelão et al., 2010).

This method has also been used in the preparation of infant formulas that could be used as substitutes of breast milk (Maduko et al., 2007). A SL, similar to human milk fat, composed mainly of palmitic, oleic and linoleic acid was produced by enzymatic interesterification of tripalmitin and coconut, safflower and soybean oils, using the immobilized enzyme from *R. miehei,* Lipozyme RM IM. The milk fat substitute with highest resemblance to human milk fat was obtained after 12h reaction at 55°C with a molar ratio of 1:1 tripalmitin to vegetable oil blend. This blend was incorporated to skim caprine milk to obtain a human milk analogue based on goat milk.

2.4 Oils enriched with ω-3 PUFA

Using the appropriate enzyme, long chain polyunsaturated fatty acids, such as EPA and DHA, can be introduced to vegetable oils in order to increase their nutritional value. Blends of (i) palm stearin and soybean oil (Osorio et al., 2001), (ii) palm stearin and palm kernel oil (Osório et al., 2006; Osório et al., 2008; Pires et al., 2008; Osório et al., 2009b; Osório et al., 2009a) were efficiently enriched with ω-3 PUFA in the absence of solvent, using commercial immobilized lipases or immobilized C*. parapsilosis* enzyme, either in batch or in continuous reactors. The immobilized *C. antarctica* lipase (Novozym 435) was also used to incorporate EPA ethyl ester into primrose oil (Akoh et al., 1996). After a 24 h interesterification reaction, the content of EPA increased to 43%. DHA was also incorporated into primrose oil using

Novozym 435 (Senanayake and Shahidi, 2004). The highest incorporation of DHA was obtained after 24h and a molar ratio of 1:3 oil to DHA, obtaining 37.4% of DHA in the SL.

The enrichment of borage oil with DHA, using Novozym 435, was optimized using response surface methodology (Senanayake and Shahidi, 2002b). The predicted model gave maximal incorporation of DHA using the minimum amount of enzyme possible. Predicted optimal conditions were an enzyme activity of 165U, a reaction time of 24h at 50°C which produced a DHA incorporation of 34.1%. The results obtained under the predicted conditions gave a DHA incorporation of 35.6%. Borage oil was also modified by the incorporation of capric acid and EPA using Novozym 435, and *R. miehei* (Lipozyme IM60) (Akoh and Moussata, 1998). *R. miehei* lipase incorporated 10.2% of EPA and 26.3% of capric acid in the *sn*-1,3 positions, while *C. antarctica* lipase incorporated 8.8% of EPA and 15.5% of capric acid in the three positions.

Using the lipase of *R. miehei,* ω-3 PUFA were introduced into nut oil (Sridhar and Lakshminarayana, 1992) and soybean oil (Akimoto et al., 2003), reaching a concentration of 9.5% EPA and 8% DHA and 10.1% EPA and 34.1% DHA, respectively. The enrichment of coconut oil with ω-3 and ω-6 PUFA, using *R. miehei* lipase, was optimized by response surface methodology (Rao et al., 2002). Predicted optimal conditions for the incorporation of ω-3, were a molar ratio of 1:4 coconut oil to PUFA after a reaction of 34h at 54°C. For ω-6 incorporation, predicted optimal conditions were a molar ratio of 1:3 coconut oil to PUFA, a reaction time of 48.5h with a temperature of 39°C. Under these predicted conditions the maximal incorporation of ω-3 was of 13.65% and of 45.5% for ω-6. Response surface methodology was also used to optimize the acidolysis of soybean oil with FFA from sardine oil using *R. miehei* lipase (Lipozyme RM IM) (de Araujo et al., 2011). Highest incorporation of EPA and DHA reached 9.2% with a molar ratio of 3:1 FFA to oil, 12h reaction, 40°C and 10% enzyme load.

Another long chain polyunsaturated fatty acid that can be introduced in vegetable oils is the α-linolenic acid, ALA. This PUFA was introduced into rice bran oil using *R. miehei* immobilized lipase (Chopra et al., 2011). The highest incorporation obtained was of 18% under optimal conditions, such as temperature 37.5°C, reaction time 4.5h, a substrate ratio of 1-1.9 and an enzyme load of 1-2%.

The lipase of *Pseudomonas* sp. (PS-30) was used to catalyze the acidolysis reaction between high laurate canola oil and the ω-3 PUFA DHA and EPA (Hamam et al., 2005;

Hamam and Shahidi, 2005b). The system was optimized by response surface methodology. Optimal conditions for DHA incorporation were 4.79% enzyme load, 46.1°C and 31.1h, giving an incorporation of 37.3%. Regarding EPA, the optimal conditions were 4.6% enzyme load, 40°C and 26.2h, which gave an incorporation degree of 61.6%.

Different enzymes were tested for their ability to incorporate long chain fatty acids into triolein (Hamam and Shahidi, 2007), trilinolein and trilinolenin (Hamam and Shahidi, 2008). The studied lipases were *C. antarctica* (Novozym 435), *R. miehei* (Lipozyme-1M), *Pseudomonas* sp. (PS-30), *A. niger* (AP-12), and *C. rugosa* (AY-30). The incorporation of stearic, α-linolenic, γ-linolenic, arachidonic acids and DPA, into triolein, was higher with *R. miehei* lipase. However, the highest incorporation of linoleic acid, EPA and DHA into triolein was found with *Pseudomonas* sp. lipase. Also, this lipase incorporated the highest amount of stearic acid (C18) and ω-6 fatty acids into trilinolein showing preference for the C18 and for the γ-linolenic acid (ω-6). With *C. antarctica* and *R. miehei* lipases, the highest incorporation of ω-3 PUFA into trilinolein with preference for the α-linolenic acid was observed. Regarding trilinolenin, *R. miehei* and *Pseudomonas* sp. lipases showed to be the best biocatalysts for the incorporation of C18 and ω-3 fatty acids, preferring stearic acid (C18) and EPA (ω-3). The better incorporation of ω-6 was found with *Pseudomonas* sp., *C. rugosa* and *M. miehei* lipases.

Enzymatic acidolysis has also been used to enrich fish oils with ω-3 polyunsaturated fatty acids. The *sn-1,3* lipase from *R. miehei* was used to catalyze the acidolysis reaction between ω-3 PUFA and menhaden oil under supercritical carbon dioxide conditions, in order to increase their PUFA content (Lin et al., 2006). Using *R. miehei* lipase, an increase of 10% of ω-3 PUFA in cod liver oil was achieved (Yamane et al., 1993). EPA and DHA were introduced into sardine oil using the lipase from *Pseudomonas* sp., producing an oil with 65% EPA and DHA (Adachi et al., 1993).

2.5. Structured lipids type MLM

MLM are SL which have medium chain fatty acids (MCFA), between 6 and 10 carbons, in the *sn*-1 and *sn*-3 position, and long chain fatty acids (LCFA), with more than 12 carbons, in the *sn*-2 position. These SL do not show the health problems related with the long chain TAG (LLL) and have desired nutritional, energetic and pharmaceutical properties (Huang and Akoh, 1996). The pancreatic lipase preferably hydrolyzes the *sn*-1 and *sn*-3 position with MCFA over the LCFA, thus the *sn*-2 monoacylglycerols are easily absorbed in the intestine

(Iwasaki and Yamane, 2000). MLM are used as easily accessible energy sources for patients with absorption problems (Huang and Akoh, 1996) since their hydrolysis and absorption rate is faster than for LLL triacylglycerols (Jandacek et al., 1987). In addition, since MCFA present lower caloric value than the long chain fatty acids (5 kcal/g against 9 kcal/g) and are metabolized as glucose, therefore not stored as fat tissue in the human body, MLM can be used in low caloric foods.

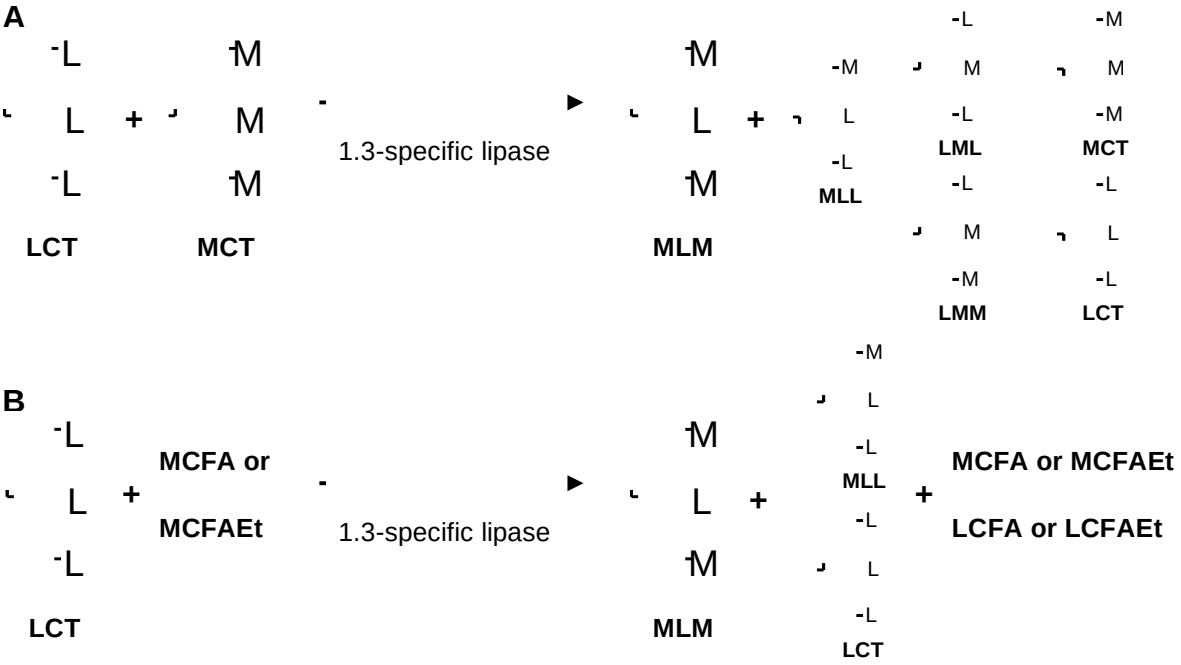

Figure 29. Synthesis of MLM (A) Interesterification between LLL and MMM. (B) Acidolysis of LLL and MCFA or MCFA esters. (Iwasaki and Yamane, 2000)

MLM can be produced from a mixture of medium chain TAG (MMM) and LLL, using *sn*-1,3 lipases. This reaction generates a mixture of TAG which is easily recovered as a TAG fraction by removing the catalyst. However, the TAG fraction has several species (MLM, LML, MLL, LMM, MMM and LLL) which are hard to isolate. So, this method is preferred for the modification of the physical properties of a mixture (Iwasaki and Yamane, 2000). Another strategy for the production of MLM is the lipase catalyzed reaction between LLL and an excess of MCFA of their ethyl esters (*Figure 29*). This reaction will specifically substitute the FA in the *sn*-1 and *sn*-3 position with MCFA without modifying the FA in the *sn-2* position, producing triacylglycerols with one MCFA and two LCFA or triacylglycerols with two MCFA and one LCFA (Gunstone, 2001). The TAG obtained from the reaction can be separated from the FA or methyl esters by molecular distillation (Iwasaki and Yamane, 2000). Between FA and their ethyl esters, the reaction rate as acyl donor is higher for the ethyl esters. In addition, the interesterification rate is higher for long chain alcohols, followed by triacylglycerols, methyl esters and glycerol (Huang and Akoh, 1996).

2.5.1 MLM enriched with caprylic acid

One of the most interesting MLM is the SL with caprylic acid in the *sn-1,3* positions and an unsaturated or polyunsaturated fatty acid in the *sn-2* position (*Figure 30*). Using the commercial immobilized lipases from *R. miehei* (IM60) and *C. antarctica* (SP435) this SL was obtained by interesterification of caprylic acid ethyl ester and triolein in a solvent system (Huang and Akoh, 1996). The reaction product, using with *R. miehei* lipase as catalyst, had 41.7% dicaprylolein, 46% monocaprlylolein and 12.3% triolein; while with *C. antarctica* lipase the reaction produced 62% dicaprylolein, 33.5% monocaprlylolein and 4.5% triolein.

The immobilized *sn-1,3* specific lipase from *R. delemar* was used to catalyze the reaction between safflower or linseed oil and caprylic acid at 30°C (Shimada et al., 1996b). Under these conditions, between 45-50%mol of the TAG fatty acids were substituted by caprylic acid and the enzyme remained active for 55 cycles of 48h. Recovering the TAG and repeating the reaction with caprylic acid increased the incorporation of this fatty acid. After three cycles, all the *sn*-1,3 positions were substituted with caprylic acid.

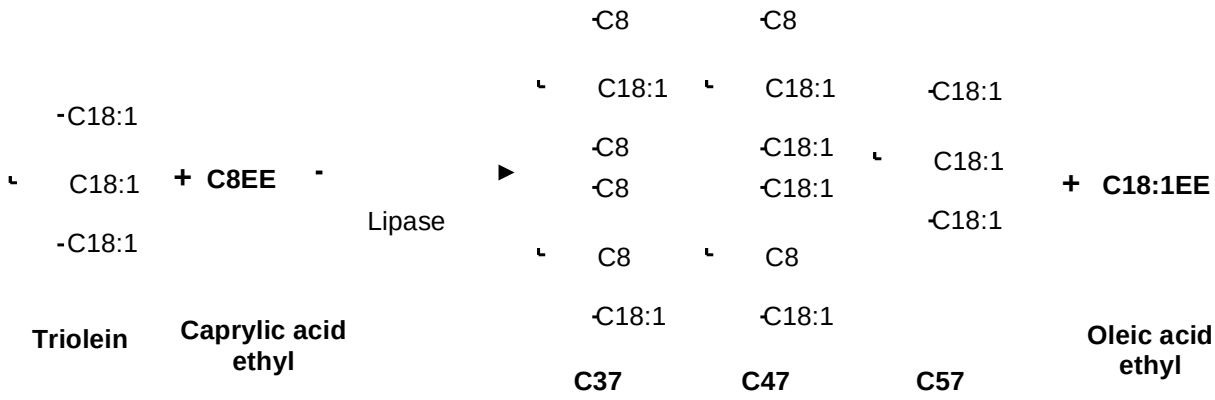

Figure 30. Production of MLM with a non specific lipase (Huang and Akoh, 1996).

This SL can also be produced by incorporating caprylic acid intro perilla oil (Kim et al., 2002). Using the commercial immobilized enzymes from *R. miehei* (Lipozyme IM) and *T. lanuginosus* (Lipozyme TL IM) in a hexane system, after 24h the incorporation was of 48.5% mol and 51.4% mol respectively. Lipozyme TL IM was used to catalyze the acidolysis reaction between soybean oil and caprylic acid (Li et al., 2008). The reaction was carried out in a solvent-free system and the optimal conditions to obtain a caprylic incorporation of 27%mol, were 16% enzyme load, molar ratio of 1:4 caprylic acid to soybean oil, 4% of water, with a temperature of 40°C, agitation of 150rpm and a reaction time of 20 h.

The commercial immobilized enzymes from *R. miehei* (IM 60) was used to catalyze the acidolysis reaction between peanut oil and caprylic acid (Lee and Akoh, 1998). The best conditions found were 50°C, reaction time of 72h and molar ratio of 1:2 peanut oil to caprylic acid reaching 30% incorporation. Immobilized lipase from *R. miehei* (Lipozyme RM IM) and from *Pichia lynferdii* NRRL Y-7723 were used to produced a MLM by the acidolysis reaction between borage oil and caprylic acid (Kim et al., 2010). Incorporation of caprylic acid was of 48.7% with Lipozyme RM IM, at 40°C, and of 47.5% for NRRL Y-7723 at low temperatures between 10°C and 15°C.

The heterologous enzyme of *R. oryzae* immobilized in Eupergit was used to produce a MLM from olive oil and caprylic or capric acids (Nunes et al., 2011b). The reaction was carried out at 40°C, with a molar ratio of 1:2 olive oil to FFA and after 24h, caprylic acid incorporation was of 21.6% and 34.8% for capric acid. Caprylic acid was also introduced into olive oil using a bench-scale continuous packed bed reactor with the lipase from *R. miehei* (Lipozyme IM 60) (Fomuso and Akoh, 2002). Optimal production was obtained with a flow rate of 1mL/min, residence time of 2.7h, temperature of 60°C and molar ratio oil 1:5, olive oil to caprylic acid. This reactor system was also used to catalyze the reaction between palm olein and caprylic acid (Lai et al., 2005). After 24h reaction in the reactor the incorporation of caprylic acid was of 30.5%. A pilot continuous packed bed reactor was used for the incorporation of caprylic acid into rapeseed and safflower oil catalyzed by *R. miehei* lipase (Lipozyme IM) (Xu et al., 1998).

The incorporation degree of caprylic acid into vegetable oils depends on the composition of the original oil (SilRoy and Ghosh, 2011). Silroy and Ghosh (2011) analyzed the incorporation of caprylic acid to rice bran, ground nut and mustard oils using *C. antarctica* lipase (Novozym 435). Incorporation after 72h was of 30.8%, 34.2% and19.5% for rice bran, ground nut and mustard oils, respectively.

Other applications of enzymatic acidolysis include the production of SL rich in 1,3-dicapryloy-2-γ-linolenoyl glycerol from borage oil rich in γ–linolenic acid and caprylic acid, using the immobilized lipase from *R. oryzae* (Kawashima et al., 2002). Using a ratio of 1:2, oil to caprylic acid, in a continuous reactor with 15g of immobilized lipase from *R. oryzae* at 30°C, the reaction produced 44.5%mol of the desired SL and after purification the concentration increased to 56.6%mol. This reaction was also carried out in a packed bed continuous reactor using *R. delemar* lipase in a solvent-free system (Shimada et al., 1999). At 30°C, with a flow rate of 4.5 mL/h with 8g of the immobilized enzyme, the incorporation of caprylic acid was of 50-55% mol and the reactor was stable for 60 days. The products were

separated by molecular distillation and further analysis showed that the caprylic acid was only incorporated in the *sn-1* and *sn-3* positions.

Structured lipids rich in caprylic acid and conjugated linoleic acid, CLA, are also of interest. A SL rich in caprylic acid in the *sn-1* and *sn-3* positions and CLA in the *sn-2* position were synthesized by acidolysis of TAG rich in CLA with caprylic acid, using *R. miehei* immobilized lipase and a molar ratio of 1:10, triglyceride to fatty acid (Kawashima et al., 2004). This SL was also produced using coconut oil rich in CLA and tricaprylin, catalyzing the reaction with *R. miehei* immobilized lipase at 65°C for 48h, under nitrogen (Rocha-Uribe and Hernandez, 2004).

Caprylic acid was also introduced into chicken fat using *Carica papaya* latex as lipase (Lee and Foglia, 2000). Optimal conditions were 1:2 molar ratio, chicken fat to caprylic acid, and temperature of 65°C obtaining an incorporation of 23.4%. *C. papaya* lipase latex was also studied for its ability to incorporate MCFA esters into tripalmitin (Gandhi and Mukherjee, 2001).

SL rich in caprylic acid can also be produced using two triacylglycerols as substrates. Soumanou et al. (1997) used tricaprylin and peanut oil and the immobilized microbial lipases from *R. miehei*, *Candida* sp. and *C. viscosum* as catalysts. The best results were obtained with *R. miehei* lipase, at 50°C, having a yield of SL of 79%. Using a two-step process, a MLM with caprylic acid in the *sn-1,3* positions and oleic or linoleic acid in the *sn-2* position, was produced from peanut oil and caprylic acid (Soumanou et al., 1998). The first step consisted in the production of 2-MAG by ethanolysis of peanut oil with immobilized *R. delemar* lipase. The second step was the esterification of the 2-MAG, producing a SL that had 90% of the caprylic acid in the *sn-1,3* positions and 98.5% of the *sn-2* position had unsaturated fatty acid.

2.5.2 MLM enriched with capric acid

Another MLM of interest is the SL with capric acid in the *sn-1,3* positions and an unsaturated or polyunsaturated fatty acid in the *sn-2* position. Capric acid was introduced into lard using the commercial immobilized lipase TL IM from *T. lanuginosus*, reaching an incorporation percentage of 50.14% mol (Zhao et al., 2006; Zhao et al., 2007). The optimal conditions were, 5-10% of enzyme load, a reaction time of 24h, with a molar ratio of 1:2, lard to capric acid, and a temperature of 50-55°C. The commercial immobilized lipase Lipozyme TL IM was also used to introduce capric acid into olive oil (Oh et al., 2009). The reaction was

carried out with a molar ratio of 1:3 olive oil to capric acid and a temperature of 50°C and after 8h an incorporation of 50% mol was obtained.

This SL was produced using the commercial immobilized lipases IM 60 from *R. miehei* and SP 435 from *C. antarctica* to catalyze the reaction between tricaprin and trilinolein (Lee and Akoh, 1997). This reaction produced two types of SL: one with two molecules of capric acid and another with two molecules of linoleic acid. A similar SL was produced from tricaprin and tristearin using immobilized *R. miehei* lipase (Lipozyme IM 60) with an enzyme load of 10% and a molar ratio of 1:1 (Akoh and Yee, 1997). Under the best reaction conditions the product had 84.7% of modified TAG.

MLM containing capric acid were obtained by acidolysis of virgin olive oil, in hexane or in solvent-free media, using commercial immobilized lipases (Nunes et al., 2011a) or in the absence of a solvent using a heterologous lipase from *R. oryzae* immobilized in different supports, as catalysts (Nunes et al., 2011b; Nunes et al., 2012a; Nunes et al., 2012b).

2.5.3 MLM enriched with polyunsaturated fatty acids.

Other structured lipids of interest are the MLM enriched with polyunsaturated fatty acids like EPA and DHA in the *sn-2* position. The polyunsaturated fatty acids present a higher absorption in the organism when they are present as triacylglycerols rather than as their methyl or ethyl esters forms. Also, their absorption is higher when the PUFA are located in the *sn*-2 position, position that is not hydrolyzed by the pancreatic lipase (Lawson and Hughes, 1988). It has also been shown than the methyl and ethyl ester forms of the PUFA are hydrolyzed four times slower than the corresponding triacylglycerols (Yang et al., 1989).

This type of SL has been produced by a two step process, production of 2-monoacylglycerols (2-MAG) rich in PUFA from fish oils by ethanolysis, with a *sn-1,3* specific lipase, followed by a lipase catalyzed esterification with caprylic acid or its ester (Irimescu et al., 2001a; Muñío et al., 2009) . This reaction produces a SL rich in caprylic acid in the *sn-1* and *sn-3* positions and a PUFA in the *sn-2* position. Irimescu et al. (2001) produced the 2-MAG from fish oil using immobilized *C. antarctica* lipase (Novozym 435) with a yield of 92.5% and 43.5% of the fatty acids in the *sn*-2 position was DHA. Using the immobilized *R. miehei* lipase (Lipozyme RM IM) the reaction between the 2-MAG and ethyl caprylate gave 85.3% of TAG with two caprylic acids in the *sn*-1,3 positions, 13% TAG with one caprylic acid and 1.7% of tricaprylin. From the di-substituted TAG 51%wt had DHA in the *sn-2* position. To increase the purity of the SL, the *sn*-2-MAG were produced by ethanolysis of

tridocosahexaenoylglycerol or trieicosapentaenoylglycerol, with immobilized *C. antarctica* lipase (Novozym 435) followed by re-esterification with immobilized *R. miehei* lipase (Lipozyme RM IM) (Irimescu et al., 2001b). Muñío et al. (2009) also produced the *sn*-2-MAG rich in PUFA using Novozym 435, with a reaction yield of 65%, and the esterification reaction, using immobilized lipase D from *R. oryzae*, showed an incorporation percentage of 64%.

Kawashima et al. (2001) modified the two-step process by changing the production of *sn*-2-MAG by the production of TAG rich in PUFA in the three positions (Kawashima et al., 2001). *C. antarctica* lipase was used for the production of TAG rich in PUFA and *R. delemar* lipase was used for their acidolysis with caprylic acid, reaching an incorporation of 41%mol. After three successive acidolysis reactions the content of caprylic acid reached 66% mol. Following the two step process, 1,3-dicapryloyl-2-eicosapentaenoylglycerol was synthesized from tri-eicosapentaenoylglycerol (tri-EPA) and ethyl caprylate (Irimescu et al., 2000). Immobilized *C. antarctica* lipase was used for the production of tri-EPA and *R. miehei* immobilized lipase for the esterification reaction of tri-EPA and ethyl caprylate, which had a yield of 91%. Nagao et al. produced the same type of SL by producing oil rich in arachidonic acid in the *sn*-2 position using *C. rugosa* lipase, followed by an acidolysis with *R. oryzae* lipase that produced a SL with an incorporation degree of caprylic acid of 44% (Nagao et al., 2003).

The immobilized lipase from *R. delemar* was also used for the synthesis of MLM rich in functional fatty acids in the *sn*-2 position in a one step process (Shimada et al., 1996a; Shimada et al., 1997c). The desired MLM with caprylic acid in the *sn*-1,3 positions and DHA in the *sn*-2 position was obtained from the lipase catalyzed reaction between tuna oil and caprylic acid. This enzyme was capable of substituting 65% of the tuna oil FA in the *sn*-1,3 positions with caprylic acid and all the resulting TAG were mono of disubstituted. Using a packed bed reactor in a solvent-free system, the incorporation of caprylic acid into tuna oil was of 45% and 91% of the caprylic acid was incorporated in the *sn*-1,3 positions (Hita et al., 2007).

Similar experiments were carried out using *R. miehei* immobilized lipase to catalyze the acidolysis reaction between menhaden oil and caprylic acid (Akoh and Moussata, 2001). Under optimal conditions, the SL had 29.5% of caprylic acid and the PUFA in the fish oils remained unmodified. This SL was also produced in a packed bed reactor at 65°C, a molar ratio of 4-5 and a residence time of 180-220min obtaining a caprylic acid incorporation of 38.8% (Xu et al., 2000). A packed bed reactor was also used by Camacho Paez et al. (2002)

to produced a SL from cod liver oil and caprylic acid using immobilized *R. miehei* lipase (Lipozyme RM IM) (Camacho Paez et al., 2002). The produced SL had 57% caprylic acid, 5.1%, EPA, 10% DHA and 6.3% palmitic acid.

A similar SL was produced by acidolysis reaction between capric acid and fish oil rich in EPA and DHA, using the immobilized *R. miehei* lipase (Jennings and Akoh, 1999; Senanayake and Shahidi, 2002a). Jennings and Akoh (1999) reached after 24h, in a hexane system, a capric acid incorporation of 43% which was higher than that obtained in the solvent free system, which only reached 31.8%. After optimization, capric acid incorporation reached 65.4%, in the hexane system, and 56.4% in the solvent-free system. The optimal reaction conditions found by Senanayake and Shahidi (2002a) were molar ratio 1:3 oil to fatty acids, temperature of 45°C, reaction time of 24h and an enzyme load of 10%(w/w of substrates) obtaining a SL with 2.3% EPA, 7.6% DHA and 27.1% capric acid.

The lipases from *R. miehei* and *Pseudomonas* sp. KWI-56 were used to catalyse the reaction between single cell oils, rich in DHA and DPA, with caprylic acid (Iwasaki et al., 1999; Yankah and Akoh, 2000). The incorporation degree of caprylic acid was of 23% mol with *R. miehei* lipase, while with *Pseudomonas* lipase was of 65% (Iwasaki et al., 1999). These results prove that each lipase has different specificity toward different PUFAs. With *R. miehei* lipase, Yankah and Akoh (2000) obtained a caprylic acid molar incorporation of 47.6%.

The incorporation of capric acid into single cell oils rich in DHA and DPA were studied using five commercial lipases, *C. antarctica*, *R. miehei*, *Pseudomonas* sp., *A. niger* and *C. rugosa* (Hamam and Shahidi, 2005a). The highest incorporation of capric acid was obtained with *Pseudomonas* sp. lipase (27.9%) and this acid was esterified mainly in the *sn*-1,3 positions, while DHA and DPA were found in the *sn*-2 position.

2.6 Other Structured Lipids

A reduced calorie SL was produced by the incorporation of caproic and butyric acid into triolein, obtaining a mixture of MLM and a lipid with short chains fatty acids (SCFA) in the *sn-1,3* positions and a LCFA in the *sn-2* position (SLS) (Fomuso and Akoh, 1997). The optimal condition were found with a molar ratio of 1:4:4 triolein, caproic acid and butyric acid, with 10% enzyme load of *R. miehei* lipase (IM 60) at 55°C. After 24h reaction the product had 49% of di-substituted and 38% of mono-substituted TAG. SL rich in caproic acid can also been obtained by interesterification between trilinolein and tricaproin with *R. miehei* lipase

(IM 60) and *C. antarctica* lipase (SP 435) (Fomuso and Akoh, 1998). Reaction was carried out with a molar ratio of 1:2 trilinolein to tricaproin, in hexane at 45°C, catalyzed by Lipozyme IM 60, and 55°C for SP 435. The products obtained with IM 60 had 53.5% of TAG with two molecules of caproic acid and 22.2% of TAG with one molecule of caproic acid, and the products from SP 435 reaction had 41% and 18% respectively. The reaction was optimized by changing tricaproin as acyl donor for caproic acid.

SL can also be produced for coating applications in the food industry (Sellappan and Akoh, 2000). These lipids were produced by acidolysis of tristearin with oleic and lauric acid in a hexane solvent system, using Lipozyme IM60 as lipase. The reaction product was more effective than cocoa butter in the prevention of moisture absorption.

Other SL that have been successfully produced are: 1,3-dilauroyl-2-oleoylglycerol (LaOLa), obtained with a purity of 70% after enzymatic acidolysis between triolein and lauric acid with catalyzed by immobilized *R. miehei* lipase (Miura et al., 1999); 1,3-distearoyl-2-oleoylglycerol and 1(3)-2-dioleoyl-1(3)-monostearoyl glycerol with purities of 36% and 27% respectively, produced from the acidolysis of rapeseed oil with stearic acid or methyl stearate, catalyzed by immobilized *R. arrhizus* (Gitlesen et al., 1995); monoleyl-1(3)-cinnamate and dioleyl-2-cinnamate synthesized with Novozym 435 from cinnamoylated lipids and triolein (Karboune et al., 2005); nutraceutical phenolic lipids synthesized from dihydrocaffeic acid and flaxseed oil (Sabally et al., 2006); phospholipids obtained from the reaction between soybean phospholipids and free fatty acids, catalyzed by Lipozyme TL IM (Peng et al., 2002) and low calorie SL produced by acidolysis of stearic acid with triacetin, incorporating the stearic acid into the *sn-1* and *sn-3* positions, using Chirazyme L-2 as catalyst and obtaining 88% of the desired SL (Yang et al., 2001).

3. Conclusions

SL are of great interest since they avoid health problems related with long chain TAG and have targeted nutritional, pharmaceutical and energetic properties. MLM represent one of the most interesting SL since they present lower caloric value than the natural fats and can be used as easily accessible energy sources for patients with absorption problems. Enzymatic synthesis, using specific lipases, represents the most effective method mainly due to the high specificity of the process. In this work we study the ability of the immobilized lipase Lip2 from Y. lipolytica to produce MLM type structured lipids.

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Chapter II: Results

Publication 2

Enzymatic trans-esterification of a highly concentrated long chain ω3 polyunsaturated fatty acid ethyl ester with a group B pro-vitamin alcohol for prevention and treatment of cardiovascular diseases

This part of the thesis presents the functionalization of polyunsaturated fatty acids Omega-3 (ω3-PUFAs). We studied the production of a pharmaceutical molecule, which is in clinical trial for the treatment of cardiac arrhythmia by the french company Laboratoires Pierre Fabre. This molecule, the DHA-nicotinol, is an ester enzymatically-synthesised by the transesterification of *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) ethyl ester with nicotinol.

Omega-3 PUFAs are of interest since *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) and *cis*-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA), the most important Omega-3, present anti-thrombotic and anti-inflammatory properties that reduce risk factors of arthritis rheumatoid, cancer, cardiovascular diseases, myocardial infarction, bronchial asthma, inflammatory intestinal diseases, diabetes and Alzheimer's disease.

The co-substrate is the nicotinol (3-hydroxymethylpyridine), an alcohol from the group B pro-vitamins. Nicotinol is the alcohol derived from nicotinic acid, also known as niacin (Vitamin B3) which after absorption, is rapidly converted into nicotinic acid. The nicotinic acid presents the ability to substantially decrease plasma free fatty acid, triglyceride, VLDL (very-low-density lipoprotein) and LDL (low-density lipoprotein) levels and to raise the plasma concentration of protective HDL (high-density lipoprotein). DHA-nicotinol would present the cumulative properties of the two reactants.

In addition to enzyme and reaction medium selection, the enzymatic trans-esterification of DHA ethyl esters with nicotinol was optimised by varying the medium, working temperature, enzyme/substrate and ester/alcohol ratios. Finally we maximised both the kinetics and the conversion obtained at equilibrium.

Enzymatic trans-esterification of a highly concentrated long chain ω3 polyunsaturated fatty acid ethyl ester with a group B pro-vitamin alcohol for prevention and treatment of cardiovascular diseases.

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Abstract

Consumption of Omega-3 polyunsaturated fatty acids (ω3-PUFAs), especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), reduces the incidence of cardiovascular diseases. Nicotinol, an alcohol from the group B pro-vitamin, is recommended in dyslipidemia, hypercholesterolemia and hyperlipidemia treatment due to its degradation in nicotinic acid. The enzymatic trans-esterification of highly concentrated ω3-PUFAs ethyl esters with nicotinol was optimised in order to synthesise an ester presenting the cumulative properties of the two reactants. Commercially immobilised lipase B from *Candida antractica*, Novozyme 435, used at a temperature of 60°C, was demonstrated to be the best catalyst. An eco-compatible solvent free system enabled enzyme activity, conversion at thermodynamic equilibrium and volumetric productivity to be maximized. From both kinetic and thermodynamic points of view, it was demonstrated crucial to evacuate ethanol co-product from the reaction medium. Using nitrogen bubbling, 97% conversion of DHA ethyl ester to DHA-nicotinol was obtained in 4 hours using 45 $g.L^{-1}$ of enzyme. In these conditions, a productivity of 4.2 g of product $\cdot h^{-1} \cdot$g of $enzyme^{-1}$ was obtained.

1. Introduction

Several epidemiological studies among populations consuming high quantities of fish have demonstrated an inverse relationship between its consumption and cardiovascular diseases. Omega-3 polyunsaturated fatty acids (ω3-PUFAs), especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have been presumed to be the active compounds. These fatty acids are essential for mammalians growth and development, and as their synthesis in the organism from α-linolenic acid is low, a dietary intake is therefore indispensable (Caballero et al., 2006). Dietary uptake of ω3-PUFAs can be provided as a triglyceride or a phopsholipid form, as found in fish or fish oil, or as a purified and concentrated ethyl ester form. In both cases, EPA and DHA exhibit a positive effect in prevention and/or treatment of cardiovascular diseases and in modulating the corresponding risk factors (Connor, 2000).

EPA and DHA benefits are multiple and independent. Particularly, they are known to lower the plasma triglyceride, very low density lipoprotein (VLDL) -cholesterol and low density lipoproteins (LDL) -cholesterol levels and to slowly raise the high density lipoproteins (HDL) -cholesterol level (Nestel et al., 1984; Singer et al., 1984; Phillipson et al., 1985; Kinsella, 1986; Sullivan et al., 1986; Singh and Chandra, 1988; Sacks and Katan, 2002). Thus, they are used in the treatment of hyperlipidemia and hypercholesterolemia (Goodfellow et al., 2000; Castaño et al., 2006; Ros and Laguna, 2006). The mechanism of this lowering effect is thought to be caused by a decrease in the triglyceride synthesis in the liver following the inhibition of acyl-coenzyme A (1,2 diacylglycerol-o-acyl-transferase) and the induction of the peroxisomal β-oxydation in the liver (Rustan et al., 1988).

Moreover, several clinical studies conducted with high concentrated EPA/DHA supplements concluded that they induce a reduction of coronary disease risks (Balk et al., 2006) and mortality due to coronary heart attacks (Leaf et al., 2003; Harris and von Schacky, 2004; Harris et al., 2008; Lavie et al., 2009). The principal cause of these deaths is persistent ventricular arrhythmias, usually ventricular fibrillation (Leaf et al., 2003; Harris et al., 2008). EPA and DHA have the ability to modulate cardiomyocyte electrical activity (Leaf et al., 1999). In addition, the refractory period of the cardiac cycle is also prolonged. These two effects affect directly myocyte's activity, stabilizing them and therefore making them resistant to arrhythmias.

Finally, EPA and DHA present other advantages: reduction of blood pressure, decrease of platelet aggregation, induction endothelial relaxation (Pownall et al., 1999; Geleijnse et al., 2002; Balk et al., 2006; Harris et al., 2008), non steroidal anti-inflammatory properties. Contrary to Omega-6 fatty acids (ω6-PUFAs), ω3-PUFAs are precursors of 3-series prostanoids and 5-series leukotrienes, both associated with anti-inflammatory and anti-thrombotic properties (Calder, 2001; Simopoulos, 2002; Mori and Beilin, 2004; Ton et al., 2005).

In the present study, we propose the synthesis of esters combining ω3-PUFAs and nicotinol (3-hydroxymethylpyridine), an alcohol chosen among pro-vitamins belonging to the group B. Nicotinol is the alcohol derived from nicotinic acid, also known as niacin (Vitamin B3) (Szapary and Rader, 2001). After absorption, nicotinol is rapidly converted into nicotinic acid. At relatively high doses, not covered by the endogenous production from tryptophan via the kynurenine pathway, nicotinic acid has the ability to substantially decrease plasma free fatty acid, triglyceride, VLDL and LDL levels and to raise the plasma concentration of protective HDL (Harris et al., 1997). Nicotinic acid could be used in treatment of dyslipidemia, hypercholesterolemia and hyperlipidemia (Szapary and Rader, 2001).

We hypothesized that EPA, DHA and nicotinol positive effects would be additional and even synergistic, after adsorption in the organism and lipase-catalysed hydrolysis. Here, we investigated the possibility of catalysing the trans-esterification reaction between DHA ethyl ester and nicotinol using an enzymatic route with triacylglycerol lipases (EC.3.1.1.3). The mild temperature used in enzyme processes will prevent the polyunsaturated fatty acid oxidation. A preliminary study will be carried out in a given reaction medium to choose the most promising commercially available triacylglycerol lipase. Then the reaction will be optimised by varying the medium, working temperature, enzyme/substrate and ester/alcohol ratios. Finally the enzymatic process will be optimised to maximise both the kinetics and the conversion obtained at equilibrium.

2. Materiel and Methods

2.1 Materials

Commercial immobilised lipases Novozyme 435 (immobilized form of *Candida antarctica* lipase), Lipozyme RM IM (immobilized form of *Rhyzomucor miehei* lipase) and Lipozyme TL IM (immobilized form of *Thermomyces lanuginosa*) were a gift from Novozyme (Denmark). Lipase PS Amano IM was a gift from Amano (Japan), (Table 1). High concentrated ω3-PUFAs ethyl esters (from tuna oil) containing 80% molar of DHA and 12% molar of EPA (OMEGAVIE®) were purchased from Polaris (France). Nicotinol was purchased from Acros organics (Geel, Belgium). 3 Å molecular sieve was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents (2methyl 2butanol, hexane, 5-methyl 2 hexanone, acetonitrile and acetone) were of purity higher of 99% and purchased from Acros Organics (Geel, Belgium). Solvents and nicotinol were dried using 3Å molecular sieves activated overnight at 350°C.

Table 1. Characteristics of the immobilized lipases tested for transesterification between DHA-ethyl ester and nicotinol.

Commercial name	Enzyme origin	Support	Hydrophobicity/ philicity	Source
Novozym 435	*Candida antarctica form B*	Lewatit VP OC 1600	Medium hydrophobic	Novozymes (Denmark)
Lipozyme RM IM	*Rhizomucor miehei*	Duolite A568	Hydrophilic	Novozymes (Denmark)
Lipozyme TL IM	*Thermomyces lanuginosa*	Silica granules	Hydrophilic	Novozymes (Denmark)
Lipase PS Amano IM	*Burkholderia cepacia*	Diatomaceous earth	Hydrophilic	Amano (Japan)

2.2 Reaction

2.2.1 In solvent

For enzyme selection, reactions were carried out in glass tubes under magnetic agitation, containing ω3-PUFAs ethyl esters (0.1 M, 0.36 g) and nicotinol (0.15 M, 0.16 g) (1.5 molar ratio) in 10 ml of 2-methyl-2-butanol (2M2B). Reactions were started by the addition of 25 mg of the enzyme, representing 7% w/w of the DHA ethyl ester. The trans-esterification was conducted at 40°C for enzyme selection (Lipozyme RM IM®, Lipozyme TL IM, Lipase PS Amano IM and Novozyme 435). In all further experiments with Novozyme 435 the reaction temperature was of 60°C.

Samples (150µL) were withdrawn at various time intervals and centrifuged at 13000 rpm during 3 minutes for enzyme removing, using an Eppendorf centrifuge 5415D. The samples were appropriately diluted in hexane and analysed by gas chromatography.

2.2.2 In solvent free medium

Reactions were carried out in glass tubes containing 3ml of a mixture of ω3-PUFAs ethyl esters and nicotinol. A molar ratio between substrates of 1, 1.5 and 3 were used corresponding to DHA ethyl ester 1.98, 1.8 and 1.43 M and nicotinol 1.98, 2.7 and 4.3 M respectively. Reactions were started by the addition of 135 mg of Novozyme 435, representing 7% w/w of the DHA ethyl ester. The trans-esterification was conducted at 60°C with Novozyme 435. Reactant compositions were determined by taking samples (75µL) from the reaction medium at different times. A fraction (25µL), previously centrifuged 3 minutes at 13000 rpm, is diluted 500-fold in n-hexane for gas chromatography analysis.

2.3 Analysis of the samples

Samples were analysed by gas chromatography with a GC device 6890N, Agilent technologie. Separation was ensured by a HP-5 column (30 m length x 0.32 mm internal diameter and 0.25 µm thickness, Variant Inc., USA) connected to a flame ionization detector (FID). The following conditions were used: carrier gas He (25 ml/min), air and hydrogen flow of 300 mL/min and 30 mL/min. The temperature program used for the ethyl estes analysis was the following: 180ºC for 15 minutes, increase from 180ºC to 250ºC at 6°C/ min, hold for 10 minutes at 250ºC, increase from 250°C to 280°C at 10°C/ min and hold for 8 minutes.

3. Results and Discussion

3.1 Enzyme selection

As the objective of the study was the rapid development of an industrial process with economical pertinence for the trans-esterification of DHA ethyl ester with nicotinol (pyridin-3-ylmethanol), only commercial immobilised lipases were tested. Four lipases were selected, Novozyme 435, Lipozyme RM IM, Lipozyme TL IM and Lipase PS Amano IM, and assessed in 2-methyl-2-butanol (2M2B) with a 1.5 molar ratio nicotinol/DHA ethyl ester and 7% enzyme/ester (w/w). This first enzyme screening was performed at 40°C, temperature at which the four enzymes are reported to be stable. Lipozyme RM IM and Lipozyme TL IM showed low activity, with DHA ethyl ester conversion of only 2% and 8%, respectively, after 72 hours of reaction. Lipase PS Amano IM was more active but still only 22% of DHA ethyl ester conversion was achieved in 72 hours. Finally, Novozyme 435 was found to be the most efficient enzyme leading to 19% of DHA ethyl ester conversion in 1 hour. This enzyme is well-known for its high temperature stability and 60°C is a common working temperature over long period (Slotema et al., 2003). In these conditions, 26% of DHA ethyl ester conversion is reached in 1 hour, which represents an increase of 38% compared to the result obtained at 40°C. From these results, Novozyme 435 was selected for further improvements, choosing 60°C as the working temperature.

3.2 Reaction medium selection

The choice of the reaction medium is crucial because it will influence the enzyme activity and stability, conversion at thermodynamic equilibrium, solubility of substrates and products and consequently enzyme reuse and productivity and the stability of the reactor. In our case, selection of the reaction medium was first dictated by the difficulty to solubilise two substrates of different polarities. Indeed DHA ethyl ester is very hydrophobic whereas nicotinol is very polar. Nicotinol is not soluble at 40°C in n-hexane, cyclohexane and heptane and therefore no reaction was observed in such hydrophobic media (data not shown). This phenomenon was attributed to the adsorption of the nicotinol on enzyme support leading to mass transfer limitations. Different solvents of medium polarity and usually well-tolerated by enzymes were then tested: 5-methyl-2-hexanone, 2 methyl 2 butanol (2M2B) and acetonitrile. Undoubtedly, the ideal medium would consist in using a solvent free system (SFS) only composed by the reactants: high volumetric productivities would be obtained in an eco-compatible environment. Even if DHA ethyl ester and nicotinol are not miscible, it was decided to test this reaction system. The same enzyme / DHA ethyl ester weight ratio

(g/g) was used with and without solvent in order to be able to compare the results. Table 2 shows the percentage of DHA ethyl esters esterified under the tested conditions.

It was demonstrated that it is crucial to use dry solvents to avoid a parasite reaction, the hydrolysis of the ethyl ester. This one is largely reduced in the solvent free system (data not shown).

Table 2. Percentage of DHA ethyl ester conversion to DHA-Nicotinol. DHA:nicotinol ratio was 1:1.5 and the temperature 60°C. Novozyme 435: 7 % w/w of the DHA ethyl ester.

Solvent	Boiling point (°C)	log P*	DHA ethyl ester Conversion (%)	
			30 minute	6 hours
Acetonitrile	82	-0.34	10.8	35
2M2B	102	0.89	12.4	38
5-methyl-2-hexanone	145	1.88	25.4	41
Solvent free system	-	-	34.5	43

* log P : n-Octanol/WaterPartition Coefficient

The highest enzyme activity (after 30 minutes of reaction) and final conversion (after 6 hours of reaction) were obtained with the solvent free system (43% of conversion in 6 hours). If a solvent was used, the highest conversions were obtained with 5-methyl-2-hexanone (41%), closely followed by 2M2B (38%). Even if 5-methyl-2-hexanone enabled better results to be obtained, 2M2B was estimated to be the best solvent due to a lower bowling point which will minimise the energy costs of the purification process and the higher flammability hazard of 5-methyl-2-hexanone (Sciencelab, 2011). The solvent free system and 2M2B were thus selected for further improvements.

3.3 Improvement of enzyme kinetic and conversion at equilibrium

At thermodynamic equilibrium, the reaction conversion reached only 38 % and 43 % in 2M2B and in the solvent free-system respectively. This equilibrium might be shifted by either evaporating the formed ethanol or/and by increasing the ratio between alcohol and ester. The first strategy would have the extra advantage of decreasing a possible ethanol inhibition, largely described in the literature (Marty et al., 1997). To confirm this hypothesis the reaction was carried out in open tubes in order to favour ethanol evaporation (Table 3).

Table 3. Percentage of DHA conversion to DHA-Nicotinol in closed and open system. DHA:nicotinol ratio was 1:1.5 and the temperature 60°C. Novozyme 435: 7 % w/w of the DHA ethyl ester.

Solvent	DHA ethyl ester Conversion (%)			
	Closed tube		Open tube	
	30 min in 2M2B 15 min in SFS	6 hours	30 min in 2M2B 15 min in SFS	6 hours
2M2B	12.4	37.5	19.8	71.5
Solvent free system	19.5	43.0	22.3	74.2

In both reaction media, enzyme activity was higher using open tubes (60 and 14 % increase in 2M2B and SFS respectively). This result could be attributed to ethanol inhibition. The fact that ethanol evaporation seems to be less efficient in SFS could be attributed to the high solubility of ethanol in the nicotinol phase leading to a reduction of its thermodynamic activity. Use of open tubes enabled the thermodynamic equilibrium to be largely shifted, reaching 71.5% and 74.2 % in 6 hours in 2M2B and in SFS respectively.

The value of the ratio DHA ethyl ester:nicotinol was also investigated to optimize both kinetic and thermodynamic equilibrium. The tested ratios were 1:1, 1:1.5 and 1:3 in 2M2B and SFS, with Novozyme 435 at 60°C (Figure 1). In 2M2B, higher DHA ethyl ester:nicotinol ratios lead to both less enzyme activity and less conversions at thermodynamic equilibrium, indicating that nicotinol might act as an inhibitor. The highest conversion obtained in 2M2B using a ratio 1:1 was of 95% after 24h, while whit the ratios 1:1.5 and 1:3 the conversions obtained after 24h were of 90% and 83% respectively.

In SFS, the reaction is twice more efficient than in 2M2B, 40 % conversion being obtained in 30 minutes. Moreover, the kinetic is poorly affected by the reactant ratio. At equilibrium, the best performances were obtained with a stoechiometric ratio and a 1.5 ratio leading to 97% conversion in 24 h. For the ratio 1:3, conversion is lower (83%) which could be explained by a less efficient ethanol evaporation due to nicotinol excess. In addition it can be postulated that after 30 minutes of reaction, when the kinetic progress is largely reduced, the limiting step is shifting from the enzyme activity to the ethanol removal from the medium.

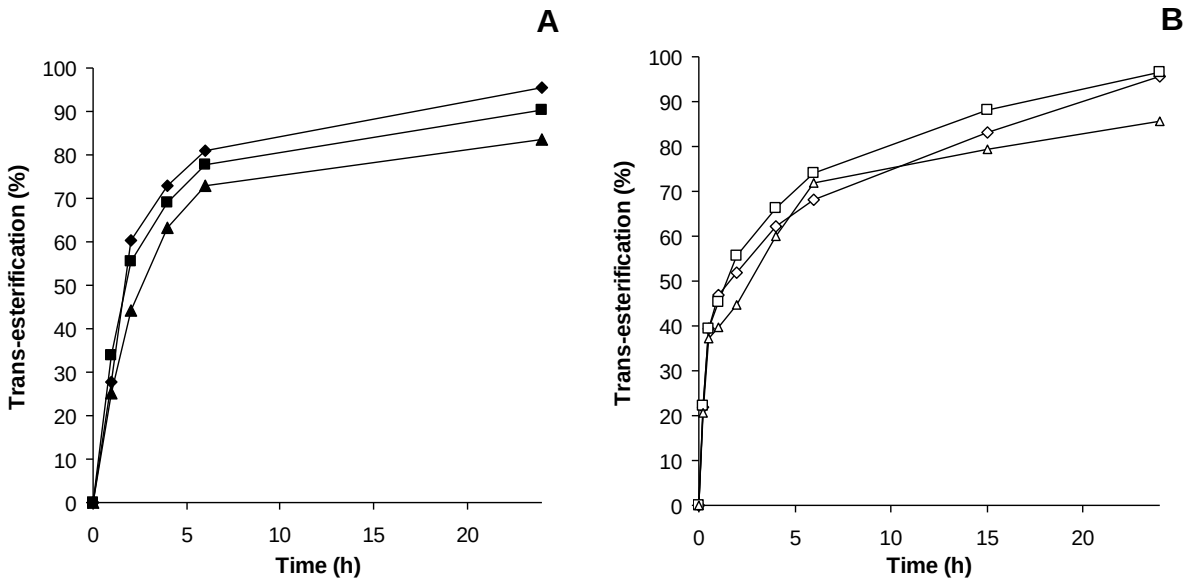

*Figure 1. Percentage of esterification of DHA ethyl ester to DHA-Nicotinol with different DHA ethyl ester : Nicotinol ratios in the 2M2B (**A**) and solvent free systems (**B**). (◆) 2M2B ratio 1:1, (■)2M2B ratio 1:1.5, 2M2B ratio 1:3 (▲), (◇) solvent free 1:1, (□) solvent free 1:1.5 and (△) solvent free 1:3. Temperature 60°C. Novozyme 435: 25mg in 2M2B and 135mg in SFS (7% w/w).*

The solvent free system appears to be the optimal reaction system, as it enables the conversion obtained at thermodynamic equilibrium to be maximised (97%) using low DHA ethyl ester:Nicotinol ratio. In addition, the high concentration of DHA ethyl ester will permit high volumetric productivity to be obtained and the development of a friendly environmental process, since the absence of solvent is undoubtedly a crucial advantage. This reaction system was consequently chosen for further process improvement.

3.4 Optimization of the solvent free system

The selected conditions were a temperature of 60°C, a Novozyme 435 concentration of 45 g/L (7% w/w enzyme/DHA ethyl ester) and a ratio DHA ethyl ester:Nicotinol of 1:1 or 1:1.5. Ethanol removal was found crucial for this reaction both from a kinetic point of view and to maximise the conversion at the thermodynamic equilibrium. As it was postulated that the limiting phenomenon during the reaction becomes the ethanol removal, another strategy for ethanol removal was investigated: nitrogen bubbling. This process would present the extra advantage of avoiding the oxidation of the DHA ester.

Undoubtedly, our hypothesis was verified: removal of ethanol from the reaction medium with nitrogen bubbling enabled very high conversions (superior to 94%) to be achieved in only 4 hours (Figure 2). In the open system reactor, 48 hours were required to obtain the same yield. A 1.5 DHA ethyl ester:nicotinol ratio appeared optimal with 99% conversion in 4 hours, since at a lower ratio (1:1) the reaction only reached 94% in 6h. At the end of the reaction, it is easy to get rid of the residual nicotinol by simple decantation In addition, in the reactor under nitrogen bubbling no oxidation of the ω3-PUFAs was observed.

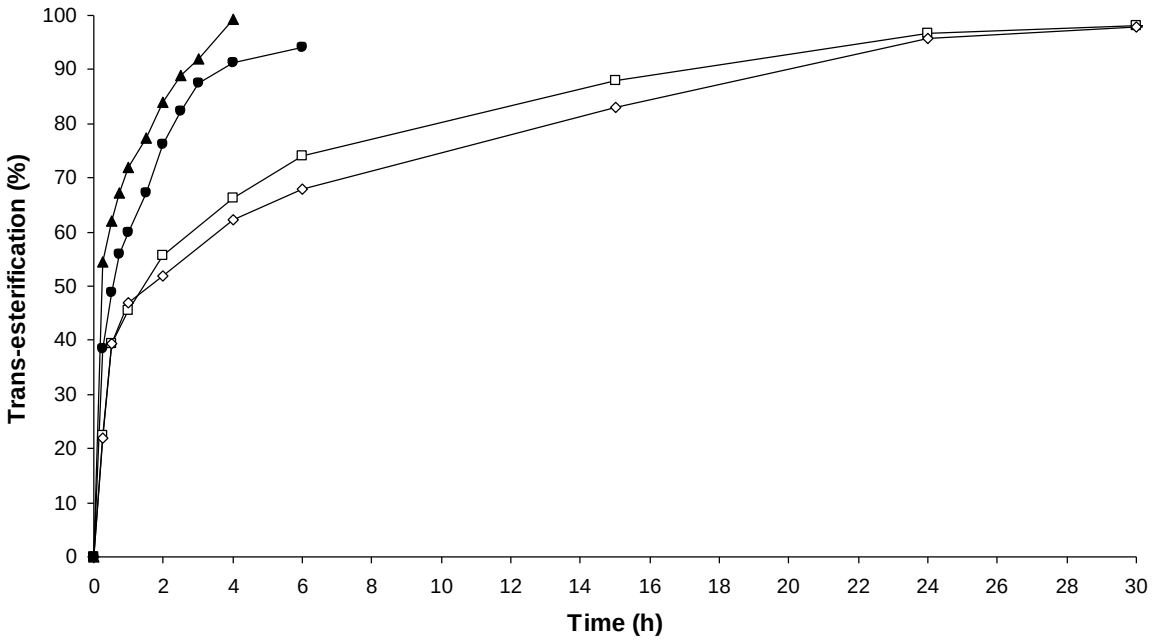

Figure 2. Percentage of DHA ethyl ester conversion with a solvent free system using two different reactors and two reaction ratios of DHA ethyl ester : nicotinol: (●) under N_2 bubbling ratio 1:1, (▲) under N_2 bubbling ratio 1:1.5, (◇) open reactor ratio 1:1 and (□) open reactor ratio 1:1.5. Temperature 60°C. Novozyme 435: 135mg (7% w/w).

4. Conclusions

Using Novozyme 435 for the transesterification reaction between DHA-ethyl ester and nicotinol is an excellent option for the production of DHA-Nicotinol, a pharmaceutical product that can be used in prevention and treatment of cardiovascular diseases. We found a system which is solvent free, has 99% yield in 4 hours, works at 60° C and the nitrogen bubbling highly reduces the oxidation of ω3-PUFAs. The absence of solvents and the short reaction times allow the development of an economical process. Indeed, in these conditions, a productivity of 4.2 g of product $.h^{-1}$.g of $enzyme^{-1}$ was obtained.

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Publication 3

Yarrowia lipolytica Lipase Lip2: an efficient enzyme for the production of Docosahexaenoic Acid Ethyl Esters Concentrates

The efficient production of the targeted molecule DHA-nicotinol required the development of a supply route of high purity DHA ethyl ester. The selectivity of lipases was studied to produce Omega-3 polyunsaturated fatty acid concentrates rich in DHA in the form of ethyl ester. Enzymatic purification was chosen for the production of concentrates since this method enables the purification to be operated under mild conditions, which is preferable since DHA is susceptible to oxidation. Lipases are able to discriminate between fatty acids in function of their chain length and/or saturation degree in three types of reactions: hydrolysis, trans-esterification, and esterification. Lipases act by kinetic resolution, reacting more efficiently with the bulk of saturated and mono-unsaturated fatty acids than with the more resistant PUFAs. Indeed, the 5 and 6 double bonds, in EPA (*cis*-5, 8, 11, 14, 17-eicosapentaenoic acid) and DHA respectively, enhance steric hindrance in the active site of the lipases. Enzymatic hydrolysis was the chosen reaction.

Screening of lipases led to the discovery of a more specific enzyme for PUFAs purification, the lipase Lip2 from *Yarrowia lipolytica*, which can be compared with the lipases identified in the bibliography as efficient, *Thermomyces lanuginosus* lipase and the lipases from *Candida rugosa*. These lipases were studied by comparing their ability to concentrate DHA-EE in the ester fraction by hydrolysing a tuna oil ethyl ester mixture (FOEE) with a high reaction yield. An analysis of the different ethyl esters hydrolysis allowed us to better understand the specificity of the tested lipases.

Yarrowia lipolytica Lipase Lip2: an Efficient Enzyme for the Production of Docosahexaenoic Acid Ethyl Esters Concentrates

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Abstract

The production of Omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) concentrates rich in *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) was studied using lipase-catalyzed hydrolysis of a tuna oil ethyl ester mixture. The lipases from *Yarrowia lipolytica* (YLL2), *Thermomyces lanuginosus* (TLL) and *Candida rugosa* (CRL1, CRL3 and CRL4) were tested. *Candida rugosa* lipases discriminate principally esters in function of their chain length, with a low discrimination of DHA versus γ-linolenate, 11-eicosenoate, arachidonate, EPA and DPA ethyl esters. On the contrary, YLL2 and TLL enable a better discrimination to be obtained, enzyme selectivity being principally due to the positioning of the double-bond the closest from the carboxylic group. YLL2 enables the highest concentrations of DHA (77%) and ω3 esters (89.5 %) to be obtained. YLL2 is consequently the most effective described lipase for DHA purification both from kinetic, purity and yield points of view.

Key words: Lipases, enzymatic hydrolysis, PUFA concentrates, docosahexaenoic acid, DHA

1. Introduction

Omega-3 (ω–3) polyunsaturated fatty acids (PUFAs) interest has increased due to their beneficial effects on human health. In particular, *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA, C22:6) and *cis*-5, 8, 11, 14, 17-eicosapentaeonic acid (EPA, C20:5) which present anti-thrombotic and anti-inflammatory properties that reduce risk factors of arthritis rheumatoid, cancer, cardiovascular diseases, myocardial infarction, bronchial asthma, inflammatory intestinal diseases, diabetes and Alzheimer's disease (Carvalho et al., 2009; Okada and Morrissey, 2008; Rubio-Rodriguez et al., 2010). DHA is an important structural component of brain gray matter, eye retina and hearth tissue, and it is required during pregnancy for appropriate fetus development (Castro-Gonzalez, 2002; Ward and Singh, 2005). Deficiency of ω–3 can provoke fatigue, dry skin, heart problems, poor circulation, depression and memory loss, among others.

An optimal ratio of Omega-6 (ω-6) over ω-3 of 4:1 is recommended in diet. However, in most diets, especially in Western diets, the consumption of ω-3 is higher than optimal, reaching ratios higher than 10. The appropriate intake of ω-3 is of 1.6g/day for men and 1.1g/day for women (IOM, 2005). The most important sources of ω-3 are fish oils, but their triacylglycerols contain more than 50 different fatty acids. Therefore, is recommended to consume concentrated forms of ω–3 in order to minimize daily lipid intake and decrease saturated and mono-saturated fatty acid consumption.

Several methods were developed to concentrate ω-3 PUFAs, including adsorption chromatography, molecular distillation, low temperature crystallization, urea complexation, supercritical fluid extraction and enzymatic reaction (Rubio-Rodriguez et al., 2010). This last method enables the purification to be operated under mild conditions, which is preferable since EPA and DHA are susceptible to oxidation. Lipases are able to discriminate between fatty acids in function of their chain length and saturation degree in three types of reactions: hydrolysis, trans-esterification, and esterification (Carvalho et al., 2003; Shahidi and Wanasundara, 1998). Lipases act by kinetic resolution, reacting more efficiently with the bulk of saturated and mono-unsaturated fatty acids than with the more resistant PUFAs (Shahidi and Wanasundara, 1998). Indeed, the 5 and 6 double bonds, in EPA and DHA respectively, enhance steric hindrance in the active site of the lipases. Lipases present different discrimination depending of the reaction used for ω-3 purification. Reactions can be classified in their order of efficiency: hydrolysis of tri-acylglycerides, esterification of free fatty acids and the most efficient, hydrolysis of fatty acid ethyl esters (Mbatia et al., 2010;

Shimada et al., 1997a; Shimada et al., 1997b). Several lipases have been used to concentrate ω-3 PUFAs. Some examples include *Thermomyces lanuginosus* (Hoshino and Yamane, 1990; Lyberg and Adlercreutz, 2008; McNeill et al., 1996), *Candida rugosa* (Byun et al., 2007; Hoshino and Yamane, 1990; Koike et al., 2007; McNeill et al., 1996; Okada and Morrissey, 2007; Sun et al., 2002; Tanaka et al., 1992; Wanasundara and Shahidi, 1998; Yan et al., 2002), *Aspergillus niger* (Carvalho et al., 2009; Hoshino and Yamane, 1990; Okada and Morrissey, 2007; Sun et al., 2002; Tanaka et al., 1992; Wanasundara and Shahidi, 1998), *Pseudomonas* sp. (Byun et al., 2007; Koike et al., 2007; Kojima et al., 2006; Lyberg and Adlercreutz, 2008; Sun et al., 2002; Tanaka et al., 1992; Wanasundara and Shahidi, 1998), *Rhizopus javanicus* (Carvalho et al., 2009; Tanaka et al., 1992), *Rhizomucor miehei* (Byun et al., 2007; Koike et al., 2007; Lyberg and Adlercreutz, 2008; McNeill et al., 1996; Ustun et al., 1997; Wanasundara and Shahidi, 1998), *Rhizopus niveus* (Byun et al., 2007; Koike et al., 2007; McNeill et al., 1996), *R. oryzae* (Sun et al., 2002; Wanasundara and Shahidi, 1998) and *Mucor javanicus* (Okada and Morrissey, 2007).

Lipases can also discriminate between EPA and DHA, which is required due to the specific medical application of each fatty acid (Shimada et al., 1998b). Most lipases such as lipases from *Geotrichum candidum*, *C. rugosa* and *T. lanuginosus* prefer EPA over DHA, due to a higher steric hindrance with DHA, cause by the two additional carbons and mainly to the presence of a double bond one carbon closer from the carboxyl group in DHA (Halldorsson et al., 2003; Lyberg and Adlercreutz, 2008). Nevertheless, lipases from *Pseudomonas* species showed DHA preference over EPA, which can be considered as an inconvenient (Lyberg and Adlercreutz, 2008).

Discovering more specific enzymes for PUFAs purification is still a great challenge. In this paper, the potentialities of the lipase Lip2 from *Yarrowia lipolytica* (YLL2) are investigated, in comparison with the lipases identified as efficient, *T. lanuginosus* lipase (TLL) and Lip1 (CRL1), Lip3 (CRL3) and Lip4 (CRL4) from *C. rugosa*. These lipases were studied by comparing their ability to concentrate DHA-EE and EPA-EE in the ester fraction by hydrolysing a tuna oil ethyl ester mixture (FOEE). Discrimination between PUFAs and especially between EPA and DHA will be considered. A special attention will be given to the recovery yield of ω−3 esters, especially DHA ethyl ester.

2. Material and methods

2.1 Materials

Tuna oil ethyl esters mixture with 25% DHA and 5% EPA was kindly donated by Pierre Fabre (France). The ethyl ester mixture composition was analyzed with gas chromatography using the GC method described below. Commercial ethyl esters standards were bought from Nu-Chek-Prep, Inc. (Minnesota, USA). Peptone, tryptone and yeast extract were purchased from (Difco, Paris, France). Unless stated other chemicals of commercial grade were purchased from Sigma/Aldrich.

2.2 Lipases

The extracellular lipase Lip2 from *Y. lipolytica* was expressed in *Y. lipolytica* strain JMY1212 under the control of the POX2 promoter inducible by oleic acid (Bordes et al., 2007). Lipases from *T. lanuginosus* and *C. rugosa* were expressed in *Y. lipolytica* strain JMY1212 with the plasmid JMP62-TEF-*Ura-Ex,* a derivative of JMP62 (Nicaud et al., 2002) where the POX2 promoter was substituted by the constitutive TEF promoter inducible by glucose (Muller et al., 1998). Methods for the construction and lipase expression in *Y. lipolytica* are described elsewhere (Piamtongkam et al., 2011).

2.3 Lipases production

YLL2, TLL, CRL1, CRL3 and CRL4 were produced in Erlenmeyer flasks (500 mL) containing 50 mL medium $Y_1T_2O_3$/ $Y_1T_2D_5$ made of yeast extract (10 g/L), bactotryptone (20 g/L), and either oleic acid (30 g/L) or glucose (50 g/L), buffered with phosphate buffer (100 mM, pH 6.8) and inoculated with an overnight preculture grown in YPD (yeast extract 10 g/L, bactopeptone 10 g/L, and glucose 10 g/L) at an initial cell density of OD_{600} = 0.5. Cells were incubated at 28 °C until complete oleic acid/glucose consumption. Cells were removed by centrifugation (10 000 rpm for 10 min) and supernatants were directly used in the reactions.

2.4 Lipase activity assay

Lipase activity of the culture supernatant was determined by monitoring the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) into butyric acid and *p*-nitrophenol. The method was optimized using 2-methyl-butan-2-ol (2M2B) as solvent to solubilise *p*-nitrophenyl butyrate. Lipase activity was measured in 96-well microplates filled with 20 µL of the lipase supernatant, 175

µL of a 100 mM phosphate buffer pH 7.2 containing 100 mM NaCl. The reaction started with the addition of 5 µL *p*-NPB (40 mM in 2M2B) and activity was measured by following absorbance at 405 nm at 25 °C for 10 min using the VersaMax tunable microplate reader (Molecular Devices, Rennes, France). One unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of butyric acid per min at 25 °C and pH 7.2.

2.5 Hydrolysis reaction

The reaction was carried out at room temperature in 1.5ml eppendorf tubes containing 0.5 ml of 100 mM fish oils ethyl esters mixture (FOEE) in decane containing 25% DHA and 5% EPA and 0.5 ml of aqueous enzymatic solution. The mixture was shaken in a Vortex Genie 2 (D. Dutscher, Brumat, France). The progress of the reaction was followed at regular time intervals by taking samples from the organic phase. 50 µL of organic phase were taken and dissolved in 300 µL of hexane, followed by saponification of the free fatty acids (FFA) with 500 µL of saturated Na_2HCO_3. The resulting organic phase was analysed with a GC device (6890N, Algilent technologie) equipped with a capillary HP-5 column (30 m length x 0.32 mm internal diameter and 0.25 µm thickness, Variant Inc., USA) connected to a FID detector. Injector, in split mode ratio 20, and detector temperatures were set at 250°C and 270°C respectively.The following conditions were used: carrier gas He (25 ml/min), air and hydrogen flow of 300 mL/min and 30 mL/min. The temperature program used for the ethyl esters analysis was the following: 180ºC for 15 minutes, 180ºC to 250ºC at 7 °C/ min, and hold for 10 minutes at 250ºC.

2.6 Successive hydrolysis

Successive hydrolysis was carried out with a final reaction volume of 10ml equally distributed in 2ml eppendorf tubes preserving the same FOEE-enzyme solution ratio as before, at room temperature and agitated by vortex. The progress of the reaction was followed by taking samples from the organic phase at regular time intervals and they were treated and analysed as specified in section 2.4. The reaction was stopped after 5h for YLL2 and TLL and the organic phase recovered. FFA were removed by saponification with saturated Na_2HCO_3 and the reaction was re-started by the addition of fresh enzyme with a FOEE-enzyme solution ratio of 1:1(v/v).

3. Results and discussion

3.1 Characterization of fish oil ethyl esters preparation

The tuna oil ester mixture composition is given in Table 1. The main components, representing 89.6% in mole of the mixture, are in order of quantity in molar percentage, ethyl esters of DHA (23.6%), palmitic acid (21.4%), oleic acid (13.2%), palmitoleic acid (6.7%), stearic acid (5.6%), EPA (5.2%), myristic acid (4.7%), alpha linolenic acid (2.2%), arachidonic acid (1.7%), linoleic acid (1.6%), gamma linolenic acid (1.2%), DPA (1.0%) and 11-eicosanoate (0.9%). Other esters represent each less than 0.9%.

3.2 Enzyme production using Y. lipolytica expression system.

The main extracellular lipase from the yeast *Y. lipolytica* (YLL2) the lipase from *T. lanuginosus* (TLL) and the three main lipases from *C. rugosa* (CRL1, CRL3 and CRL4) were cloned in the strain JMY1212 of *Y. lipolytica,* dedicated to enzyme expression and enzyme activity comparison (Bordes et al., 2007; Cambon et al., 2010). In this strain, the expression cassette containing the lipase gene under the control of POX2 (YLL2) or TEF (TLL, CRL1, CRL3, CRL4) promoters is integrated in the yeast genome at a specific site by homologous insertion at the LEU2 locus. This method avoids multiple integrations of the lipase gene and differences in expression level due to a random insertion. For each construction, after yeast transformation, five independent clones were cultivated for enzyme production. A standard deviation inferior to 10% was obtained for the five clones of each enzyme, indicating that all clones owned a single copy of the lipase gene. Enzyme activities were measured using the classical test of hydrolysis of the *p*-nitro phenol butyrate and are shown in Table 2.

Table 2. Characteristics of microbial lipases used.

Source	Lipase	Abbreviation	Activity (U/ml)[a]
Yarrowia lipolytica	Lip2	YLL2	38.7
Thermomyces lanuginosus	-	TLL	26.2
	Lip1	CRL1	42.3
Candida rugosa	Lip3	CRL3	1.8
	Lip4	CRL4	11.3

[a] µmol of *p*-nitrophenol liberated per minute and ml of enzyme.

Table 1. Composition of the mixture of ethyl esters from tuna oil (FOEE).

Ethyl esters				
Scientific name	*Common name*	*Abbreviation Δ double bond positions Omega family*	*MW*	*%Mol*
Ethyl tetradecanoate	Ethyl myristate	C14:0	254.41	4.7
Ethyl Hexadecanoate	Ethyl palmitate	C16:0	284.5	21.4
Ethyl 9-Hexadecenoate	Ethyl palmitoleate	C16:1, Δ 9, ω-7	282.48	6.7
Ethyl octadecanoate	Ethyl stearate	C18:0	312.48	5.6
Ethyl 9-Octadecenoate	Ethyl oleate	C18:1, Δ 9, ω-9	310.48	13.2
Ethyl 9,12 Octadecadienoate	Ethyl linoleate	C18:2, Δ 9,12, ω-6	308.5	1.6
Ethyl 9,12,15 Octadecatrienoate	Ethyl alpha linolenate	αC18:3, Δ 9,12,15, ω-3	306.5	2.2
Ethyl 6,9,12 octadecatrienoate	Ethyl gamma linolenate	γC18:3, Δ 6,9,12, ω-6	306.48	1.2
Ethyl Eicosanoate	Ethyl arachidate	C20:0	340.6	0.1
Ethyl 11-Eicosenoate		C20:1, Δ 11, ω-9	338.54	0.9
Ethyl 11,14 Eicosadienoate		C20:2, Δ 11,14, ω-6	336.48	0.5
Ethyl 11,14,17 Eicosatrienoate	Ethyl ETA	C20:3, Δ 11,14,17, ω-3	334.5	0.1
Ethyl 8,11,14 Eicosatrienoate	Ethyl hommogamma linolenate	C20:3, Δ 8,11,14, ω-6	334.48	0.1
Ethyl 5,8,11,14-eicosatetraenoate	Ethyl arachidonate, Ethyl ARA	C20:4, Δ 5,8,11,14, ω-6	332.48	1.7
Ethyl 5,8,11,14,17 Eicosapentaenoate	Ethyl EPA	C20:5, Δ 5,8,11,14,17, ω-3	330.5	5.2
Ethyl Docosaenoate	Ethyl behenate	C22:0	368.6	0.2
Ethyl 13,16 Docosadienoate		C22:2, Δ 13.16, ω-6	364.57	0.4
Ethyl 7,10,13,16 Docosatetraenoate		C22:4, Δ 7,10,13,16, ω-6	360.59	0.6
Ethyl 7,10,13,16,19 Docosapentaenoate	Ethyl DPA	C22:5, Δ 7,10,13.16,19, ω-3	358.5	1.0
Ethyl 4, 7, 10, 13, 16, 19-Docosahexaenoate	Ethyl DHA	C22:6, Δ 4,7,10,13.16,19, ω-3	356.5	23.6
Ethyl 15-Tetracosenoate	Ethyl nervonate	C24:1, Δ15, ω-9	394.6	0.4
Others*			329.37	8.4

* Calculated using an average molecular weight of 329.37

Three enzymes, CRL1, YLL2 and TLL, presented high activities, 42.3, 38.7 and 26.2 U/mL, respectively. CRL4 showed a medium activity, approximately the quarter of the most active enzyme CRL1. Finally, CRL3 was the less active enzyme with only 4% of the activity of the most efficient enzyme. On a SDS protein gel, the level of expression is similar for the five

lipases (data not shown). These differences in activity are specific of the *p*-NPB substrate and do not foresee of their respective activities during the reaction of interest.

3.3 Analysis of enzyme performances for DHA and/or ω3 purification

Hydrolysis of the FOEE was carried out in a biphasic system (FOEE in decane / enzyme in water, v/v) with the five studied enzymes. During the hydrolysis reaction, the lipases are expected to efficiently hydrolyse the saturated and mono-, di and tri-unsaturated ethyl esters into free fatty acids, leaving under ester form the more resistant ones, the poly-unsaturated esters, especially EPA-EE and DHA-EE. A more ambitious objective would be to be able to discriminate between poly-unsaturated esters in order to obtain DHA-EE with a high purity. For each couple ester/enzyme, the initial rate of hydrolysis was determined. However, because the concentration of esters in the mixture is very different, ranging from 0.75 to 19.1 mM for the thirteen main esters, initial rate is not the appropriate parameter to compare enzyme efficiencies versus the different esters. Considering that substrate concentrations are largely inferior to affinity constants, the reaction was considered to follow a first-order kinetic. The efficiency coefficient, initial rate divided by initial ester concentration, for the different couples enzyme/substrate are given in the Table 3.

Table 3. Efficiency factor (ratio reaction rate / initial concentration) of the five enzymes against the thirteen main ethyl esters. C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, α C18:3, γ C18:3, C20:1, ARA, EPA, DHA, DPA stand for myristate, palmitate, palmitoleate, stearate, oleate, linoleate, α-linolenate, γ–linolenate, 11-eicosenoate, arachidonate, eicosapentaenoate, docosahexaenoate, docosapentaenoate ethyl esters, respectively.

Enzyme	Efficiency factor (1/d)												
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	αC18:3	γC18:3	C20 :1	ARA	EPA	DHA	DPA
YLL2	9.6	8.6	20.7	6.8	23.1	13.8	5.8	0.5	5.5	2.8	2.9	0.9	6.4
TLL	6.7	6.1	6.9	5.7	6.3	5.2	4.8	1.3	5.4	1.9	2.1	1.1	3.0
CRL1	0.29	0.32	0.78	0.09	0.61	0.61	0.24	0.03	0.01	0.00	0.06	0.02	0.12
CRL3	0.28	0.36	0.81	0.11	0.49	0.44	0.16	0.03	0.07	0.03	0.04	0.04	0.00
CRL4	0.33	0.62	0.73	0.40	0.74	0.45	0.21	0.05	0.02	0.00	0.13	0.00	0.04

Another way to analyse these results is to calculate the competitive factor α, which evaluates the capacity of one enzyme to discriminate between the different ethyl esters (Lyberg and Adlercreutz, 2008). The competitive factor is defined by the following equation (1):

$$\alpha = \frac{\log\left([P]_o / [P]\right)}{\log\left([Ester]_o / [Ester]\right)} \quad (1)$$

where $[P]_o$ is the initial ethyl palmitoleate concentration, taken as reference substrate since it is one the ethyl ester most hydrolysed by all the enzymes, [P] is the ethyl palmitoleate concentration at time t, $[Ester]_o$ are the initial ethyl esters concentrations and [Ester] are their concentrations at time t. A high α indicates a low activity toward a specific ethyl ester and consequently a higher discrimination versus this ethyl ester. The competitive factors α are shown in Table 4.

Table 4. Competitive factor α for the different lipases calculated after 6 hours reaction for YLL2 and TLL and after 24 hours for the three lipases from C. rugosa.

Enzyme	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	αC18:3	γC18:3	C20 :1	ARA	EPA	DHA	DPA
YLL2	2.5	2.9	1	3.7	0.9	1.7	2.5	13.5	3.2	10.3	9.1	29.4	3.9
TLL	1.0	1.2	1	1.3	1.1	1.4	1.5	5.9	1.3	4.1	3.6	10.5	1.4
CRL1	4.4	3.9	1	16.9	1.6	1.6	5.5	46.6	>100	>100	26.3	82.8	12.0
CRL3	5.1	3.7	1	14.1	2.5	2.8	9.6	47.0	22.5	48.5	39.4	36.7	>100
CRL4	3.3	1.4	1	2.6	1.0	2.2	5.6	27.6	78.0	>100	9.1	>100	32.1

Whatever the considered ester, YLL2 is the most efficient enzyme, being in average 2 times more active than the lipase TLL, the second most efficient enzyme. In comparison, YLL2 was 1.5 more active than TLL during hydrolysis of *p*NPB. Nevertheless, this activity ratio between the two enzymes depends of the considered ester, demonstrating differences in enzyme selectivities. YLL2 presents a largely higher activity than TLL for ethyl-oleate, palmitoleate and linolenate (3.7, 3.0 and 2.7 times more active respectively), whereas for ethyl myristate, palmitate, stearate, α linolenate, arachidonate and EPA, the ratio is inferior at 2 (between 1.2 and 1.4). For ethyl 11-eicosenoate and DHA, the two enymes present approximately the same activity. Finally, γ linolenate is less recognised by YLL2 than TLL. The three lipases from *C. rugosa* are one or two orders of magnitude less active than YLL2. Surprisingly, CRL3, which presented a low *p*-NPB hydrolysis activity, is here as efficient as its two homologous enzymes.

Both factors, efficiency coefficient and α, show that YLL2 presents a large preference for mono unsaturated esters, ethyl palmitoleate and oleate being 141% and 240% better hydrolysed than their corresponding saturated esters (Tables 3 and 4). In the family of C18 esters, additional double bonds have a negative effect on the enzyme activity, e.g. -40% and -75% for C18:2 and C18:3 respectively, compared to activity versus ethyl oleate. In this family, ethyl γ linolenate (γ C18:3, Δ6ω6) stands out, with an hydrolysis 10 times less efficient than the one obtained with ethyl α linolenate (αC18:3, Δ9ω3). After DHA, γ linolenate is the most recalcitrant ester with an α factor of 13.5. The presence of a double bond at position 6 (*Δ6*), six carbons from the carboxyl group, causes steric hindrance unfavourable for enzyme activity. Regarding TLL, it presents no large differences in specificities for esters with chain lengths from C14 to C18, except for the γ C18:3, as observed with YLL2.

Ethyl ARA and EPA, from the C20 ester family , show the same behaviour for both lipases activity. They are hydrolysed 8 and 3 times less efficiently than ethyl oleate respectively for the two enzymes and 2 and 2.5 times than α-linolenate. Their α factors are around 10 and 4 for YLL2 and TLL respectively. The presence of an extra double in position 17 of ethyl EPA (C20:5, Δ5,8,11,14,17) compared with ethyl ARA (C20:4, Δ5,8,11,14) has no influence on the two enzymes activities. Other C20 esters, including the saturated C20:0 and the three unsaturated C20:1 (Δ11), C20:2 (Δ11,14) and C20:3 (Δ11,14,17), present a high efficiency coefficient around 6 and 5 day^{-1} for YLL2 and TLL respectively, in the same order of magnitude with the one obtained with α-linolenate esters (data not shown). Consequently, the low activity of both enzymes versus ethyl ARA and EPA is principally due to the presence of the double bond the closest of the ester group in position Δ5.

DHA-EE is the poorest hydrolysed ester, 26 and 6 times less efficiently hydrolysed than ethyl oleate for YLL2 and TLL respectively. The α factor is of 29 and 11 for the two enzymes, respectively. The high number of carbon is not responsible of enzyme selectivity since DPA (Δ7,10,13,16,19) (Table 3), C22:0, C22:2 (Δ13,16), C22:4 (Δ7,10,13,16) and even C24:1 (Δ15) (data not shown) present efficiency coefficient higher than 5.4 day^{-1}. The presence of the double bond the closest to the ester group, at position Δ4, appears more important.

In summary, for the two tested mucorales lipases, discrimination versus esters is principally due to the position of the double bond the closest from the carboxylic group (Table 5). If the double bond the closest to the ester group is at least at the position 7, reactivity is high with an optimum with mono-unsaturated esters. On the contrary, a double bond at positions 4, 5 and 6 are unfavourable for YLL2 and TLL enzyme activities. DHA the only member of the Δ4

family is the most resistant ester for both enzymes. Surprisingly, ethyl gamma linolenate, the only member of the Δ6 family, is more resistant than the two members of the Δ5 families, ethyl ARA and EPA, for both enzymes. It has been previously reported that lipases show higher discrimination against fatty acids with their first double bond at a carbon with an even number (*cis*-4, *cis*-6) than the rest of them (*cis*-5, *cis*-9) (Jachmanian et al., 1996; Lyberg and Adlercreutz, 2008; Mbatia et al., 2010; Mukherjee et al., 1993). It was suggested that this lipase discrimination might be caused by an *anti*-orientation of the fatty acids with *cis*-4, *cis*-6 unsaturation (Jachmanian et al., 1996; Mukherjee et al., 1993). In addition, YLL2 presents higher discrimination of DHA in comparison with TLL (α of 29 against 10). This is a crucial advantage to obtain a high purity DHA-EE concentrate.

Table 5. Efficiency factor of YLL2 and TLL against the fish oil ethyl esters classified according to the position of the double bond the closest from the ester group.

	Efficiency factor (day^{-1})								
Enzyme	**Δ4**	**Δ5**	**Δ6**	**Δ7**	**Δ9**	**Δ11**	**Δ13**	**Δ15**	**Saturated FA**
YLL2	0.9	2.4	1.2	4.4	19.9	4.5	2.9	4.3	8.4
TLL	1.1	1.9	0.8	2.5	6.2	4.6	4.3	3.9	6.1

Concerning the lipases from *C. rugosa,* the most important result is the low average activity observed, representing only 3-4% of the activity of YLL2. This low activity can be related to the special topology of their active sites. Indeed, the binding pocket of *C. rugosa* lipases is exceptional: it is a tunnel of 25 Å length, with the catalytic triad located at the mouth of the tunnel (Grochulski et al., 1994). In consequence the fatty acyl chain has to be introduced into the tunnel, which can be less effective than the positioning in a crevice at the surface of the protein, like in mucorales lipases. A general trend is that CRL1 and CRL3 present a marked preference for mono and di-unsaturated esters. CRL4, on the contrary, is not so selective from this point of view. This result was previously observed during hydrolysis of sardine oil with commercial *C. rugosa* lipase, in its free form and immobilized in chitosan-alginate-$CaCl_2$ (Okada and Morrissey, 2008; Okada and Morrissey, 2007) and during esterification of sardine oil fatty acids (Jonzo et al., 2000).

Another clear trend is that the three lipases from *C. rugosa* show a strong preference for esters with chain length smaller than C20, due to the tunnel topology of their active site. For CRL1 and CRL3, after 24 hours of reaction, 40% of the esters with chain length between 14 and 18 carbons (except ethyl γ linolenate) were converted, compared to only 4% of esters with chain length of 20 carbons and higher, including ethyl γ linolenate. For CRL4, the

selectivity for these two groups of esters is higher, hydrolysing 58% of the first group (C14:0 to C18:2) and 1% of the second group. The positioning of the double bond the closest to the carboxylic group is also of importance for esters with chain length smaller than C20, being the ethyl γ linolenate one order of magnitude less recognised than α ethyl linolenate. Indeed γ-linolenic acid has been previously reported as a poor substrate for *C. rugosa* lipase (Shimada et al., 1998a).

The highest DHA purity (41.8%) was obtained with the lipase YLL2 after 6 hours of reaction (Table 6). A recovery of DHA of 89.1% was obtained. TLL arrived in second position in term of performances with 39.5% of DHA-EE purity and 89% of DHA recovery. With lipases of *C. rugosa*, the DHA recovery is higher, superior to 95%, but the purity is lower, even after 24 h reaction.

From this analysis of the enzymes performance it can be concluded that *C. rugosa* lipases are only efficient to purify esters with a number of carbon higher than 20, but this mixture will be contaminated with ethyl γ linolenate. They would be useful to purify a mixture rich in ω3 esters, especially CRL4 since it is unable to hydrolyse DHA-EE. The yield of ω3 recovery will be high, close theoretically to 93% (α ethyl linolenate being consumed). However, high purities of DHA will not be obtained since the lipases from *C. rugosa* present low reactivities versus γ linolenate, ARA, EPA and DPA. Considering a perfect separation between esters with carbon number lower and higher (plus γ linolenate) than C20, a maximum purity of ω3 esters of 78% would be achieved, 60% for DHA and 73% for a mixture EPA/DHA.

On the other hand, the two mucorales lipases can recognize some ω3 esters such as α ethyl linolenate, ethyl ETA, ethyl DPA and even ethyl EPA. Therefore the yield of ω3 recovery will be lower than with CR lipases (Table 6). However, the expected DHA purity is higher with mucorales lipases than with CR lipases, since they consume the main part of esters containing more than 18 carbons, being γ linolenate the ester which would be the most difficult to separate from DHA. YLL2 is more efficient than TLL because ARA, EPA and DPA ethyl esters are better recognised and DHA discrimination is higher.

In consequence, it was chosen to optimise the reaction only with *Y. lipolytica* and *T. lanuginosus* lipases. In addition, this choice is supported by the higher activities of these two enzymes compared with CRL activities, which would decrease the cost of purification.

Table 6. Purity and recovery of EPA-EE, DHA-EE and ω3 ethyl ester mixture with the five different enzymes. Reaction time 6 hours for YLL2 and TLL and 24 hours for the three lipases from C. rugosa.

Enzyme	DHA purity (%)	DHA recovery (%)	EPA purity (%)	EPA recovery (%)	ω3 ester purity (%)	ω3 ester recovery (%)
YLL2	41.8	89.1	5.9	56.5	50.0	78.3
TLL	39.5	89.0	6.5	66.6	48.6	80.5
CRL1	31.4	98.2	6.7	94.4	41.7	95.8
CRL3	30.3	95.6	6.7	95.9	41.0	95.1
CRL4	37.2	100.0	7.0	86.6	48.5	97.6

3.4 Optimisation of DHA and ω3 purification

For the two mucorales lipases, the kinetic profile is similar, the concentration of the best recognised esters decreases rapidly and then remains constant. For instance, for YLL2 after two hours of reaction, 70% of palmitoleyl and oleyl esters were hydrolysed and even after 24 hours, no more reaction was observed. The time at which for a specific ester, the reaction stops, depends on its recognition by the enzyme; lower recognition of an ester is translated in higher reaction time to complete the hydrolysis. For instance, the decrease in concentration of EPA, ARA, DHA continues for 24 hours. These observations led us to believe that each individual reaction stops due to a thermodynamic equilibrium and not to a problem of inhibition by fatty acids or ethanol.

One technique previously used to further increase the purity of ω-3 from fish oils is successive hydrolysis with removal of side-products between each reaction (Okada and Morrissey, 2008; Shimada et al., 1998a; Shimada et al., 1994). Therefore, in order to increase the purity of DHA-EE in the mixture, three successive hydrolysis were performed with optimal reaction times (5h for YLL2 and TLL). Between each phase of reaction, fatty acids were removed by saponification and fresh enzyme was added. During this process, most of the ethanol was also removed.

Each hydrolysis increased the percentage of DHA-EE to a different degree (Figure 1). After three hydrolysis, the highest purity of DHA-EE was obtained with YLL2, 73%, against 65% for TLL. This is in agreement with the results previously obtained. In addition, DHA-EE recovery was higher with YLL2, 89%, than with TLL, 85%.

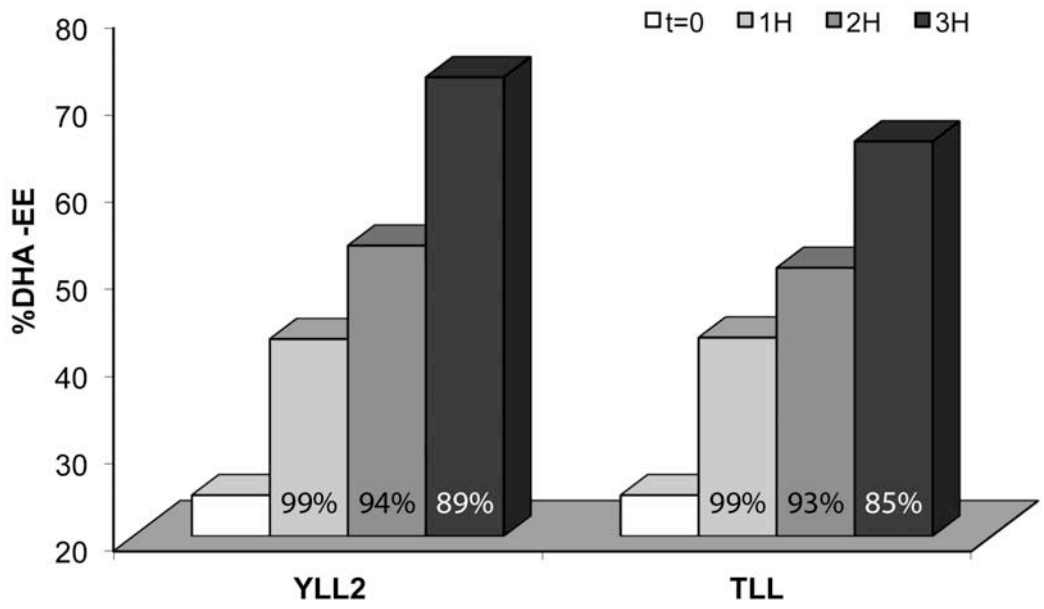

Figure 1. Percentage of DHA-EE after three hydrolysis with YLL2 and TLL; recovery percentage for each hydrolysis is shown at the base of each column. Reaction time for each hydrolysis is 5 hours.

Most of the esters were hydrolysed with a conversion superior to 90% (e.g. 94% for C14:0, C16:0, C16:1, C18:1; 93% for C18:0; 92% for C18:3 with YLL2 and 1% less in relative with TLL). Some esters are more resistant: 70% and 65% for C18:2 with YLL2 and TLL respectively; 50% and 33% for γC18:3. YLL2 and TLL hydrolysed respectively more than 80% and 70% of EPA-EE reducing its concentration to 3% and 4.8% respectively. YLL2 enables the ratio ω3/ω6 to be increased from 6.1 to 14.4 (8.6 for TLL) with a percentage of ω3 in the final mixture of 84.5% (79.4% for TLL). Another important result is that the content in saturated esters was considerably reduced from 34.4% to 6.7 % (7.9 % with TLL).

Even if efficient, a process consisting of successive reactions, with intermediate elimination of side-products, is complex from an industrial point of view. In order to better understand the reasons explaining why the reaction stops, ethanol (50 mM) was added in the initial reaction mixture. Addition of 50 mM ethanol decreased the conversion at equilibrium by 36%, In consequence, an experiment with an open reactor was tested in order to favour ethanol evaporation as it is formed. With this method an 89.5% purity of ω3 esters and 77.1% of DHA were obtained. Ethyl EPA, γ-linolenate, palmitate, linoleate, ARA, oleate represent 3.2%, 2.4%, 2%, 1.7%, 1.2% and 0.8 % respectively.

4. Conclusions

The lipase Lip2 from the yeast *Yarrowia lipolytica* is here described for the first time for purification of ω3 esters and especially DHA. It was demonstrated that this lipase is the most efficient from both a kinetic and selectivity point of view. A 90 % ω3 and 77 % DHA concentrate was obtained.

We are currently working on the determination of key structural positions involved in PUFAs discrimination at a molecular level in order to select targets for mutagenesis and obtain variants with improved selectivity.

Acknowledgements

Leticia Casas-Godoy acknowledges the support from CONACYT (grant number 305884) for the scholarship to complete her PhD studies. Rungtiwa Piamtongkam acknowledges the Royal Golden Jubilee PhD program, Thailand Research Fund (grant number PHD/0235/2547) and French Embassy for financial support.

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Publication 4

Site directed mutagenesis improved specificity of Lip2 from *Yarrowia lipolytica* towards DHA ethyl ester purification

The screening of lipases for DHA ethyl ester purification showed that the extracellular lipase 2 (YLL2) from the oleaginous yeast *Yarrowia lipolytica* is very efficient for the enrichment of DHA ethyl ester. Using wild-type enzyme of YLL2 a DHA purity of 73 % was obtained during ethyl ester mixture from tuna oil hydrolysis (initial DHA purity 23.6%, with 89 % DHA recovery. Lower performances were obtained with one of the best enzymes described to purify DHA, the *T. lanuginosa* lipase (65% DHA purity; 85 % DHA recovery).

However these lipases are not sufficiently active and selective to fulfil the industrial requirements, DHA purity higher than 85% with high yields of DHA recovery. In consequence, it was considered to improve the selectivity of YLL2 using enzyme engineering tools. In order to produce a mutant of YLL2 highly selective enzyme evolution was carried out using site directed mutagenesis. Site directed mutagenesis targeted to the active site is generally the easiest and the most efficient method to improve an enzyme selectivity. Positions in the substrate binding site, the lid and the hydrophobic crevice and dent were selected for lipase selectivity improvements. Each one of these targets was substituted by two amino acids of different sizes and analysed by comparing their performance with the wild type enzyme.

Site directed mutagenesis improved specificity of Lip2 from *Yarrowia lipolytica* towards DHA ethyl ester purification

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Abstract

Lipase 2 from *Yarrowia lipolytica* (YLL2) was shown to be an efficient catalyst for the purification of Omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) especially cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), an important molecule in the pharmaceutical industry, from the complex lipidic mixture found in fish oil. On the basis of alignment with homologous lipases of known 3D-structure, 13 amino acid residues forming the hydrophobic substrate binding site of the lipase were selected for site-directed mutagenesis. The objectives were to improve enzyme activity and selectivity. Three amino acids of the lipase lid were proven to be important for enzyme activity and specificity: variants I98V and R99Q are 37% and 38% respectively more active than the WT lipase and more efficient to eliminate two recalcitrant esters, ARA and EPA (66 and 51 % respectively. Variant I100L is the most active tested enzyme during hydrolysis of polyunsaturated ARA and EPA esters (260% and 186 % respectively more active than the WT lipase). Variant V285L presents an affinity towards DHA ester lower than the WT lipase (competitive factor of 208 against 150 for WT-YLL2). Finally, position 235 appears crucial for selectivity, variant V235F being the enzyme presenting the highest competitive factor for DHA of 411. The highest DHA-EE purities were obtained with I100L (44.0%), followed by L290A (43.9%), and V235L (43.3%),after 6 hours reaction. DHA-EE recovery yield for these variants was of 89.7%, 90.4% and 97.3% respectively.

1. Introduction

Omega-3 (ω−3) polyunsaturated fatty acids (PUFAs) interest has increased due to their beneficial effects on human health. In particular, *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA, C22:6) and *cis*-5, 8, 11, 14, 17-eicosapentaeonic acid (EPA, C20:5) which present anti-thrombotic and anti-inflammatory properties that reduce risk factors of arthritis rheumatoid, cancer, cardiovascular diseases, myocardial infarction, bronchial asthma, inflammatory intestinal diseases, diabetes and Alzheimer's disease (Okada and Morrissey, 2008; Carvalho et al., 2009; Rubio-Rodriguez et al., 2010). Deficiency of ω−3 can provoke fatigue, dry skin, heart problems, poor circulation, depression and memory loss, among others.

An optimal ratio of Omega-6 (ω-6) over ω-3 of 4:1 is recommended in diet. However, in most diets, especially in Western diets, the consumption of ω-3 is higher than optimal, reaching ratios higher than 10. The appropriate intake of ω-3 is of 1.6 g/day for men and 1.1 g/day for women (IOM, 2005). The most important sources of ω-3 are fish oils, but their triacylglycerols contain more than 50 different fatty acids. Therefore, it is recommended to consume concentrated forms of ω−3 in order to minimize daily lipid intake and decrease saturated and mono-saturated fatty acid consumption. Moreover, their use as substrates for pharmaceutical products requires high purity. For instance, nicotinyl DHA esters, synthesized via transesterification of DHA with nicotinol, presents cardia anti-arrhythmic properties (Brune et al., 2007; Séverac et al., 2012).

Lipases are capable of concentrating ω−3 PUFAs, allowing the purification of fish oil to be operated under mild conditions, which is preferable since EPA and DHA are susceptible to oxidation. Lipases can discriminate between fatty acids in function of their chain length and/or saturation degree (Shahidi and Wanasundara, 1998; Carvalho et al., 2003). Enzymes with an active site in form of tunnel such as *Candida rugosa* lipases discriminate preferentially in function of the chain length of the acyl moiety. On the other hand, lipases with a open active site at the surface of the protein, such as *Thermomyces lanuginosa* lipase, discriminate in function of the position of the double bond closest from the carboxylic group (Casas-Godoy et al., 2012). In consequence, this last class of enzyme is the most efficient to obtain a high purity DHA ethyl ester concentrate(DHA-EE), since DHA is the only fatty acid presenting a double bond at Δ4 position. Recently, the extracellular lipase Lip2 from the oleaginous yeast *Yarrowia lipolytica* (YLL2) was demonstrated to be very efficient to enrich DHA. Using wild-type enzyme a DHA purity of 73 % was obtained during ethyl

ester mixture from tuna oil hydrolysis (initial DHA purity 23.6%, with 89 % DHA recovery (Casas-Godoy et al., 2012). Lower performances were obtained with one of the best enzymes described to purify DHA, the *T. lanuginosa* lipase (65% DHA purity; 85 % DHA recovery).

YLL2 is consequently a good candidate to develop a highly selective catalyst using enzyme evolution. Site directed mutagenesis targeted to the active site is generally the easiest and the most efficient method to improve enzyme selectivity. Since only closed structure of YLL2 is available (PDB code: 3O0D) (Bordes et al., 2010), homology with the related lipase from *T. lanuginosa* (sequence identity 31%, sequence homology 47%, gap 14%; PDB ID: 1GT6 (Yapoudjian et al., 2002) was used to select amino acid targets for mutagenesis.

2. Material and methods

2.1 Materials

Tuna oil ethyl esters mixture with 25% DHA and 5% EPA was kindly donated by Pierre Fabre (France). The ethyl ester mixture composition was analyzed elsewhere (Casas-Godoy et al., 2012). Commercial ethyl esters standards were bought from Nu-Chek-Prep, Inc. (Minnesota, USA). Peptone, tryptone and yeast extract were purchased from (Difco, Paris, France). Unless stated other chemicals of commercial grade were purchased from Sigma/Aldrich.

2.2 Construction of Lip2 variants

The extracellular lipase Lip2 from *Y. lipolytica* was expressed in *Y. lipolytica* strain JMY1212 (Bordes et al., 2007; Cambon et al., 2010). The plasmid JMP8 containing the expression cassette carrying the wild type LIP2 gene is described elsewhere (Scheib et al., 1999). The LIP2 gene encoding the extracellular lipase YLL2 was placed under the transcriptional control of the strong promoter POX2 inducible by oleic acid.

The derivative plasmids carrying single amino acid changes in the LIP2 gene were constructed by site-directed mutagenesis using the QuikChangeTM site-directed mutagenesis kit (Stratagene). The procedure used the JMP8 double-stranded DNA vector and two synthetic complementary oligonucleotide primers with the desired mutation. The following primers and their complementary reverse complements were used to construct the variant enzymes:

T88**S**: 5'-C CTT GTT ATT CGA GGA **TCC** CAC TCT CTG GAG G-3'; V94**A**: 5'-T CGA GGA ACC CAC TCT CTC GAG GAC **GCC** ATA ACC GAC ATC CG-3'; V94**L**: 5'-T CGA GGA ACC CAC TCT CTC GAG GAC **CTC** ATA ACC GAC ATC CG-3'; D97**A**: 5'-GAC GTC ATA ACC **GCC** ATC CGA ATC ATG CA-3'; D97**V**: 5'-GAC GTC ATA ACC **GTC** ATC CGA ATC ATG CA-3'; I98**A**: 5'-GTC ATA ACC GAC **GCC** CGA ATC ATG CAG GC-3'; I98**V**: 5'-GTC ATA ACC GAC **GTC** CGA ATC ATG CAG GC-3'; R99**K**: 5'-ATA ACC GAC GTC **AAG** ATC ATG CAG GCT CC-3'; R99**Q**: 5'-ATA ACC GAC GTC **CAG** ATC ATG CAG GCT CC-3'; I100**A**: 5'-ATA ACC GAC ATC CGA **GCC** ATG CAG GC-3'; I100**L**: 5'-ATA ACC GAC ATC CGA **CTC** ATG CAG GC-3'; F129**I**: 5'-CAC AAT GGC **ATC** ATC CAG TCC TAC-3'; I231**F**: 5'-CGA GGA GAT **TTC** GTC CCT CAA GTG C-3'; I231**V**: 5'-CGA GGA GAT **GTC** GTC CCT

CAA GTG C-3'; V232**A**: 5'-GGA GAT ATC **GCC** CCT CAA GTG CCC TTC TGG GAC GGC TAC CAG CAC TGC-3'; V232**L**: 5'-GGA GAT ATC **CTC** CCT CAA GTG CCC TTC TGG GAC GGC TAC CAG CAC TGC-3'; V235**A**: 5'- C GTC CCT CAA **GCC** CCC TTC TGG G-3'; V235**F**: 5'- C GTC CCT CAA **TTC** CCC TTC TGG G-3'; V235**L**: 5'- C GTC CCT CAA **CTC** CCC TTC TGG G-3'; D239**E**: 5'-G CCC TTC TGG **GAG** GGT TAC CAG C-3'; D239**K**: 5'-G CCC TTC TGG **AAG** GGT TAC CAG C-3'; V285**A**: 5'-CTC CAG CAG GTC AAT **GCC** ATT GGT AAC CAT CTG CAG TAC-3'; V285**L**: 5'-CTC CAG CAG GTC AAT **CTG** ATT GGT AAC CAT CTG CAG TAC-3'; L290**A**: 5'-GGA AAC CAT **GCC** CAG TAC TTC GTC AC-3'; L290**F**: 5'-GGA AAC CAT **TTC** CAG TAC TTC GTC AC-3'.

Mutations were confirmed by DNA sequencing (GATC Biotech, Konstanz, Germany).

Escherichia coli DH5α strain was used to produce the desired plasmids. After *E. coli* transformation the different plasmids were extracted and digested by Not1 to release the expression cassette. The expression cassette, flanked by zeta regions and composed of URA3 marker (ura3d1), POX2 promoter (pPOX2), and LIP2 gene was used for transformation of *Y. lipolytica* strain JMY1212 described elsewhere (Bordes et al., 2007). This strain enables single integration of the expression cassette into the genome at a defined locus: the zeta docking platform.

For each construction, after yeast transformation, five independent clones were cultivated for enzyme production. Clones were cultivated in 100mL Erlenmeyer flasks containing 10mL of YTO medium made of yeast extract (10 g/L), bactotryptone (20 g/L), and either oleic acid (10 g/L), buffered with phosphate buffer (100 mM, pH 6.8). Stock solution of oleic acid (200 g of oleic acid/L, 5 g of Tween 40) was subjected to sonication three times for 1 min on ice for emulsification purposes. Cultures were stopped after total consumption of oleic acid, which was checked by centrifugation of the culture and visual analysis of the supernatant opacity. A standard deviation inferior to 10% was obtained for the five clones of each enzyme, indicating that all clones owned a single copy of the lipase gene. Enzyme activities were measured using the classical test of hydrolysis of the *p*-nitro phenol butyrate as decribed below.

2.3 Lipases production

YLL2 and its variants were produced in Erlenmeyer flasks (500 mL) containing 50 mL medium $Y_1T_2O_3$ or $Y_1T_2D_5$ made of yeast extract (10 g/L), bactotryptone (20 g/L), and either

oleic acid (30 g/L) or glucose (50 g/L), buffered with phosphate buffer (100 mM, pH 6.8) and inoculated with an overnight preculture grown in YPD (yeast extract 10 g/L, bactopeptone 10 g/L, and glucose 10 g/L) at an initial cell density of OD_{600} = 0.5. Cells were incubated at 28 °C until complete oleic acid consumption. Cells were removed by centrifugation (10 000 rpm for 10 min) and supernatants were directly used in the reactions.

2.4 Lipase activity assay

Lipase activity of the culture supernatant was determined by monitoring the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) into butyric acid and *p*-nitrophenol. The method was optimized using 2-methyl-butan-2-ol (2M2B) as solvent to solubilise *p*-nitrophenyl butyrate. Lipase activity was measured in 96-well microplates filled with 20 µL of the lipase supernatant, 175 µL of a 100 mM phosphate buffer pH 7.2 containing 100 mM NaCl. The reaction started with the addition of 5 µL *p*-NPB (40 mM in 2M2B) and activity was measured by following absorbance at 405 nm at 25 °C for 10 min using the VersaMax tunable microplate reader (Molecular Devices, Rennes, France). One unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of butyric acid per min at 25 °C and pH 7.2.

2.5 Hydrolysis reaction

The reaction was carried out at room temperature in 1.5mL eppendorf tubes containing 0.5 mL of 100 mM fish oils ethyl esters mixture (FOEE) in decane containing 25% DHA and 5% EPA and 0.5 mL of aqueous enzymatic solution. The mixture was shaken in a Vortex Genie 2 (D. Dutscher, Brumat, France). The progress of the reaction was followed at regular time intervals by taking samples from the organic phase. 50 µL of organic phase were taken and dissolved in 300 µL of hexane, followed by saponification of the free fatty acids (FFA) with 500 µL of saturated Na_2HCO_3. The resulting organic phase was analysed with a GC device (6890N, Algilent technologie) equipped with a capillary HP-5 column (30 m length x 0.32 mm internal diameter and 0.25 µm thickness, Variant Inc., USA) connected to a FID detector. The following conditions were used: carrier gas He (25 mL/min), air and hydrogen flow of 300 mL/min and 30 mL/min. The temperature program used for the ethyl esters analysis was the following: 180ºC for 15 minutes, 180ºC to 250ºC at 7 °C/ min, and hold for 10 minutes at 250ºC.

3. Results and discussion

3.1 Amino acid selection as target for site-directed mutagenesis

Open 3D structure of YLL2 lipase with the lid in a position that allows accessibility to the catalytic serine is not available. The three-dimensional model was previously built by homology modelling techniques by using the structures of lipases from *Rhizomucor miehei* (4TGL) and *T. lanuginosa* (1GT6) as templates (Bordes et al., 2009) (Figure 1).

```
Y.l: 1   VYTSTETSHIDQESY-NFFEKYARLANIGY---C--VGPGTKIFKPFNC-GLQCAH--FPNVELIEEFHDPRLIF 66
T.l: 3              SQDLFNQFNLFAQYSAAAY---CGKNNDAPAG-TNITCTGNACPEVEKADATFLYSFED-SGVG 61
R.m: 1    SINGGIRAATSQEI-NELTYYTTLSANSY---CRTVIPGAT----WDC--IHCDA--TEDLKIIKTWST--LIY 60
R.n: 1    SDGGKVVAATTAQI-QEFTKYAGIAATAY---CRSVVPGNK----WDC--VQCQKWVPDGKIITTFTS---LLS 60
USW           ASTQGISEDLYNRLVEMATISQAAYADLCNIPST--------IIKGEKIYNAQT------------ 46

Y.l: 67   DVSGYLAVDHASKQIYLVIRGTHSLEDVITDIRIMQAPLTN--FDLAANISSTATCDDCLVHNGFIQSYNNTYN 138
T.l: 62   DVTGFLALDNTNKLIVLSFRGSRSIENWIGNLNFDLKEIND------------ICSGCRGHDGFTSSWRSVAD 122
R.m: 61   DTNAMVARGDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPP--------------VSGTKVHKGFLDSYGEVQN 120
R.n: 61   DTNGYVLRSDKQKTIYLVFRGTNSFRSAITDIVFNFSDYKP--------------VKGAKVHAGFLSSYEQVVN 121
USW       DINGWILRDDTSKEIITVFRGTGSDTNLQLDTNYTLTPFDT-----------LPQCNDCEVHGGYYIGWISVQD
1put                SKVVYVSHDGTRRQLDVADGVSLMQAAVSNGIYDIVGDCGGSASCATCHVY

Y.l: 139 QIGPKLDSVIEQYPD------YQIAVTGHSLGGAAALLFGINLK--VNGH---DPLVVTLGQ---------PIVG 193
T.l: 123 TLRQKVEDAVREHPD------YRVVFTGHSLGGALATVAGADLR--GNGY---DIDVFSYGA---------PRVG 177
R.m: 121 ELVATVLDQFKQYPS------YKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQGQ---------PRVG 180
R.n: 122 DYFPVVQEQLTAHPT------YKVIVTGHSLGGAQALLAGMDLYQREPRLSPKNLSIFTVGG---------PRVG 181
USW       QVESLVKQQASQYPD------YALTVTGHSLGASMAALTAAQLS--ATYD---NVRLYTFGE---------PRSG

Y.l: 194 NAGFANWVDKLFFGQENPDVSKVSKDRKLYRITHRGDIVPQV-PFWDGYQHCSGEVFIDWPLIHPP-LSNVVMCQ 266
T.l: 178 NRAFAEFLTV-------------QTGGTLYRITHTNDIVPRLPPREFGYSHSSPEYWIKSGTLVPVTRNDIVKIE 239
R.m: 181 NPAFANYVVST--------------GIPYRRTVNERDIVPHLPPAAFGFLHAGSEYWITDN---SP--ETVQVCT 236
R.n: 182 NPTFAYYVEST--------------GIPFQRTVHKRDIVPHVPPQSFGFLHPGVESWI---KSGTS---NVQICT 238
USW       NQAFASYMNDAFQVSS-------PETTQYFRVTHSNDGIPNLPPADEGYAHGGVEYWSVD----PYSAQNTFVCT

Y.l :267 GQ-SNKQCSAGNTLLQQVNVIGNHLQYF-VTEGVC 299
T.l: 240 GI-DATGGNNQPNI---PDIP-AHLWYFGL-IGTC 268
R.m: 237 SDLETSDCS--NSIVPFTSVL-DHLSYFGINTGLC 268
R.n: 240 SEIETKDCS--NSIVPFTSIL-DHLSYFDINEGSC 268
USW       GD-EVQCCEAQGGQ---GVND-AHTTYFGMTSGACTW
```

Figure 1. . Multiple sequence alignment of Lip2 from Yarrowia lipolytica (Y.l.), Thermomyces lanuginosa (T.l.), Rhizomucor miehei (R.m.), Rhizopus niveus (R.n.), the feruloyl esterase from Aspergillus niger (1USW) and a fragment from a putidaredoxin from Pseudomonas putida (1put). Residues forming part of α helices and β strands are coloured in magenta and green, respectively. The three catalytic residues are coloured in red, the two catalytic residue of the oxyanion hole in orange, the cysteines are blue-coloured and the lid is underlined.

The overall structures are similar, the core of the fold is conserved and as expected, the most significant differences are seen in the regions of the surface loops. The three catalytic residues (S162, D230 and H289) and the two amino acids involved in the oxyanion hole

(T88 and L163) are perfectly superposed in these enzymes. The substrate binding site appears as a hydrophobic crevice located at the protein surface, with the catalytic triad exposed to the solvent. The hydrophobic crevice of YLL2 would consist of T88, V94, D97, I98, R99, I100, F129, L163, P190, V232, V235, P236, and Y241 (Scheib et al., 1999; Bordes et al., 2009; Bordes et al., 2010). The hydrophobic dent, where the *sn-2* substituent of the glycerol backbone binds, is formed by I231, V283, V285 and L290.

T88 is one of the two amino acids from the oxianion hole, which consists in two residues that give their backbone amide protons to stabilize the tetrahedral intermediate. Like other mucorales lipases, Lip2 belongs to the “GX” type lipase, which presents specificities for medium and long chain fatty acids (Pleiss et al., 2000). In mucorales lipases, this first residue of the oxyanion hole is either a threonine or a serine and it was previously demonstrated that other amino acids substitutions of this residue result in an inactive enzyme (Bordes et al., 2009). In consequence, the sole variant T88S was tested.

V94, D97, I98, R99 and I100 belong to the lid, α-helix formed by residues comprised between T88 and L105 (Bordes et al., 2010). Amino acid residues I98-R99-I100 form a supplementary α-helical turn at the C-terminus of the lid that is not observed in any of the homologous fungal lipase. Variants V94A, V94L, V232A and V232L were already constructed in order to open up or to further restrain the active site topology in order to alter the enantiopreference of the enzyme during resolution of 2-bromo-arylacetic acid esters (Bordes et al., 2009).

Position 97 was already saturated in a previous study and was identified as crucial for enzyme activity. Variants D97A and D97V being the most active variants (Bordes et al., 2009) were tested in this study. Variants I98A, I98V, R99K, R99Q, I100A, I100L, F129I, V235A, V235F and V235L were constructed to open up or to restrain the active site. P190 and P236 are very well-conserved in the family of mucoral lipases. It was decided to not mutate these two positions. Amino acids of the hydrophobic dent were also targeted and the following variants were constructed: I231V, I231F, V285A, V285L, L290A and L290F.

Variants activities were measured using the classical method of *p*-nitrophenyl butyrate (*p*-NPB) hydrolysis (Table 1). All the variants produced presented activity, none of the mutations led to a complete loss of *p*-NPB activity. Variants D97A, D97V, F129I, displayed the lowest activities, approximately 25% of WT activity. On the contrary, variants I100A, V235A, and D239K presented a largely higher activity than the WT enzyme (1.6 to 2.1-fold

increase). On a SDS protein gel, the level of expression is similar for all the tested lipases (data not shown). These differences in activity are specific of the *p*-NPB substrate and do not foresee their respective activities during the reaction of interest, therefore, all variants were further tested for purification of DHA from FOEE.

Table 1. p-Nitrophenol butyrate hydrolysis activity of wild type YLL2 and its variants.

Enzyme	Activity (U/mL)[a]	Enzyme	Activity (U/mL)[a]	Enzyme	Activity (U/mL)[a]
WT	38.7	**R99K**	13.9	**V235A**	67.0
T88S	12.2	**R99Q**	36.1	**V235F**	11.9
V94A	12.8	**I100A**	62.5	**D239E**	20.7
V94L	14.0	**I100L**	14.2	**D239K**	80.0
D97A	8.5	**F129I**	9.9	**V285A**	62.7
D97V	9.5	**I231F**	18.0	**V285L**	29.1
I98A	23.8	**I231V**	18.5	**L290A**	53.1
I98V	45.4	**V232A**	21.6	**L290F**	33.3
		V232F	47.6		

[a] µmol of *p*-nitrophenol liberated per minute and mL of enzyme.

3.2 Variant activity and selectivity towards FOEE

Hydrolysis of the FOEE was carried out in a biphasic system (FOEE in decane / enzyme in water, v/v) with YLL2 and its variants. For each couple ester/enzyme, two factors were analyzed to evaluate the performance of YLL2 variants: efficiency coefficient (initial velocity divided by initial ethyl ester concentration (Table 2) and competitive factor α (Casas et al. 2012). The competitive factor is defined by the following equation:

$$\alpha = \frac{\log\left([P]_o / [P]\right)}{\log\left([Ester]_o / [Ester]\right)} \quad (1)$$

where $[P]_o$ is the initial ethyl palmitoleate concentration, taken as reference substrate since it is the ethyl ester most hydrolysed by all the enzymes, [P] is the ethyl palmitoleate concentration at time t, $[Ester]_o$ are the initial ethyl esters concentrations and [Ester] are their concentrations at time t. A high α indicates a low activity toward a specific ethyl ester and consequently a higher discrimination versus this ethyl ester. The competitive factors α are shown in Table 3.

Table 2. Efficiency factor of YLL2 (WT) and its variants against the twenty main ethyl esters. C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, α C18:3, C20:1, ARA, EPA, DHA, DPA stand for myristate, palmitate, palmitoleate, stearate, oleate, linoleate, α-linolenate, γ–linolenate, 11-eicosenoate, arachidonate, eicosapentaenoate, docosahexaenoate, docosapentaenoate ethyl esters, respectively.

Enzyme	Efficiency factor (1/d)											
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	αC18:3	C20 :1	ARA	EPA	DHA	DPA
WT	9.6	8.6	20.7	6.8	23.1	13.8	5.8	3.9	2.0	2.9	0.17	6.4
I98A	3.7	3.5	11.1	2.8	10.0	8.2	2.8	2.9	1.1	1.2	0.1	2.0
I98V	11.8	10.2	24.6	8.2	26.4	10.3	7.3	7.1	4.0	4.1	0.36	6.9
R99Q	12.7	11.3	26.9	9.0	28.5	12.3	6.0	6.4	3.0	4.1	0.39	7.9
I100A	2.5	2.3	3.6	2.0	3.4	3.1	2.1	2.7	1.2	1.3	0.07	1.49
I100L	4.4	4.2	7.9	3.6	7.4	5.6	3.6	3.3	5.2	5.4	0.41	3.0
V232A	1.5	1.1	2.8	0.7	4.2	0.5	0.6	0.6	0.8	0.9	0.17	1.18
V232F	1.8	1.6	3.8	1.3	4.8	1.6	1.5	0.9	1.2	1.5	0.17	1.2
V235A	2.58	2.07	4.54	2.11	4.60	2.20	0.7	1.1	1.53	2.07	0.29	1.1
V235F	1.1	1.0	2.1	1.0	1.9	1.4	1.0	1.2	0.9	1.0	0.01	0.8
V285A	12.5	12.1	27.5	10.5	26.3	19.3	9,3	7.5	3.9	4.1	0,61	6.1
V285L	7,3	7,3	19,7	6,3	18,1	14,1	5,5	4,6	2,4	2,5	0,12	3,5
L290A	10.7	12.0	20.8	9.2	25.6	14.6	4.5	2.3	3.3	5.8	0.18	6.2

Variants were classified in function of their performances from both a kinetic and selectivity points of view, in comparison with wild-type YLL2 performances. From all the variants tested, variants V94A and D239K showed the same behaviour than WT-YLL2 (data not shown). The other two variants of these positions, V94L and D239E, showed reduced hydrolytic activity but preserved WT-YLL2 selectivity profile (data not shown).

We find in a second group, variants with low hydrolysis activity (data not shown). D97A and D97V are part of it, with less than 5% of the average WT-YLL2 activity, which confirms that aspartic acid at position 97 is crucial for activity. Variant L290F, even if its *p*-NPB activity was good, it presents a low activity during ester hydrolysis (15% of the average WT-YLL2 activity). It can be suspected that the bulky phenylalanine is unfavourable for the positioning of the fatty acid in the active site whatever the chain length. This was not the case for *p*-NPB catalysis, since activity of this variant is almost the same than WT-YLL2 activity, probably due to the lower steric hindrance of the substrate compared to component of FOEE. A smaller amino acid at this position, like an alanine, led on the contrary to a 30% global increase of the hydrolysis of all esters compared to WT-YLL2 activity.

Table 3. Competitive factor α for YLL2 (WT) and its variants.

Enzyme	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	αC18:3	C20 :1	ARA	EPA	DHA	DPA
WT	2.5	2.9	1	3.7	0.9	1.7	2.5	3.2	10.3	9.1	150	3.9
I98A	3.72	3.98	1	4.98	1.15	1.48	4.95	4.88	13.02	11.8	152	7.29
I98V	2.54	3.00	1	3.82	0.90	2.99	4.85	4.46	9.44	8.02	114	4.62
R99Q	2.67	3.04	1	3.93	0.91	2.79	4.68	5.69	17.80	9.26	102	4.54
I100A	1.69	1.94	1	2.25	1.09	1.49	2.27	1.61	4.44	3.87	62	3.15
I100L	2.36	2.48	1	3.02	1.11	1.69	3.01	3.27	1.86	1.76	48	3.72
V232A	1.99	2.88	1	4.63	0.62	2.33	3.65	7.08	4.8	3.32	18	2.57
V232F	2.05	2.40	1	3.01	0.85	2.43	4.80	4.43	3.19	2.60	26	3.55
V235A	1.96	2.51	1	2.46	0.98	2.35	6.28	5.88	3.48	2.51	21	8.05
V235F	1.96	2.30	1	2.25	1.15	1.53	2.17	1.81	2.65	2.19	401	2.93
V285A	2.83	2.91	1	3.44	1.07	1.65	3.92	4.96	10.11	9.48	67	6.27
V285L	3.22	3.21	1	3.77	1.11	1.52	4.36	5.19	10.37	9.93	208	7.07
L290A	2.25	1.99	1	2.68	0.75	1.57	5.84	4.41	11.76	8.00	152	4.12

Surprisingly, it was found in this group all variants of position V232, *i.e.* V232A, V232C, V232F, V232L, V232S and V232T, which presented a poor hydrolytic activity (15%, 23%, 19%, 6%, 7% and 35% of the average WT-YLL2 activity, respectively) whereas their *p*-NPB hydrolysis activity was correct (data not shown except for V232A and V232F). Concerning selectivity, variants V232A, V232C, V232F, V232L and V232S presented a lower α factor than WT-YLL2 for long chain polyunsaturated fatty esters. For instance, the ratio of the hydrolysis rate of ethyl EPA versus ethyl linoleate one is of 41%, 22%, 38%, 54% and 53% respectively against 14% for the WT-YLL2. It is noticeable that variant 232C seems to be no more capable of hydrolysing ethyl DHA. Variant I100A presents the same behaviour than V232A and V232F with low efficiency factors (25% of the WT-YLL2 one) and a ratio of the hydrolysis rate of ethyl EPA versus ethyl linoleate of 36%. In addition competitive factors α for saturated esters, di and tri-unsaturated esters are decreased indicating a better affinity for these substrates compared to monounsaturated esters. Another variant with low hydrolytic activity was F129I, representing only 25% of WT-YLL2 activity and no change in specificity.

Other variants with lower activity than WT-YLL2 are T88S, I98A, R99K, I231F, I231V and D239E (data not shown except for I98A). The efficiency factor shows that all the members of this group showed higher preference versus C16:1 over C18:1, which is inversed selectivity

that the one presented by WT-YLL2. However, selectivity is globally the same for all these variants to the WT-YLL2 one.

The last group consists of the most interesting variants. Variant V290A presents 11% higher activity than the WT-YLL2 (Table 2). Analysis of the competitive factor shows that its selectivity is globally the same than the WT-YLL2 (Table 3). It can be mentioned a better affinity for saturated esters, oleate and linolenate esters. On the contrary, as already mentioned, the presence of a bulky amino acid, a phenylalanine, at this position is detrimental for the positioning of all the esters in the active site.

Variant V285A is more efficient than V290A, being 35% more efficient than WT-YLL2. Nevertheless, this variant is more active versus DHA ethyl ester than WT-YLL2 which is underlined by the competitive factor decreasing from 150 to 67. The second variant of these position, variant V285L, is slightly less efficient than WT-YLL2 (90% of its activity) (Table2). However it presents interest because its affinity versus DHA ester was found lower, with competitive factor of 208 against 156 for WT-YLL2.

Variants I98V and R99Q have a similar behaviour; they are 17% and 24% more active in average than WT-YLL2 respectively (Table2). However, this increase in activity is especially high for ARA and EPA esters (respectively 69% and 44% increase in average for these two esters). Nevertheless, this positive effect is counterbalanced by the fact that DHA was also well-recognised which is underlined by the decrease of the competitive factor from 150 to 114 and 102 respectively.

Variant I100L is one of the most active tested enzymes during hydrolysis of polyunsaturated ARA and EPA esters (256 and 188 % respectively). In addition, it is accompanied by a large decrease in the hydrolysis of short and medium chain esters (42% in average of the WT-YLL2). Unfortunately, catalysis of DHA ester is 2.5 times higher than the WT-YLL2.

Finally, position V235 appeared important. Variants V235A and V235F are largely less active than the WT-YLL2 (25% and 13% respectively) (Table 2). On the contrary, variant V235L is equivalent to WT-YLL2 (data not shown). From the point of view of selectivity this variant are very different for DHA ester recognition. Variant V235A present a reduced competitive factor compared to WT-YLL2 (21 against 150) whereas variant V235F is less efficient with DHA-ester with a competitive factor of 401.

3.3 Purification of ω-3 ethyl esters

The last factor analyzed was DHA-EE, EPA-EE and ω3 ethyl ester mixture purity and recovery (Table 4). Variants from positions D97, V232 and variants I100A and V235F did not produced high concentrations of DHA-EE due to a low hydrolytic ability of the enzymes with all the ethyl esters, including EPA-EE. The highest DHA-EE purities were obtained with I100L (44.0%), followed by L290A (43.9%), V235L (43.3%), D239K (43.0%) and V285L (43.0%) after 6 hours reaction. A recovery of DHA-EE over 88% was obtained with these five variants. R99Q gave a good concentration of DHA-EE (40.4%) but was the variant with the highest hydrolysis of DHA-EE. Regarding EPA-EE, it was best hydrolyse by I100L, followed by R99Q, V235L, I98V, L290A and D239K.

Table 4. Purity and recovery of DHA-EE, EPA-EE and ω3 ethyl ester mixture with YLL2 and its variants. Reaction time 6 hours.

Enzyme	DHA purity (%)	DHA recovery (%)	EPA purity (%)	EPA recovery (%)	ω3 ester purity (%)	ω3 ester recovery (%)
WT	41.8	89.1	5.9	56.5	50.0	78.3
D97A	24.7	97.0	5.4	97.4	33.6	97.0
D97V	24.9	98.6	5.4	99.3	33.8	98.5
I98A	40.2	97.9	5.9	65.7	45.8	91.2
I98V	42.1	87.4	4.4	41.6	49.0	74.7
R99Q	40.4	76.5	4.2	36.6	47.2	65.6
I100A	33.5	95.3	6.1	79.1	42.8	90.4
I100L	44.0	89.7	3.4	31.4	50.0	75.1
V232A	28.9	95.3	5.4	80.6	38.0	93.0
V232F	32.4	96.1	5.0	66.9	41.2	90.6
V235A	32.9	91.8	4.8	61.3	41.4	85.7
V235F	29.4	98.3	5.1	78.4	37.9	93.2
V235L	43.3	88.2	4.4	40.9	50.3	75.6
D239K	43.0	90.8	4.7	45.9	50.2	77.9
V285A	42.5	84.1	4.8	43.5	48.9	88.5
V285L	43.0	97.3	5.6	54.5	47.2	91.4
L290A	43.9	90.4	4.6	43.9	50.8	77.2

To summarise, variants from positions D97 and V232 show low hydrolytic activity, higher concentrations of DHA-EE were obtained with variants of position V232 after long reaction times (data not shown), while with variants of position D97 further concentration was not accomplish. Variant I98V showed a similar profile than WT and did not show improved

discrimination of DHA-EE. A similar behavior was found in D239K and L290A, two variants that slightly improved discrimination of DHA-EE. Variant R99Q is the variant that showed the highest hydrolysis of DHA-EE and it also increased its selectivity of EPA-EE. Variants of positions I100 and V235 showed important changes in the α factors for DHA-EE, differences in selectivity and good performance in purity and recovery of DHA-EE. In addition they showed an increase in EPA-EE selectivity.

4. Conclusions

Site directed mutagenesis allowed us to study the effect of specific position in the chain length selectivity of YLL2. Amino acids of the active site, the hydrophobic dent and the lid of the lipase have an important effect in the selectivity profile of this lipase and in its ability to discriminate DHA-EE. Enzyme selectivity is principally due to the positioning of the double-bond the closest from the carboxylic group. Changes in the selectivity profile of the mutants and increased discrimination of DHA-EE were obtained. In addition inverse selectivity of certain ethyl esters was observed. Double substituted variants are now under construction to further improve enzyme selectivity versus DHA ester.

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Publication 5

Optimization of medium chain length fatty acid incorporation into olive oil catalysed by immobilized Lip2 from *Yarrowia lipolytica*

The last objective studied in the thesis was the production of structured lipids (SL) by enzymatic acidolysis between virgin olive oil and caprylic or capric acids using immobilized Lip2 from *Y. lipolytica*, lipase that has not been previously used for this application. The obtained SL should be rich in oleic acid at the *sn-2* position while C8:0 and C10:0 should be mainly esterified at the *sn-1,3* positions. Lip2 from *Y. lipolytica* was immobilized on Accurel MP 1000 and tested in a solvent-free system. In addition, the acidolysis reaction of olive oil with C8:0 or C10:0 catalyzed by immobilized YLL2 was optimized by response surface methodology (RSM) as a function of the molar ratio free fatty acids/triacylglycerols (FFA/TAG), temperature and reaction time.

Results can be compared to incorporation of C8:0 in similar reactions using the commercial lipases Lipozyme RM IM and Lipozyme TL IM. The batch reactions were modeled and optimized using RSM. An excess of free fatty acids in the reaction mixture lead to acidic substrate inhibition, decreasing the initial reaction rate and the final incorporation degree. The best reaction conditions were the same for SL production with both fatty acids: molar ratio of 2:1 FFA/TAG, reaction temperature of 40°C and reaction time of 48h. Under these conditions, the SL produced had 25.6%mol of C8:0 and 21.3%mol of C10:0, which confirmed the validity of the model. Improvements of this reaction using the same enzyme could be reached by increasing the range of the parameters used in the RSM study.

Optimization of medium chain length fatty acid incorporation into olive oil catalysed by immobilized Lip2 from *Yarrowia lipolytica*

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Abstract

Triacylglycerols (TAG) enriched with medium-chain fatty acids (M) present specific nutritional, energetic and pharmaceutical properties. Structured lipids (SL) were produced by acidolysis between virgin olive oil and caprylic (C8:0) or capric (C10:0) acids in solvent-free media, catalyzed by the main extracellular lipase from *Yarrowia lipolytica* lipase 2 (YLL2), immobilized in Accurel MP 1000. Response surface methodology was used for modeling and optimization of the reaction conditions catalyzed by immobilized YLL2. Central composite rotatable designs were performed as a function of the reaction time (2.5-49.5 h) and the molar ratio of medium chain fatty acid/TAG (MR; 0.6-7.4), for both acids, and also of temperature (32-48 ºC) for C8:0 experiments. As for capric acid, the incorporation of caprylic acid in olive oil showed not to depend of the temperature, within the tested range. The response surfaces, fitted to the experimental data, were described by a first-order polynomial equation, for C8:0 incorporation, and by a second-order polynomial equation for C10:0 incorporation. Under optimized conditions (48h reaction at 40°C, with a molar ratio of 2:1 M/TAG) highest incorporation was reached for C8:0 (25.6%mol) and C10:0 (21.3%mol).

1. Introduction

Structured lipids (SL) can be defined as triacylglycerols (TAG) that have been (i) restructured to change the fatty acids (FA) positions in the glycerol backbone, (ii) modified by the incorporation of new fatty acids or (iii) synthesized *de novo* to yield novel TAG, either chemically or enzymatically (Iwasaki et al., 1999; Iwasaki and Yamane, 2000; Osborn and Akoh, 2002). MLM type lipids are SL with medium chain length fatty acids (M), containing between 6 and 10 carbons, in the *sn*-1 and *sn*-3 positions, and long chain fatty acids (L), with more than 12 carbons, at the *sn*-2 position. This type of SL avoid health problems related with long chain TAG and have targeted nutritional, energetic and pharmaceutical properties (Huang and Akoh, 1996). MLM are used as easily accessible energy sources for patients with absorption problems (Huang and Akoh, 1996), because their hydrolysis and absorption rates are faster than those for long chain TAG (Jandacek et al., 1987), since gastric lipase prefers short and medium-chain length TAG, over long-chain length triacylglycerols (Reis et al., 2009).

SL can be produced chemically or enzymatically using lipases as catalysts (triacylglycerol acylhydrolases, E.C. 3.1.1.3.) (Marangoni and Rousseau, 1995; Willis and Marangoni, 1999). Both chemical and enzyme catalysis processes can be performed in continuous and do not require the presence of co-factors. However, enzyme catalysis has several advantages over chemical processes, since it can be carried out under mild conditions of temperature and at atmospheric pressure (Xu, 2000; Neklyudov and Ivankin, 2002; de Castro et al., 2004). In addition, lipases present (i) high selectivity (regio-, stereo- and typo-selectivities), leading to a decrease in side product synthesis (ii) high stability in organic solvents and (iii) their activity and selectivity can be improved by genetic engineering (Willis and Marangoni, 1999; Xu, 2000; de Castro et al., 2004; Kazlauskas and Bornscheuer, 2008).

By designing a SL with a precise chemical structure, the nutritional and pharmaceutical properties can be controlled. Triacylglycerols of MLM type can be produced by lipase-catalyzed acidolysis between TAG and free fatty acids (FFA), either in solvent or in solvent-free media (Lee and Akoh, 1998; Willis and Marangoni, 1999; Kawashima et al., 2001; Camacho Paez et al., 2002; Camacho et al., 2007; Hita et al., 2007; Li et al., 2008; Kim et al., 2010; Laura Foresti and Lujan Ferreira, 2010; Palla et al., 2012). The main problem of this method is the price of commercial enzymes, however, the use of immobilized and low-cost non-commercial lipases has made this method potentially viable (Slotema et al., 2003; Wilkes, 2006; Severac et al., 2011b). The immobilization process of the enzyme may

increase its operational stability and enables a continuous process to be developed. It would also improve the cost efficiency and environmental impact of the process, since less energy is required, due to the low temperatures used and less product purification steps required (Holm and Cowan, 2008). Moreover, the immobilized biocatalyst is easily removed from the reaction medium.

Commercial *sn-1,3* immobilized lipases have been used for the modification of different oils such as olive, peanut, safflower, linseed and soybean oils, aimed at the production of MLM (Shimada et al., 1996; Lee and Akoh, 1998; Xu, 2000; Fomuso and Akoh, 2002; Kim et al., 2002; Lai et al., 2005; Li et al., 2008; Nunes et al., 2011a). The most interesting MLM have caprylic (C8:0) or capric acid (C10:0) at the *sn-1,3* positions and a monounsaturated (oleic acid, in general) or polyunsaturated fatty acid at the *sn-2* position. These lipids have been synthesized using the commercial immobilized lipases from *Rhizomucor miehei* (Lipozyme RM IM) (Huang and Akoh, 1996; Lee and Akoh, 1998; Xu et al., 1998; Fomuso and Akoh, 2002; Kim et al., 2002; Lai et al., 2005; Nunes et al., 2011a), *Thermomyces lanuginosa* (Lipozyme TL IM) (Jaeger et al., 1999; Kim et al., 2002; Zhao et al., 2007; Li et al., 2008; Nunes et al., 2011a), *Candida antarctica* (Novozym 435) (Huang and Akoh, 1996; Lee and Akoh, 1998; Nunes et al., 2011a; SilRoy and Ghosh, 2011) and *Rhizopus oryzae* (Kawashima et al., 2002). Nonetheless the variety of available commercial immobilized lipases is limited and the need of more efficient enzymes is always crucial.

Currently, the search for new lipases, new supports and immobilization methods aimed at the production of structured lipids, is being carried out in order to lower the costs related with commercial immobilized lipases (Hita et al., 2007; Kim et al., 2010; Nunes et al., 2011b; Nunes et al., 2012; Palla et al., 2012; Rasera et al., 2012; Tecelao et al., 2012). Extracellular lipase Lip2 from *Yarrowia lipolytica* (YLL2) is a good candidate for the production of MLM since it is homologue to the *sn-1,3,* selective lipases from *R. miehei* (PDB codes: 3TGL (Brzozowski et al., 1992) and 4TGL (Derewenda et al., 1992); sequence identity 29%, sequence homology 46 %, gap 16 %) and *T. lanuginosa* (PDB ID: 1GT6 (Yapoudjian et al., 2002); sequence identity 31 %, sequence homology 47%, gap 14%) (Aloulou et al., 2007; Bordes et al., 2009; Casas-Godoy et al., 2012). This new lipase presents very good hydrolytic activity towards tricaprylin, olive oil and triolein and was found very efficient as catalyst of several reactions (Fickers et al., 2011) (Yu et al., 2007a; Yu et al., 2007b).

The objective of this study was the production of structured lipids by enzymatic acidolysis between virgin olive oil and caprylic or capric acids using this new enzyme, Lip2 from *Yarrowia lipolytica*. The SL obtained should be rich in oleic acid at the *sn-2* position while

C8:0 and C10:0 should be mainly esterified at the *sn-1,3* positions. Lip2 from *Y. lipolytica* immobilized on Accurel MP 1000 (YLL2) was tested in a solvent-free system. In addition, the acidolysis reaction of olive oil with C8:0 or C10:0 catalyzed by immobilized YLL2 was optimized by response surface methodology (RSM) as a function of the molar ratio free fatty acids/triacylglycerols (FFA/TAG), temperature and reaction time.

2. Material and methods

2.1. Materials

Extra virgin olive oil (acidity of 0.25% expressed as free oleic acid) was purchased from a local supermarket. The molar fatty acid profile of this olive oil was: 12.7%, C16:0; 2.9%, C18:0; 77.0 %, C18:1 and 7.3%, C18:2. Capric acid, caprylic acid and pure *p*-nitrophenyl butyrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Accurel MP1000 (particle size under 1500mm) was purchased from Membrana GmbH (Wuppertal, Germany). Unless stated, other chemicals and solvents were *p.a*. and purchased from Sigma/Aldrich.

2.2 Lipase production

The extracellular lipase Lip2 from *Y. lipolytica* was expressed in the multi-copy strain JMY329 of *Y. lipolytica* under the control of the POX2 promoter inducible by oleic acid (Guieysse et al., 2004). Lipase was produced according to the procedure of Leblond et al. (Leblond et al., 2009) and Lip2 was recovered from the supernatant.

2.3 Lipase Hydrolytic Activity Assay

Free lipase activity was determined by monitoring the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) into butyric acid and *p*-nitrophenol. 2-methyl-butan-2-ol (2M2B) was used as solvent to solubilise *p*-nitrophenyl butyrate. Lipase activity was measured in 96-well microplates filled with 20 µL of lipase supernatant and 175 µL of a 100 mM phosphate buffer pH 7.2 containing 100 mM NaCl. The reaction started with the addition of 5 µL *p*-NPB (40 mM in 2M2B) and the activity was measured by following absorbance at 405 nm at 30°C for 10 min using the VersaMax tunable microplate reader (Molecular Devices, Rennes, France). One unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of butyric acid per min at 25 °C and pH 7.2.

Immobilized lipase activity was also determined by monitoring the hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) into butyric acid and *p*-nitrophenol. The reaction was carried out in 2mL eppendorfs containing 2mg of immobilized enzyme and 1.425ml of a 100 mM phosphate buffer pH 7.2 containing 100 mM NaCl. The reaction started with the addition of 75 µL *p*-NPB (40 mM in 2M2B) and agitated by vortex at room temperature. The activity was measured by taking samples for 5min and measuring the absorbance at 405 using the

VersaMax tunable microplate reader (Molecular Devices, Rennes, France). One unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of butyric acid per min at 25 °C and pH 7.2.

2.4 Immobilization of Lip2 from Y. lipolytica

Lip2 supernatant was recovered from the fermentation broth by centrifugation followed by filtration with 0.45µm and 0.22µm Millipore membrane filters. Lipase activity was obtained following the *p*-NPB method described in section 2.3. Before immobilization, Accurel support MP 1000 was activated by mixing with ethanol (10mL ethanol/g support) at room temperature for 30min. Then, 10 mL of water/g support were added and the solution was mixed for 30min. The support was filtered and washed three times with 50 mL of water. Finally, the support was dried by vacuum. A total activity of 7800U of Lip2 supernatant (activity 503 U/mL) was added per gram of activated support. The support, in contact with the enzyme solution, was shaken horizontally at 4°C for 72h, following the remaining activity in the supernatant. The support loaded with Lip2 (YLL2) was filtered from the enzymatic solution and dried in a closed chamber crossed by an air flow at room temperature. Finally the water activity (a_w) was controlled at 0.52 by contact with the vapor phase of $Mg(NO_3)_2$ at room temperature. Immobilized enzyme was stored at 4°C.

2.5 Acidolysis Reaction

The substrate consisted of 3g of virgin olive oil and different amounts of caprylic (C8:0) or capric acid (C10:0) corresponding to molar ratios of FFA/TAG of 1:1 to 8:1. The immobilized lipase amount used was fixed (5 wt% of total substrates) and different temperatures (30-50°C) were tested. Reactions were carried out in solvent-free system in thermostated-capped cylindrical glass tubes under magnetic agitation.

Screening experiments of the acidolysis were carried out in a solvent free media for 24 h. At the end of the reaction, the enzyme was removed by centrifugation and the reaction medium stored at 4°C until analysis. In time-course experiments the reactions were carried out under optimal reaction conditions predicted by RSM, during which samples were taken at different time intervals and stored until analysis.

2.6 Experimental Designs and Statistical Analysis

Response Surface Methodology (Gacula and Singh, 1984) was used to model the acidolysis of virgin olive oil with caprylic or capric acids and to optimize the reaction conditions using immobilized YLL2.

2.6.1 Screening experiments: molar ratio and temperature levels

Molar ratios and temperature levels used in the experimental designs for reaction modeling and optimization of reaction conditions were chosen from the results of the 24h screening acidolysis reactions carried out with both acids. To investigate the effect of molar ratio on medium-chain fatty acid incorporation in olive oil, acidolysis experiments were performed at 40ºC, using FFA/TAG molar ratios from 2:1 to 8:1. The effect of temperature in the range of 30ºC to 50 ºC was investigated, maintaining the molar ratio FFA/TAG equal to 2:1 (the stoichiometric ratio for incorporation in positions 1 and 3 of a *sn*-1,3 lipase).

2.6.2 Modeling acidolysis and optimization of reaction conditions

For optimization with C8:0, 17 experiments (3 central points, 8 factorial points and 6 stars points) were carried out following the central composite rotatable design (CCRD), as a function of molar ratio (MR), temperature (T) and reaction time (t) (Table 1). Optimization with C10:0 was carried out with a total of 11 experiments (3 central points, 4 factorial points and 4 stars points) following the CCRD as a function of MR and reaction time (Table 2).

The incorporation values of C8:0 and C10:0 into olive oil for all the CCRD experiments were analyzed using the software ‘‘Statistica TM’’, version 6, from Statsoft, Tulsa, USA. Linear and quadratic effects of the independent factors and their linear interactions on incorporation of medium chain fatty acids into olive oil were calculated. Their significance was evaluated by analysis of variance. Response surfaces were fitted to each set of estimated values, described by first or second-order polynomial equations. First and second-order coefficients of these equations are usually unknown and, therefore, are estimated from the experimental data by using the statistical principle of least squares. The fit of the models was evaluated by the determination coefficients (R^2) and adjusted determination coefficient (R_{adj}^2). In practice, R^2 should be at least 0.75 or greater; being values above 0.90 very good (Haaland, 1989). By partial differentiation of these polynomial equations, it is possible to predict the reaction conditions required to obtain a maximal caprylic or capric acid incorporation value.

2.7 Model validation

The models obtained by RSM were confirmed by time course experiments of 48h under the predicted optimal conditions. During the reaction, 200 µL samples were taken along 48 h and stored at 4°C until analysis. Reactions were performed in duplicate and average values of incorporation are reported. Results after 24 and 48h were compared with those predicted by the model.

2.8 Analysis of reaction products

The method used in order to separate the acylglycerols and the FFA of the acidolysis reaction was adapted from Muñio et al. (Muñío et al., 2009). 100µl of reaction product were dissolved in 1.2 mL of hexane and then the FFAs were neutralized with 1.2 mL of 0.5N KOH hydroethanolic solution (20% ethanol). After vigorous shaking the hexanic phase, containing the SL, was recovered and the hydroalcoholic phase was extracted once more with 1.2mL of hexane to increase SL recovery yield. After vigorous shaking, the second hexanic phase was recovered; both hexanic phases were mixed and the hexane evaporated. An acylglycerol mixture of TAGs, diacylglycerides (DAGs) and monoacylglycerides (MAGs) was recovered.

Acylglycerols were analysed by HPLC to quantify the percentage of TAG, DAG and MAG present in the reaction products. The product free of fatty acids was analyzed using a Dionex Ultimate 3000 HPLC equipped with a 380-LC Evaporative Light Scattering Detector (Varian, USA) and a reverse-phase analytical Prontosyl C30 column (ICS, France) (250 x 4mm x 5µm) (Severac et al., 2011a). The nebulization and evaporation temperatures were kept at 35 °C and 40 °C, respectively. The nitrogen flow-rate was fixed at 1 L/min. A 40-min ternary gradient with two linear gradient steps was employed: phase A was water with 0.1% of trifluoroacetic acid (TFA), phase B acetonitrile and phase C, 2-propanol:hexane (5:4, v/v). Gradients were as follows: 30% A + 70% B in 0 min, 100% B in 15 min, 50% B + 50% C in 30 min, followed by isocratic elution with 50% B + 50% C for 10 min. The flow rate was 1 mL/min and oven temperature was set at 40 °C. The identification was performed with reference to pure standards. Elution order was: monoacylglycerols, diacylglycerols and triacylglycerols. This method allows separation of *sn1(3)*-MAG/*sn2*-MAG forms and *sn1,3*-DAG/*sn1(3),2*DAG forms.

The HPLC analysis of the samples after the neutralization of FFA, showed no presence of MAG and the percentage of DAG was less than 3%. The SL was analyzed from the acylglycerol fraction without removing the DAG because their percentage in the mixture was negligible. SL were methylated as previously described (Browse et al., 1986) using heptadecanoic acid methyl ester as internal standard. 1µL of the FAME solution was analyzed with a GC device (6890N, Agilent technology) equipped with a capillary HP-5 column (30 m length x 0.32 mm internal diameter and 0.25 µm thickness, Variant Inc., USA) connected to a FID detector. Injector, in splitless mode, and detector temperatures were set at 250°C and 260°C respectively. The following conditions were used: carrier gas He (25 mL/min), air and hydrogen flow of 350 mL/min and 35 mL/min. The temperature program used for the methyl esters analysis was the following: 60ºC for 1 minute, a temperature increase to 150ºC at 15 °C/ min, a plateau at 150°C for 1minute followed by a temperature increase to 220°C at 5°C/min and a final plateau at 220°C for 1 minute.

2.9 Incorporation degree

Results are presented as molar incorporation or incorporation degree of the desired fatty acid into the TAG. Incorporation degree is calculated using the following equation:

$$I(\%) = \left(\frac{MFA}{MT} \right) \times 100$$

where MFA are the moles of the medium chain fatty acid (C8:0 or C10:0) in the TAG and MT are the total moles of fatty acids in the acylglycerols.

3. Results and Discussion

3.1 Immobilization of YLL2

Two independent batches of lipase were produced and the load of lipase on the support was adjusted in order to get biocatalysts with the same activity. YLL2 from the fermentation had a high *p*-NPB activity of 503 U/mL, (c.f. 2.3). By following the enzyme activity in the supernatant, the amount of immobilized enzyme was calculated. After 72h, the enzyme activity in the supernatant remained constant representing between 60-65% of the initial activity. It can be assumed that the support was saturated and therefore no more enzyme could be adsorbed. The immobilized lipase was highly active with an activity of 400±10 U/g.

3.2 Screening experiments: Molar ratio and temperature levels

In the screening experiments, performed before modeling experiments, the acidolysis reactions were carried out in a solvent free system for 24h, at 40°C for different molar ratios (2:1 to 8:1;FFA/TAG) and at molar ratio of 2:1, FFA/TAG for different temperatures (30-50°C). A solvent-free system, solely composed of the mixture of reactants, was considered to maximize volume productivity, to simplify the downstream processing and to develop a clean process.

For molar ratios 1:1 to 6:1 FFA/TAG (Figure 1A), molar incorporations were higher for C8:0 than for C10:0, which could be explained by a higher reactivity of C8:0 compared to C10:0 due to its polarity (LogP=2.78 and 4.09 respectively). C8:0 incorporation decreased from 20.3%mol to 15.9%mol from molar ratio 2:1 to 4:1 FFA/TAG. In the same range of molar ratios, C10:0 incorporation remained constant (14.6%mol). With both medium chain length fatty acids, the incorporation degrees decreased with high molar ratios, probably due to an inhibitory effect caused by the high amounts of FFA in the reaction medium, which can cause a decrease in the reaction rate that would translate as lower incorporations since reaching the equilibriums takes longer reaction times. This phenomena will be studied in the time course reactions (Lee and Akoh, 1998; Li et al., 2008) (c.f. 3.4).

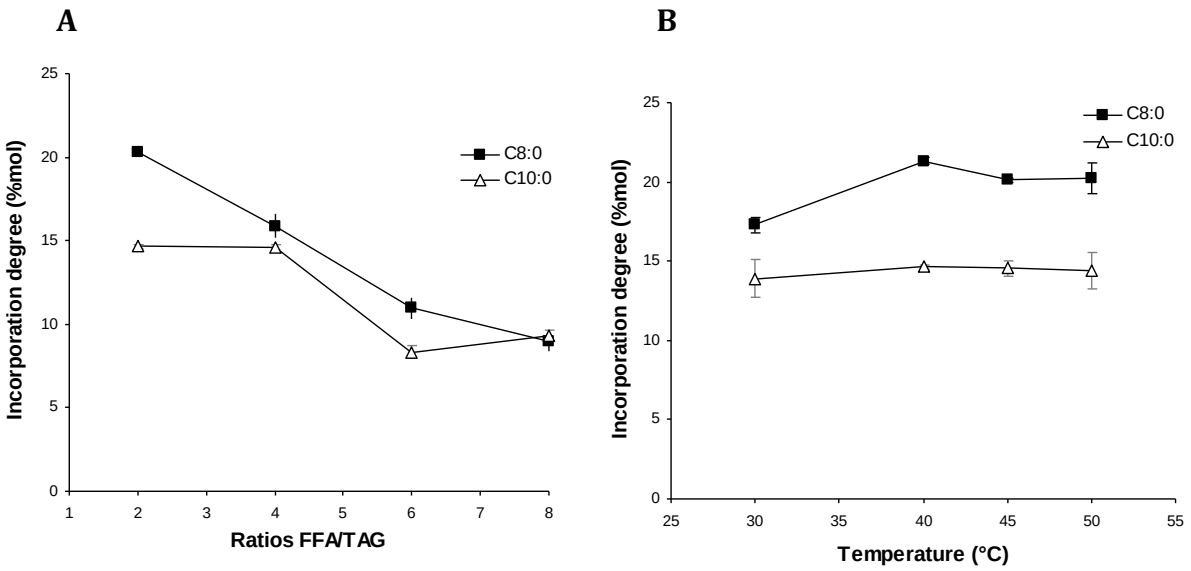

Figure 1. Caprylic (C8:0, ■) or capric (C10:0, △) acid incorporations into virgin olive oil catalyzed by immobilized YLL2, after 24h in a solvent free medium (A) Effect of molar ratio at 40°C. (B) Effect of temperature with a MR of 2:1, FFA/TAG.

The effect of temperature was also evaluated within the range of 30-50°C (Figure 1B) at a molar FFA/TAG of 2:1. Temperature has not a large influence on the incorporation degree, especially for C10:0. From these results, the levels for the central composite rotatable design of molar ratio and temperature were calculated for C8:0 (Table 1). For C10:0, the temperature was fixed at 40°C and the tested molar ratio levels are shown in Table 2. In both systems reaction time was a studied factor. For each factor, five levels were tested: -α, -1, 0, 1 and α (Tables 1 and 2).

3.3 Modeling incorporation of medium chain fatty acids into olive oil

The incorporation values of C8:0 or C10:0 into virgin olive oil, by acidolysis reaction, in solvent-free media, catalyzed by immobilized YLL2, under the conditions of the experimental designs followed, are presented in Tables 1 and 2.

Table 1. Coded and decoded experimental design matrix used (CCDR) as a function of molar ratio (MR) C8:0/TAG, temperature (T, ºC) and reaction time (t, h) and respective C8:0 incorporation values.

Experiment	X_1	X_2	X_3	MR (C8:0/TAG)	Temperature (ºC)	Reaction time (h)	Incorporation of C8:0 (% mol)
1	-1	-1	-1	2	35	12	19.2
2	-1	-1	1	2	35	40	24.3
3	-1	1	-1	2	45	12	19.8
4	-1	1	1	2	45	40	25.9
5	1	-1	-1	6	35	12	8.3
6	1	-1	1	6	35	40	14.2
7	1	1	-1	6	45	12	7.0
8	1	1	1	6	45	40	16.5
9	-1.68	0	0	0.6	40	26	19.1
10	1.68	0	0	7.4	40	26	8.5
11	0	-1.68	0	4	31.6	26	15.1
12	0	1.68	0	4	48.4	26	17.0
13	0	0	-1.68	4	40	2,5	6.1
14	0	0	1.68	4	40	49,5	23.2
15	0	0	0	4	40	26	16.4
16	0	0	0	4	40	26	15.4
17	0	0	0	4	40	26	16.0

Table 2. Coded and decoded experimental design matrix used (CCDR) as a function of molar ratio (MR) C10:0/TAG and reaction time (t, h) and respective C10:0 incorporation values.

Experiment	X_1	X_2	MR (C10:0/TAG)	Reaction time (h)	Incorporation of C10:0 (% mol)
1	-1	-1	2	10	11.5
2	-1	1	2	42	18.6
3	1	-1	6	10	8.3
4	1	1	6	42	17.3
5	-1,4	0	1.2	26	16.9
6	1,4	0	6.8	26	9.7
7	0	-1,4	4	3.4	3.0
8	0	1,4	4	48.6	19.3
9	0	0	4	26	14.6
10	0	0	4	26	14.7
11	0	0	4	26	14.5

Linear and quadratic main effects of molar ratio FFA/TAG, temperature (for C8:0 experiments) and reaction time (for both acids) and linear interactions of factors on C8:0 or C10:0 incorporation into olive oil, as well as *p* values, are presented in Table 3. A positive or a negative linear effect of a particular factor (MR, temperature or reaction time), on the incorporation degree, means that an increase in the value of that factor results in an increase or reduction in the response, respectively. A negative (or positive) quadratic effect indicates that the response is described by a convex (or concave) response surface.

Table 3. Linear and quadratic effects, linear interactions and respective *p*-values (values in parentheses) of molar ratio medium chain FFA/TAG (MR), temperature and reaction time on the acidolysis of olive oil with C8:0 or C10:0, catalyzed by immobilized YLL2.

Factor	**C8:0**	**C10:0**
MR (linear term)	-8.93 (0.0003)	-3.68 (0.0333)
MR (quadratic term)	-0.53 (0.729)	-0.45 (0.776)
Temperature (linear term)	0.92 (0.516)	-
Temperature (quadratic term)	1.03 (0.508)	-
Reaction time (linear term)	8.10 (0.0005)	9.80 (0.0006)
Reaction time (quadratic term)	0.06 (0.966)	-2.53 (0.152)
MR by Temperature (linear interaction)	-0.27 (0.883)	-
MR by Time (linear interaction)	1.02 (0.579)	0.88 (0.641)
Temperature by Time (linear interaction)	1.19 (0.520)	-

For caprylic acid, results show that temperature has no significant effect in the incorporation of this fatty acid, neither at linear nor at quadratic levels (p>>0.05) (Table 3). In addition, no significant interactions between the factors were observed. However, molar ratio and reaction time have significant linear effects on C8:0 incorporation into olive oil. The negative linear effect of MR and the positive linear effect of reaction time indicate that the incorporation of C8:0 will increase with lower MR and a longer reaction time. Thus, caprylic acid incorporation into olive oil can be fitted to a flat response surface (Figure 2), described by a first-order polynomial equation (Table 4).

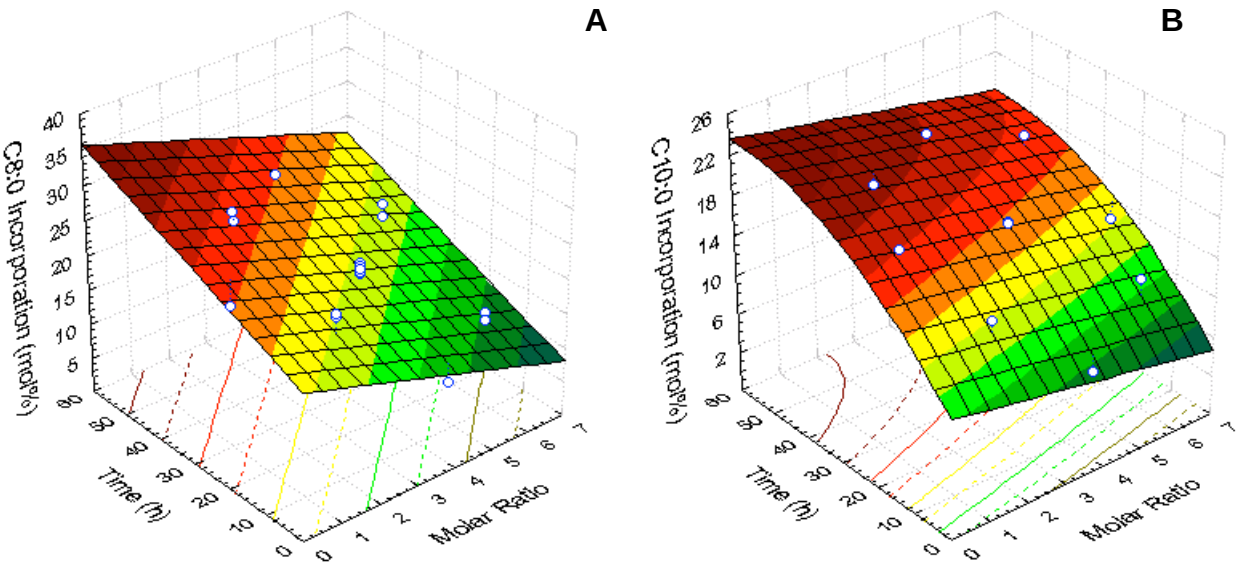

Figure 2. Response surface fitted to the incorporation of caprylic (C8:0) or capric (C10:0) acids into virgin olive oil by acidolysis catalyzed by immobilized YLL2, as a function of reaction time and molar ratio FFA/TAG.

Concerning capric acid incorporation, a negative linear effect of MR and a positive linear effect of reaction time were also observed. This system was also affected by a negative quadratic effect of reaction time generating a convex response surface (Figure 2) that can be described by a second-order polynomial equation (Table 4).

The coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{adj}) of each system are also shown in Table 4. The high values of both R^2 and R^2_{adj} of these models indicate a good fit for caprylic acid (R^2 = 0.89) incorporation and a very good fit for capric acid (R^2=0.93) incorporation (Haaland, 1989). For these models, only significant effects ($p \leq 0.05$) and those having a confidence range smaller than the value of the effect (data not shown) were considered.

Table 4. Model equations of the response surface fitted to the acidolysis of olive oil with caprylic or capric acid catalyzed by immobilized Lip2, as a function of molar ratio FFA/TAG (MR) and reaction time (t, h).

System	Model Equation	R^2	R^2_{adj}
Olive oil + C8:0	C8:0 %mol incorporation = 17,41 - 2,23·MR + 0,289·t	0.89	0.88
Olive oil + C10:0	C10:0 %mol incorporation = 6,91 - 0,92·MR + 0,55·t -0,0047·t^2	0.93	0.90

From the response-surfaces fitted to the experimental data points, no optimal points (maximum incorporation) were observed inside the considered experimental region. Thus, only the identification of the regions corresponding to higher incorporations could be achieved. It results evident that low molar ratios and high reaction times will give better incorporation degrees. For both systems, the highest incorporations inside the experimental domain should be reached at 40°C, with a molar ratio of 2:1 FFA/TAG and a reaction time of 48h. Under these conditions, 26.8%mol incorporation is expected for C8:0 and 20.6%mol for C10:0. For C8:0, the model predicted an incorporation of 22.1%mol at 24h reaction time, which can be compared with the values obtained in the screening experiments (20.3%mol) at 40°C and under similar conditions (MR= 2:1). In addition, the preliminary results also showed an incorporation of 15.9%mol at a MR of 4:1, similar to the incorporation predicted by the model (15.4%mol)..Regarding C10:0, the model predictions at a MR 2:1 and 4:1, after 24 hours, were incorporations of 15.5%mol and 13.7%mol, respectively. These values are similar to the ones obtained in the preliminary experiments (14.6%mol).

Previous studies, in solvent systems, showed that the incorporation of caprylic acid into corn (Ozturk et al., 2010) and perilla oils (Kim et al., 2002) with commercial immobilized lipases from *T. lanuginosa* (Lipozyme TL IM) (Kim et al., 2002; Ozturk et al., 2010) and *R. miehei* (Lipozyme RM IM) (Kim et al., 2002) reached maximum incorporations with high molar ratios. The optimum conditions for the incorporation of caprylic acid into corn oil with Lipozyme TL IM were: enzyme load 13.2%wt, molar ratio 3.9:1 FFA/TAG, temperature 50°C and reaction time of 3.1h (Ozturk et al., 2010). Under these conditions, the SL obtained had 21.5%mol of caprylic acid. Kim et al. (Kim et al., 2002) found that the highest incorporation of caprylic acid into perilla oil using Lipozyme TL IM and Lipozyme RM IM was obtained with a molar ratio of 6:1 FFA/TAG, at 55°C and 24h. Incorporation degrees obtained were of 48.5%mol with Lipozyme RM IM and 63.8% with Lipozyme TL IM.

However, in our system, low molar ratios are required, which has been previously reported in solvent-free reactions, with commercial (Lee and Akoh, 1998; Zhou et al., 2001; Li et al., 2008) and non-commercial immobilized lipases (Nunes et al., 2011b; Nunes et al., 2012). Using Lipozyme RM IM, caprylic acid was introduced into peanut oil (Lee and Akoh, 1998), obtaining an incorporation of 30%mol with a molar ratio of 2:1 FFA/TAG, at 50°C. With Lipozyme TL IM used to catalyze a similar reaction, a molar incorporation of 27%mol of C8:0 into soybean oil was reached after 50h reaction, at 40°C and with a molar ratio of 4:1 (Li et al., 2008). In these two systems, it was found that higher molar ratios reduced the incorporation degree due to acidic substrate inhibition. Using RSM the acidolysis reaction between caproic acid and rapeseed oil using Lipozyme RM IM was optimized (Zhou et al.,

2001). Optimal reaction conditions were molar ratio 5:1 FFA/TAG, temperature 65°C, enzyme load 14%wt, water content 10% and reaction time 17h; under these conditions the incorporation degree obtained was of 55%mol.

With the recombinant *Rhizopus oryzae* lipase (r-ROL) immobilized in Eupergit C, after 24-h acidolysis reaction of virgin olive oil with caprylic or capric acid, in solvent-free media, the maximum incorporation of caprylic (15.5%mol) or capric (33.3%mol) acids in TAG, predicted by RSM, occurs at 37ºC and 35ºC, respectively, and at C8:0/TAG of 2.8:1 or C10:0/TAG of 3:1 (Nunes et al., 2012). The fermentation conditions used in r-ROL production, highly affected hydrolytic activity and in a lesser extent interesterification activity.

3.4 Model validation

In order to validate the models, the acidolysis reactions were carried out under the selected conditions that maximize fatty acid incorporation: temperature of 40°C, molar of 2:1 FFA/TAG and reaction time of 48h. The fatty acid composition of the SL produced under these conditions is shown in Table 5. In addition, time-course reactions were carried out at 40ºC for 48 h, at molar ratios of 1:1, 2:1 and 4:1 (Figure 3). The experimental incorporation degrees of caprylic acid after 48h were 26.2%mol, 25.6%mol and 16.6%mol for MR of 1:1, 2:1 and 4:1, respectively. Results obtained with ratios of 1:1 and 2:1 are in good agreement with the predicted values by the first-order polynomial model (Table 4): 29.1%mol and 26.8%mol, respectively. However, with the molar ratio 4:1, the model predicted a caprylic acid incorporation of 22.4%mol and only 16.6%mol incorporation was reached. After 48h, capric acid incorporations were of 21.0%mol, 21.3%mol and 17.3%mol for MR 1:1, 2:1 and 4:1, respectively. In this system, all the results agree with the predicted values by the second-order polynomial model: 21.6%mol, 20.6%mol and 18.8%mol for MR of 1:1, 2:1 and 4:1, respectively.

It can also be observed in Figure 3 that, in both systems, the incorporation degrees and kinetics are similar for molar ratios of 1:1 and 2:1. The initial reaction rates at different substrate ratios were calculated using the experimental incorporation values obtained during the first 6h of reaction. For all the MR tested, reactions with C8:0 gave higher initial reaction rates than with C10:0, which confirms a higher preference of YLL2 towards caprylic acid over capric acid. However, previous reports state that YLL2 has higher activity towards methyl decanoate over methyl octanoate (Yu et al., 2007a; Yu et al., 2007b). Initial reaction rates with C8:0 (%mol incorporation of C8:0/h) were of 1.8, 1.4 and 0.5 for MR 1:1, 2:1 and 4:1, respectively. The highest initial reaction rate was obtained with the MR of 1:1.

Nevertheless, final incorporation degrees were almost the same with MR 1:1 and 2:1. Initial reaction rates with C10:0 (%mol incorporation of C10:0/h) were of 1.3, 1.1 and 0.6 for MR 1:1, 2:1 and 4:1, respectively. Again, the reaction with MR of 1:1 had a slightly higher initial rate but the final incorporation was similar with MR 1:1 and 2:1

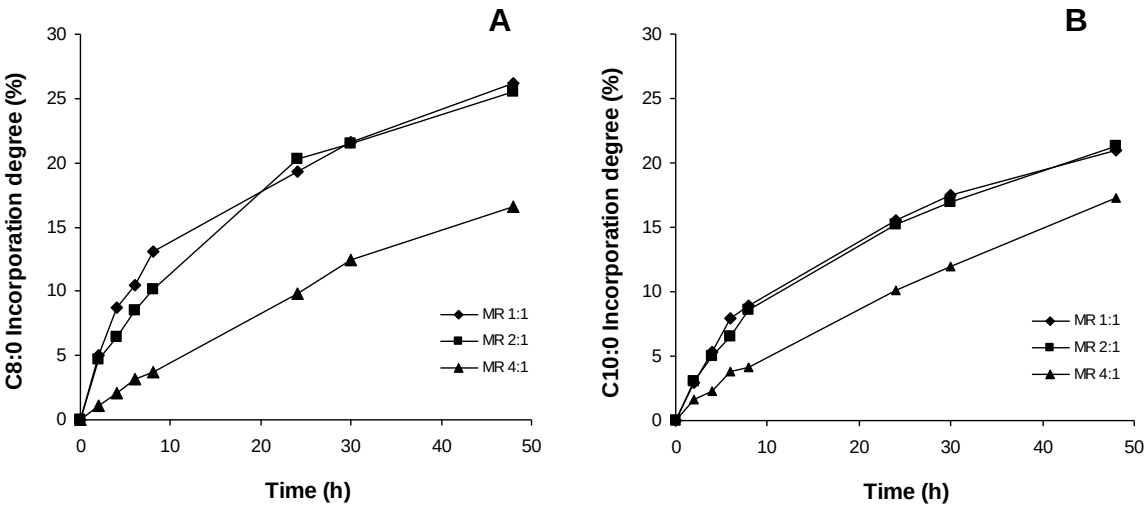

Figure 3. Time-course of acidolysis reaction of olive oil with caprylic (A) or capric (B) acids, for different MR FFA/TAG, under optimal temperature (40°C). Molar ratio FFA/TAG, 1:1 (◆), 2:1 (■) and 4:1 (▲).

Table 5. Fatty acid composition (%mol) of olive oil and the SL produced at a MR of 2:1 FFA/TAG, after 48h at 40°C.

Fatty acid	Virgin olive oil	SL	
		C8:0	C10:0
C8:0	-	25.6	-
C10:0	-	-	21.3
C16:0	12.7	8.1	9.8
C18:0	2.9	2.3	2.3
C18:1	7.7	58.6	60.7
C18:2	7.3	5.5	5.9

Figure 3 also shows that the initial reaction rates greatly decreased in both systems with MR of 4:1. The same behaviour was found in similar reactions using immobilized lipases from *R. miehei* (Lee and Akoh, 1998) and *T. lanuginosa* (Li et al., 2008), which are homologous with Lip2 from *Y. lipolytica* (Bordes et al., 2009), the recombinant *R. oryzae* lipase (Nunes et al., 2012) and *C. papaya* latex (Tecelao et al., 2012). In addition, the final incorporations of C8:0 and C10:0 with MR 4:1 were very similar, 16.6%mol and 17.3%mol, respectively. This confirms the presence of substrate inhibition, as suggested by the previous results and the negative linear effect of MR on caprylic and capric acids incorporation (c.f. 3.3). It has also been reported that increasing the amount of free fatty acids over a critical value in a substrate mixture causes acidic substrate inhibition, which leads to a reduction of the incorporation degree and of the initial reaction rate (Lee and Akoh, 1998). Also, a high content of free fatty acids generates an acidic condition of the aqueous phase around the enzyme absorbing the water of the interface required for optimal reaction activity (Zhao et al., 2007; Li et al., 2008) and increasing the solubility of FFA (Yankah and Akoh, 2000).

4. Conclusions

Production of structured lipids from olive oil and medium chain fatty acids (caprylic acid and capric acid) was successfully achieved with Lip2 from *Y. lipolytica* immobilized in Accurel MP 1000. Results can be compared to incorporation of C8:0 in similar reactions using the commercial lipases Lipozyme RM IM (30%mol)(Lee and Akoh, 1998) and Lipozyme TL IM (27.01%mol)(Li et al., 2008). Comparing these results with the recombinant *R. oryzae* lipase (r-ROL) immobilized in Eupergit C(Nunes et al., 2012), under optimal conditions, the incorporation of C8:0 was higher with immobilized YLL2 (25.6%mol) that with immobilized r-ROL (15.5%mol). Nevertheless, capric acid incorporation was higher with immobilized r-ROL (33.3%mol) than with immobilized YLL2 (21.3%mol).

The batch reactions were modeled and optimized using RSM. An excess of free fatty acids in the reaction mixture lead to acidic substrate inhibition, decreasing the initial reaction rate and the final incorporation degree. The best reaction conditions were the same for SL production with both fatty acids: molar ratio of 2:1 FFA/TAG, reaction temperature of 40°C and reaction time of 48h. Under these conditions, the SL produced had 25.6%mol of C8:0 and 21.3%mol of C10:0, which confirmed the validity of the model.

Acknowledgments

The authors are grateful to CONACYT, for a PhD fellowship for Mrs. Leticia Casas-Godoy (305884). This study was supported by the (i) Program CYTED, under the scope of the “Ibero-American Network for the Extraction and Enzymatic Transformation of Functional Ingredients and Nutraceuticals from Regional Plants and Agro-Residues (ENZNUT)”, (ii) by the Strategic Project PEst-OE/AGR/UI0245/2011 of CEER- Biosystems Engineering, a research unit supported by the national funding of Fundação para a Ciência e a Tecnologia, Portugal.

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Chapter III: General Conclusions and Perspectives

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General conclusions and Perspectives

Lipases are enzymes that have a wide range of applications in the industry. The increasing knowledge regarding this type of biocatalysts has pushed the research towards enzyme improvement in function of their applications. Enzymatic engineering allowed the improvement of lipases characteristics such as activity, thermostability and tolerance to extreme pH and organic solvents. Enzyme selectivity improvement is one of the most interesting characteristics that can be changed by enzymatic engineering.

The first objective of this thesis was to efficiently produce a pharmaceutical molecule, tried in clinical assay for the treatment of cardiac arrhythmia by the french company Laboratoires Pierre Fabre. This molecule is an ester enzymatically-synthesised by the transesterification of *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) ethyl ester with nicotinol. For the success of this application, it was necessary the development of a supply route of high purity DHA ethyl ester and an efficient process for its functionalization.

Omega-3 PUFAs are of interest since *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) and *cis*-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA), the most important Omega-3, present anti-thrombotic and anti-inflammatory properties that reduce risk factors of arthritis rheumatoid, cancer, cardiovascular diseases, myocardial infarction, bronchial asthma, inflammatory intestinal diseases, diabetes and Alzheimer's disease.

Nicotinol (3-hydroxymethylpyridine), an alcohol from the group B pro-vitamin, after absorption, is rapidly converted into nicotinic acid (Vitamin B3) that presents the ability to substantially decrease plasma free fatty acid, triglyceride, VLDL and LDL levels and to raise the plasma concentration of protective HDL. DHA-nicotinol would present the cumulative properties of the two reactants. The enzymatic trans-esterification of DHA ethyl esters with nicotinol was optimised. The screening of commercial enzymes led to the identification of the best catalyst, the immobilized lipase from *Candida antarctica*, (Novozym 435). Different solvents were tested and finally the best reactional medium was a solvent-free system, composed only of the mixture of the two reactants. The non-use of a solvent was greatly appreciated by the company. From both kinetic and thermodynamic points of view, it was demonstrated crucial to evacuate the co-product, ethanol, from the reaction medium. Using nitrogen bubbling, a conversion of DHA ethyl ester to nicotinyl-DHA superior to 97 % was obtained in 4 hours using 45 $g.L^{-1}$ of enzyme. In these conditions, a productivity of 4.2 g of product $.h^{-1}.g$ of $enzyme^{-1}$ was obtained. In addition, nitrogen bubbling prevents oxidation of the polyunsaturated ester. This system could be extrapolated for the production of similar

products using other alcohols such as panthenol, which is the alcohol derived from pantothenic acid (Vitamin B5) which has pronounced antioxidant and radioprotective effects.

The second objective was to develop a supply route of high purity DHA ethyl ester. At the beginning of the thesis, it was not possible to obtain DHA ethyl ester with purity higher than 90% at a reasonable price. In this thesis, enzymatic purification was explored. In the bibliography, lipase from *Thermomyces lanuginosa* and the lipases from *Candida rugosa* were described as the most efficient to purify ω3 esters but their selectivity is not sufficient to obtain DHA of high purity. It was then crucial to discover a new enzyme for our purpose.

The lipase Lip2 from *Yarrowia lipolytica* (YLL2) appears as a good candidate since it is homologous to one of the most efficient lipase, the lipase from *Thermomyces lanuginosus*. During this research we proved that this lipase is an efficient enzyme for Omega-3 PUFAs purification. YLL2 produced concentrates rich in DHA ethyl ester (73%) with a recovery yield of 89%. This lipase was more efficient and more selective that *T. lanuginosus* and *C. rugosa* lipases. The highest concentration obtained with *T. lanuginosus* lipase was 65%. In addition, YLL2 presented a higher specific activity that allowed short reactions.

The three lipases from *C. rugosa* show a strong preference for esters with chain length smaller than C20, due to the tunnel topology of their active site. The positioning of the double bond the closest to the carboxylic group is also of importance for esters with chain length smaller than C20, being the ethyl γ linolenate one order of magnitude less recognised than α ethyl linolenate. In consequence, a DHA ethyl ester of high purity could not be obtained, since the lipases from *C. rugosa* presented low relativities versus γ linolenate, ARA, EPA and DPA.

On the other hand, mucorales lipases discriminate esters principally in function of the position of the double bond the closest from the carboxylic group, whatever the chain length. If the double bond the closest to the ester group is at least at the position 7, there is no large difference in reactivity. On the contrary, a double bond at positions 4, 5 and 6 are unfavourable. DHA the only member of the Δ4 family is the most resistant ester for both enzymes. In consequence, the expected DHA purity would be higher with mucorales lipases than with CR lipases, since they will consume the main part of esters containing more than 18 carbons, being γ linolenate the ester which would be the most difficult to separate from DHA. YLL2 is more efficient than TLL because ARA, EPA and DPA ethyl esters are better recognised and DHA discrimination is higher.

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YLL2 is consequently the best enzyme to undertake a strategy of enzyme evolution. Site-directed mutagenesis targeted to amino acids of the active site is often the most efficient and rapid method to improve selectivity of an enzyme. Using its three dimensional structure and its alignment with the lipase from *T. lanuginosus*, which was crystallized with oleic acid in the active site, targets for site directed mutagenesis were chosen in the active site. Each one of these targets was substituted by two amino acids of different sizes and analysed by comparing their performance with the wild type enzyme. From the screening of variants two positions with important effects in specificity where chosen, positions I100 and V235. The clear effect of these two positions in the specificity of Lip2 led to saturation of both positions. This new variants showed higher discrimination of DHA-EE and different specificity profile. Further research of double mutants of positions I100 and V235 generated large knowledge of the specificity of Lip2 from *Yarrowia lipolytica.* More research regarding the positions that interact with I100 and V235 could help us to better understand the selectivity mechanism of this lipase.

Finally we achieved the synthesis of structured lipids (SL) by enzymatic acidolysis between virgin olive oil and caprylic or capric acids using immobilized Lip2 from *Y. lipolytica*, a lipase never used before for this application. The SL obtained rich in oleic acid at the *sn-2* position while C8:0 and C10:0 should be mainly esterified at the *sn-1,3* positions. Lip2 from *Y. lipolytica* immobilized on Accurel MP 1000 was tested in a solvent-free system. In addition, the acidolysis reaction of olive oil with C8:0 or C10:0 catalyzed by immobilized YLL2 was optimized by response surface methodology (RSM) as a function of the molar ratio free fatty acids/triacylglycerols (FFA/TAG), temperature and reaction time. Results can be compared to incorporation of C8:0 in similar reactions using the commercial lipases Lipozyme RM IM and Lipozyme TL IM. The batch reactions were modeled and optimized using RSM. An excess of free fatty acids in the reaction mixture lead to acidic substrate inhibition, decreasing the initial reaction rate and the final incorporation degree. The best reaction conditions were the same for SL production with both fatty acids: molar ratio of 2:1 FFA/TAG, reaction temperature of 40°C and reaction time of 48h. Under these conditions, the SL produced had 25.6%mol of C8:0 and 21.3%mol of C10:0, which confirmed the validity of the model. Improvements of this reaction using the same enzyme could be reached by increasing the range of the parameters used in the RSM study.

Further work can be carried out in several of the objectives of this thesis. First of all for the DHA-EE purification the bioinformatics tools can be used for the discovery of new enzymes. For example the sequence analysis of YLL2 shows that in the Yarrowia clade there are five lipases from *Candida phangngensis* and one lipase form *Canida galli* closely related to Lip2

from *Y. lipolytica* (*Figure 1*). In addition there are thirty-one more lipases from *C. phangngensis, C. galli and Y. lipolytica* in this clade. The identity between these lipases is high, however the results from this work show that the amino acids in the active site are essential in enzyme selectivity. These lipases could have different specificities and can help to understand the mechanism of the lipase in the hydrolysis of fish oils ethyl esters.

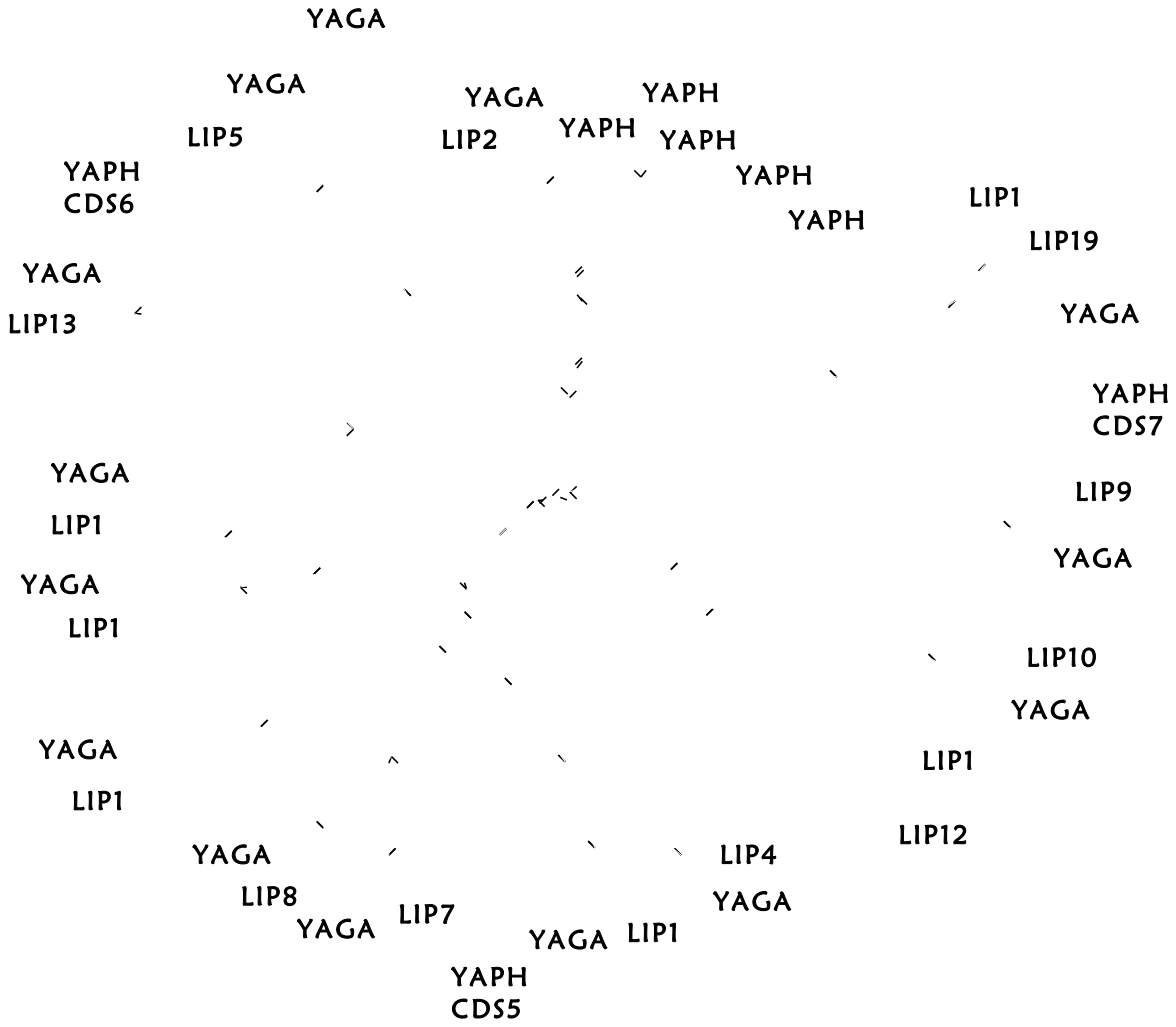

Figure 1. Yarrowia's clade, lipases form Y. lipolytica are represented as LIP followed by the number of the lipase, lipases from C. phangngensis are represented as YAPH and the lipase from C. galli as YAGA.

Enzymes could also be optimized by error prone PCR, this would require a robotic platform for the screening of mutants. In addition the robotic platform can be used for the development of a high throughout screening process for the selection of lipases with

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different specificities. A colorimetric method would help study the selectivity of the lipases versus ethyl esters with different chain length. Once the optimal enzyme is obtained it would be produced and used for the hydrolysis of fish oil ethyl esters and purification of DHA-EE. Reaction medium, temperature and reaction time would be then optimized for continuous reaction in a corning reactor. This reactor is optimal for a biphasic system since it creates micro-emulsions that allow good mass transfer.

An alternative for the purification of DHA would be the esterification of fish oil fatty acids. Preliminary results showed higher initial reaction rate and differences in the specificity of the lipases than the one observed during hydrolysis.

For the structured lipids production the reaction has to be studied using different reaction conditions. Fist of all the reaction has to be carried out with longer reaction times in order to reach the equilibrium and the amount of enzyme can be considered as a new factor. In addition some of the variants produced with changed selectivities could be used to produce SL and improve the reaction yield. Furthermore the concentrates of Omega-3 PUFAs produced by enzymatic purification could be used to enrich different vegetable oils and for the production of SL.

Résumé en Français

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Chapitre I : Etude Bibliographique

Publication 1, Partie I: Lipases

1. Définition de lipases

Les lipases sont serine hydrolases défini comme triacylglycerol acylhydrolases (E.C. 3.1.1.3) et devrait être différencié d'esterases (E.C. 3.1.1.1) par la nature de leurs substrats. En effet, le premier critère pour distinguer ces deux types d'enzymes "l'activation interfaciale", a été trouvés insuffisant puisque certaines lipases n'exposent pas ce phénomène. Plus tars, les lipases ont été défini comme des enzymes capables d'hydrolyser acylglycerol carboxyl esters de longue chaîne (≥10 atomes de carbone), tandis que les esterases hydrolyze acylglycerol carboxyl esters de courte chaîne (≤10 atomes de carbone). Comme les deux enzymes montrent une large spécificité de substrat, on doit considérer les deux critères (Verger, 1997; Chahinian et al., 2002).

2. Réactions catalysée par lipases

Les lipases catalysent l'hydrolyse de la liaison ester de tri-, di- et mono- glycerides en acides gras et glycérol. Ils sont aussi actifs sur une large gamme de substrats. Dans tous les cas, la réaction est effectuée à l'interface d'une réaction en système biphasée.

Les lipases, dans des conditions thermodynamiques favorables catalysent aussi une grande variété des réactions de synthèse qui peuvent se classer dans deux types, esterification et transesterification (Resont et al., 2009). Esterification, thio-esterification et amidation sont des réactions similaires, mais avec un acide gras, un thiol ou une amine comme des substrats. Transesterification groupes les réactions d'alcoholysis, acidolysis, aminolysis et les réactions d'interesterification. Les lipases peuvent aussi exprimer d'autres activités comme phospholipase, lysophospholipase, cholestérol esterase, cutinase ou des activités amidase, (Svendsen, 2000).

3. Les sources, rôle physiologique et règlement de l'expression de lipases

Aujourd'hui il est reconnu que les lipases sont produites par des divers organismes, y compris des animaux, des plantes et des micro-organismes (Vakhlu et Kour, 2006). Les lipases d'origine animale sont rarement assez pures pour être utilisé dans l'industrie alimentaire. Donc, le plus étudié et industriellement utilisé sont les lipases obtenues de

sources microbiennes. Les lipases de levures GRAS (Generally Recognized As Safe) sont largement acceptées et utilisées dans plusieurs industries incluant la transformation des aliments (Vakhlu et Kour, 2006).

Les lipases ont différentes fonctions physiologiques. Dans des micro-organismes l'expression est réglée par des facteurs environnementaux comme une réponse extracellulaire à un milieu pauvre. Dans la plupart des micro-organismes la présence de lipides et des acides gras comme sources carboniques incitent la production de ces enzymes extracellulaires.

4. Structure et mécanisme catalytique

Les premières structures des lipases obtenues ont été des *Rhizomucor miehei* (Brady et al., 1990) et la lipase pancréatique d'homme (Winkler et al., 1990). Des centaines de séquences de lipases se trouvent dans des bases de données et cent structures tridimensionnelles de lipases sont disponible dans Protein Data Base (http: // www.rcsb.org/pdb/home/). Cependant ces cent structures tridimensionnelles représentent lipases de seulement trente et un organismes, puisque la même lipase peut avoir plusieurs structures dans des conformations différentes, ou avec des substrats différents.

En ce qui concerne ses caractéristiques structurales, les lipases ont une motif structuraux, le repliement α / β, et une triade catalytique conservée. La plupart des lipases possèdent aussi la séquence consensus G-X1-S-X2-G. De leurs structures et les résidus formant le trou oxyanion (les acides aminés du site actif lipase qui stabilisent l'intermédiaire de réaction) et la triade catalytique, les lipases microbien et les esterases peut être groupée dans quinze superfamilles et trente-deux familles homologues (Pleiss et al., 2000a).

Le repliement α / β se compose d'un feuillet β central avec huit brins majoritairement parallèles et seulement le brin β2 antiparallèle. Les brins β3 à β8 sont connectés par hélices α arrangé sur les côtés de la feuille β central (*Figure 1*). La triade catalytique est composée d'une sérine comme nucleophile, d'un acide aspartique/glutamique comme le résidu acide et d'une histidine. Dans le repliement α / β, la sérine catalytique est placée après le brin β5, l'histidine après le brin β8 et l'acide aspartique/glutamique après le brin β7.

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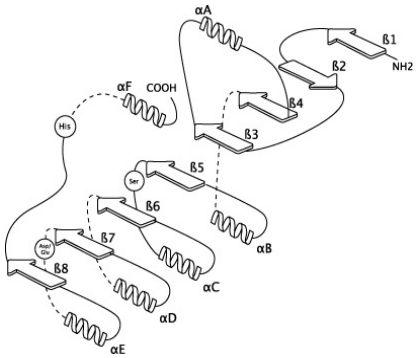

Figure 1. Le repliement α / β où les hélices α sont représenté par des spirales et les feuilles β par des flèches. Les résidus de site actif sont représentés par des cercles.

L'intermédiaire tétraédrique formé au cours de la réaction catalytique est stabilisé par au moins deux liaisons hydrogène avec deux acides aminés qui forment le trou oxyanion. Le premier résidu du trou oxyanion est situé dans la région N-terminale des lipases, dans une boucle entre le brin β3 et l'hélice αA. Pleiss et al. (2000a) ont identifié deux types de trou oxyanion *G**X*** et *GG**G**X*. Le deuxième résidu du trou oxyanion est le résidu X2 de la séquence consensus G-X1-S-X2-G. Elle est positionnée après le brin β5 dans le coude nucléophile qui est très conservée chez les lipases. Le trou oxyanion peut être préformé dans la conformation fermée de la lipase, sans la modification géométrique produite pendant l'ouverture du volet, ou formé seulement sur l'ouverture du volet.

La résolution des premières structures de lipases (Brady et al., 1990; Winkler et al., 1990) a permis l'identification d'une boucle qui couvre le site actif, le volet amphiphile. Le volet amphiphile est composé d'un ou plus hélices α, unis à la structure principale de l'enzyme par une structure flexible. C'est un élément mobile, qui découvre le site actif en présence d'une interface eau/lipide, et produit un changement conformationnel qui permet l'accès du substrat au site actif (Derewenda and Derewenda, 1991; Grochulski et al., 1993a; Grochulski et al., 1994b; Brzozowski et al., 2000). Ce mécanisme, connu comme l'activation interfaciale, explique le non Michaelis-Menten comportement observé dans la plupart des lipases. La *Figure 2* montre la lipase de *R. Miehei* dans sa conformation ouverte et fermée. Le volet amphiphile dans sa conformation fermée bloque l'entrée du substrat, diethyl phosphonate, tandis que le volet amphiphile ouvert permet l'accès au site actif (Moore et al., 2001).

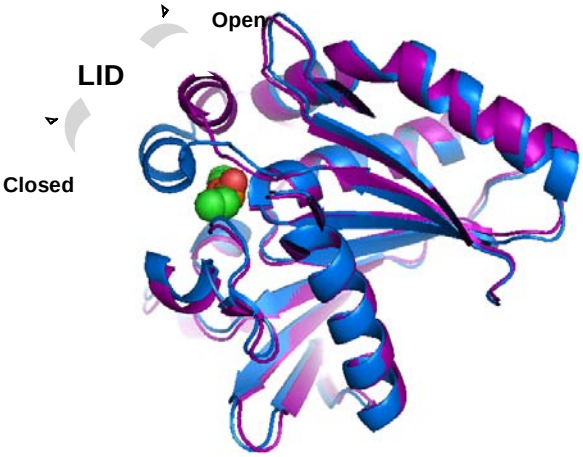

Figure 4. Rhizomucor miehei lipase. En violet sa conformation ouverte avec diethyl phosphonate, PDB 4TGL, (Derewenda et al., 1992a) et en bleu sa conformation fermée, PDB: 3TGL, (Brzozowski et al., 1992).

Le site actif de lipases est localisé dans

l'intérieur d'une poche sur le sommet de la feuille β centrale de la structure de la protéine. Les sites actifs de lipases diffèrent en leur forme, taille, profondeur de poche et caractéristiques de leurs acides aminés. Pleiss et al., (1998) ont classé les lipases en trois groupes en fonction de la topologie de leur site actif. Le premier groupe comprend des lipases avec une cavité hydrophobe proche de la surface, les lipases fongiques de type *Rhizomucor miehei* font partie de cette famille. Dans le deuxième groupe on trouve celles avec un site actif situé au fond d'un entonnoir, comme les lipases de *Candida antarctica, Pseudomonas,* la lipase pancréatique et la cutinase de mammifères. Dans le dernier groupe se trouvent les lipases avec un site actif en forme de tunnel, comme la lipase de *Candida rugosa*.

Le mécanisme catalytique des lipases commence avec une étape d'acylation. Le transfert de proton entre l'acide aspartique, l'histidine et la sérine catalytique, entraîne l'attaque nucléophile de l'hydroxyle de la sérine sur le carbonyle du substrat. Un premier intermédiaire tétraédrique est alors formé qui porte une charge négative sur l'oxygène du groupe carbonyle. Dans l'étape de dé-acylation un nucléophile attaque l'enzyme, qui libère le produit et régénère l'enzyme. Ce nucléophile peut être une molécule d'eau (hydrolyse) ou un alcool (alcoholysis).

5. Sélectivité

La sélectivité des lipases est sa préférence pour catalyser des réactions. Trois types de sélectivité peuvent être distingués: typosélectivité, régiosélectivité et enantiosélectivité. La typosélectivité c'est la sélectivité vis-à-vis du substrat, mono-di-triglycérides, et des acides gras par rapport à la longueur de la chaîne carbonée, de ses substituants et du degré d'insaturation. La régiosélectivité c'est la sélectivité par rapport à la position préférentielle d'hydrolyse sur les triglycérides. La enantiosélectivité c'est la sélectivité entre deux énantiomères ou deux molécules chirales.

6. Applications

Les lipases sont très important dans l'industrie puisque leur stabilité dans des solvants organiques, leur large variété de substrats, leur sélectivité et leur capacité de catalyser des réactions sans cofactors. De plus, ils sont aussi facilement produits et actif aux conditions ambiantes. Les lipases sont utilisent dans l'industrie alimentaire, pour la production de détergents et agents nettoyants, l'industrie pharmaceutique, pour bioremédiation et la production de biocombustibles, parmi d'autres.

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Partie II: Lip2 de *Yarrowia lipolytica*

La levure *Yarrowia lipolytica* est une levure non conventionnelle qui se caractérise par sa capacité à sécréter des protéines hydrolytiques (Guieysse et al., 2004; Fickers et al., 2011). C'est une levure qui produit une enzyme extracellulaire Lip2 responsable de toute la activité extracellulaire et deux enzymes unies à la cellule (Lip7 and Lip8) (Pignede et al., 2000a). Cette partie c'est une revue sur la lipase 2 de *Yarrowia lipolytica* en incluant ses caractéristiques structurelles et catalytiques, la spécificité du substrat, son clonage et production, l'amélioration du système d'expression et ses applications.

Partie III: Les acides gras polyinsaturés Oméga 3

Les huiles et les graisses sont deriveés d'acides gras et sont utilisés pour stocker l'énergie. Les acides gras (FA) sont des acides carboxyliques avec une chaine d'hydrocarbure de 4 à 36 carbones et avec une groupe terminal carboxyl (-COOH). Il y a des acides gras saturés et acide gras mono o polyinsaturés. La partie 1 de cette section décrit les types des acides gras, ses caractéristiques et ses fonctions. On trouve aussi les acides gras essentiels Omega-6 et Omega-3, ses caractéristiques, structures et fonctions. La partie 2 présent un revue sur les processus pour la obtention des concentrâtes de Omega-3 et les différentes techniques de purification principalement les techniques enzymatiques: estérification, transesterification et hydrolyse.

Partie IV : Lipides Structurés

Les lipides structurés sont des lipides fonctionnels qui peuvent être produit par techniques chimiques ou techniques comme les réactions enzymatiques. Les lipides structurés (SL) peuvent être définis comme triacylglycerols qui ont été modifié par techniques chimiques ou enzymatiques pour avoir des acides gras spécifiques dans les différentes positions du glycérol. Les SL ont des propriétés nutritionnelles spécifiques et des applications pour les industries alimentaires et pharmaceutiques. Cette partie c'est une revue sur les techniques enzymatiques pour la production de SL et les types des lipides structures. Une partie est dédiée aux lipides type MLM enrichis avec acide capric et caprylic, qui ont été produits pendant cette thèse.

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Chapitre II : Résultats

Ce projet de thèse s'est fixé deux objectifs principaux: premièrement, la purification et la fonctionnalisation d'acides gras poly-insaturés de type Omega-3 (PUFAs), et spécialement l'acide cis-4, 7, 10, 13, 16, 19-docosahexaénoique (DHA) et deuxièmement la production de lipides structurés. Le DHA présente des propriétés anti-thrombose et anti-inflammatoire qui permettent de réduire les facteurs de risque de l'arthrite, du cancer, de maladies cardiovasculaires, de l'asthme, du diabète et de la maladie d'Alzheimer.

Publication 2: Trans-esterification enzymatique des éthyles esters des acides gras à chaîne longue de la série Omega-3 concentrées, avec une alcool des provitamine du groupe B pour la prévention et traitement de maladies cardiovasculaires.

1. Introduction

Les acides gras polyinsaturés de la série des ω3-PUFAs, en particulier les acides docosahexaénoique (DHA) et eicosapentaenoique (EPA), sont des molécules actives. Ces acides gras ont un effet positif dans la prévention et le traitement de maladies cardiovasculaires et la modulation des facteurs de risque correspondants. Ils sont utilisés dans le traitement de l'hyperlipidémie, de l´hypercholestérolémie et le l´hypertension. Plusieurs études cliniques conduites avec des suppléments des esters éthyliques d'EPA et de DHA concentrés ont conclu qu'ils incitent une réduction de risques d'insuffisance coronarienne (Balk et al., 2006) et mortalité par crises cardiaques coronaires (Leaf et al., 2003; Harris and von Schacky, 2004; Harris et al., 2008; Lavie et al., 2009). Ces résultats ont été attribués à un effet de stabilisation des membranes cellulaires des cardiomycytes ventriculaires (Leaf et al., 1999), ce qui empêchent l'apparition d'arythmie maligne en présence de myocytes.

Finalement, le EPA et DHA présentent d'autres avantages comme: la réduction de pression artériel, la diminution d'accumulation plaquettaire (Pownall et al., 1999; Geleijnse et al., 2002; Balk et al., 2006; Harris et al., 2008) et ses non steroidal anti-inflammatoires propriétés. Contrairement à la série des ω6-PUFAs, les ω3-PUFAs sont les précurseurs de 3 séries prostanoids et 5 séries leukotrienes, molécules associés à propriétés anti-inflammatoires et anti-thrombotic (Calder, 2001; Simopoulos, 2002; Mori and Beilin, 2004; Ton et al., 2005).

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Dans cette étude on propose la production de une molécule pharmaceutique entre le ω3-PUFAs et le nictonitol, pour la obtention du nicotinyl DHA ester, actuellement en essai clinique pour le traitement des arythmies cardiaques. Le co-substrat du DHA est le nicotinol (3-hydroxymethylpyridine), un alcool appartenant au groupe de la pro-vitamine B. Après absorption, il est rapidement converti en acide nicotinique (Vitamine B3) qui possède la propriété de décroitre les acides gras libres dans le plasma, les triglycérides et d'augmenter dans le plasma la concentration des lipoprotéines bénéfiques. La trans-esterification enzymatique entre l'ester éthylique du DHA et le nicotinol a été catalysée par lipases. En plus la réaction a été optimisée en fonction de milieu, température, enzyme/substrat et ester/alcool ratio, dans le but de synthétiser un ester présentant les propriétés cumulatives des deux réactants.

2. Matériels et méthodes

Les matériels utilisés se trouvent dans la section 2.1. Également les techniques et procédures pour la réaction enzymatique avec et sans solvant et les techniques d'analyse se présentent dans les sections 2.2 à 2.3.

3. Résultats et discussion

Comme l'objectif était le développement rapide d'un processus industriel pour la transesterification de l'éthyle ester de DHA (DHAEE) avec nicotinol (pyridin-3-ylmethanol), on a seulement testé des lipases immobilisées commerciales. Quatre lipases ont été choisis, Novozyme 435, Lipozyme RM IM, Lipozyme TL IM et Lipase PS Amano IM et évalués à 40°C, dans 2-methyl-2-butanol (2M2B) avec un ratio molaire de 1.5 nicotinol/DHAEE et la relation d'enzyme/ester de 7 % (w/w). Lipozyme RM IM et Lipozyme TL IM ont montré une conversion basse. Avec PS Amano IM seulement 22% de conversion du DHAEE a été obtenu après 72h. Novozyme 435 a été l'enzyme la plus efficace avec 19% de conversion du DHAEE au bout de 1 heure. Cette enzyme est stable à 60°C. Dans ces conditions, 26% de conversion du DHAEE a été obtenu après 1 heure, qui représente une augmentation de 38% comparés au résultat obtenu à 40°C. De ces résultats, Novozyme 435 a été choisi pour améliorations, choisissant 60°C comme la température optimale.

Le choix du milieu de réaction influencera l'activité de l'enzyme et sa stabilité, la conversion à l'équilibre thermodynamique, la solubilité de substrats et des produits et par conséquence la réutilisation et la productivité de l'enzyme et la stabilité du réacteur. Les solvants testées

sont été: 5-methyl-2-hexanone, 2 méthyle 2 butanol (2M2B) et acetonitrile. Le milieu idéal consisterait en un système sans solvant (SFS) seulement composé par les réactifs. Le même ratio enzyme/DHAEE (g/g) a été utilisé avec et sans solvant pour pouvoir comparer les résultats. Table 1 a les pourcentages de DHA estérifiés avec les conditions testées.

Table 1. Pourcentage de conversion du DHAEE à DHA-Nicotinol. DHA/nicotinol ratio était 1:1.5 et la température 60°C. Novozyme 435: 7% w/w du DHAEE.

Solvant	Point d'ébullition (°C)	log P*	Conversion DHAEE (%)	
			30 minute	6 hours
Acetonitrile	82	-0.34	10.8	35
2M2B	102	0.89	12.4	38
5-methyl-2-hexanone	145	1.88	25.4	41
Système sans solvant	-	-	34.5	43

* log P : n-Octanol/Coefficient de partage d'eau

L'activité d'enzyme (après que 30 minutes de réaction) et la conversion finale (après que 6 heures de réaction) plus haute ont été obtenues avec le système sans solvant (43% de conversion au bout de 6 heures). Si un solvant a été utilisé, les conversions les plus hautes ont été obtenues avec 5-methyl-2-hexanone (41%), suivis par 2M2B (38%). Le système sans solvant (SFS) et le 2M2B ont été ainsi choisis pour améliorations.

À l'équilibre thermodynamique, la conversion de réaction a atteint 38% et 43% avec 2M2B et sans solvant respectivement. Cet équilibre pourrait être changé par l'évaporation de l'éthanol formé ou/et en augmentant le ratio entre l'alcool et l'ester. Pour confirmer cette hypothèse la réaction a été effectuée dans des tubes ouverts pour favoriser l'évaporation d'éthanol (Table 2).

Table 2. Pourcentage de conversion de DHAEE à DHA-Nicotinol dans système fermé et ouvert. Le ratio DHA:nicotinol était 1:1.5 et la température 60°C. N435: 7 % w/w de DHAEE.

Solvant	Conversion de DHA éthyle ester (%)			
	Tube ouvert		Tube fermée	
	30 min 2M2B 15 min SFS	6 heurs	30 min 2M2B 15 min SFS	6 heurs
2M2B	12.4	37.5	19.8	71.5
Système sans solvant	19.5	43.0	22.3	74.2

Pour le deux systèmes l'activité de l'enzyme été plus élevé avec les tubes ouverts. L'utilisation de tubes ouverts a permis changer l'équilibre thermodynamique atteignant 71.5% et 74.2% des conversion au bout de 6 heures avec 2M2B et sans solvant respectivement.

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Le ratio DHAEE:nicotinol a été aussi étudié pour optimiser la cinétique et l'équilibre thermodynamique. Les ratios testés étaient 1:1, 1:1.5 et 1:3 dans 2M2B et SFS, avec Novozyme 435 à 60°C (Figure 1). La conversion plus haute obtenue avec 2M2B été avec un ratio 1:1 en obtenant une conversion de 95% après 24h, tandis que avec les ratios 1:1.5 et 1:3 les conversions obtenues après 24h ont été de 90% et 83% respectivement. Pour SFS, la réaction est deux fois plus efficace que avec 2M2B, la conversion a atteint 40% au bout de 30 minutes. À l'équilibre, les meilleures performances ont été obtenues avec un ratio 1:1 et 1 :1.5 avec une conversion de 97% pour un réaction de 24 h. Le système sans solvant été optimal pour cette réaction et a été choisi pour optimisation.

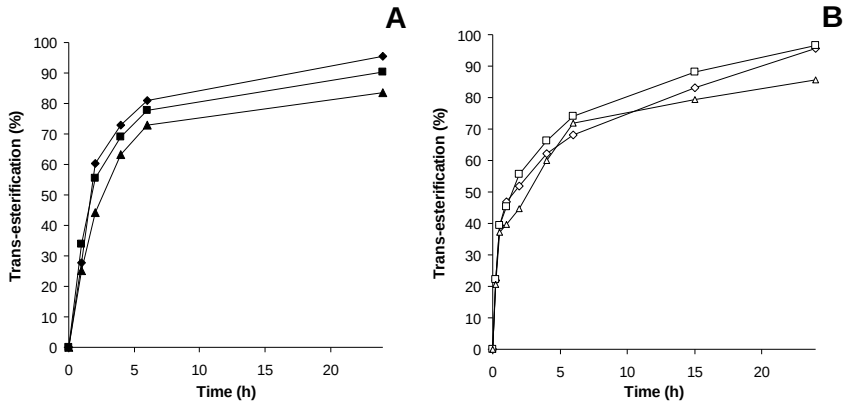

*Figure 1. Pourcentage d'estérification de DHAEE à DHA-Nicotinol avec différents ratios, dans 2M2B (**A**) et SFS (**B**). (◆) 2M2B ratio 1:1, (■) 2M2B ratio 1:1.5, 2M2B ratio 1:3 (▲), (◇) SFS 1:1, (□) SFS 1:1.5 and (△) SFS 1:3. Temperature 60°C. N435 (7% w/w).*

Les conditions choisies étaient : température de 60°C, 45 g/L de Novozyme 435 et un ratio DHA l'éthyle ester:Nicotinol de 1:1 ou 1:1.5. Enlever l'éthanol a été trouvé crucial pour cette réaction donc une autre stratégie a été examinée: bouillonnement d'azote. Ce processus présenterait l'avantage d'éviter l'oxydation de l'ester DHA. La réaction avec l'azote a donné des conversions très élevées (supérieur à 94%) au bout de 4 heures. Un ratio de DHAEE:nicotinol de 1.5 a apparu optimal avec une conversion de 99% dans 4 heures.

4. Conclusions

L'enzyme optimale a été la lipase immobilisée de *Candida antarctica*, Novozyme 435 et le choix du milieu réactionnel une milieu sans solvant. Une conversion supérieure à 99 % a été obtenue en 4 heures avec 45 g.L-1 d'enzyme. Dans ces conditions, une productivité de 4.2 g de produit h^{-1}.g d'enzyme^{-1} a été obtenue.

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Publication 3: La lipase Lip2 de *Yarrowia lipolytica*: une enzyme efficace pour la production de concentrés des éthyle esters de DHA.

1. Introduction

L'intérêt pour les acides gras polyinsaturés (PUFAs) Oméga-3 (ω–3) a augmenté en raison de leurs effets positifs sur la santé. Particulièrement l'acide docosahexaenoic (DHA, C22:6) et l'acide eicosapentaeonic (EPA, C20:5) qui présentent propriétés anti-thrombotic et anti-inflammatoires (Carvalho et al., 2009; Okada et Morrissey, 2008; Rubio-Rodriguez et al., 2010). La manque de ω–3 peut provoquer fatigue, peau sèche, des problèmes du coeur, de mauvaise circulation, dépression et perte de mémoire, parmi d'autres. Le ratio optimal d'Omega-6 et ω–3 est de 4 :1, malheureusement dans la plupart des régimes, particulièrement les régimes occidentaux, la consommation de ω-3 est plus haut que l'optimal, atteignant des ratios plus haut que 10.

Plusieurs méthodes ont été développées pour concentrer ω–3 PUFAS, y compris chromatographie d'adsorption, distillation moléculaire, cristallisation a basse température, complexation d'urée, extraction liquide supercritique et réaction enzymatique (Rubio-Rodriguez et al., 2010). D'entre elles les réactions enzymatiques sont les plus intéressantes. Les lipases peuvent discriminer entre EPA et DHA, caractéristique important pour des applications médicales spécifiques.

Un procédé de purification enzymatique a été choisi car cela permet de travailler dans des conditions à faible température ce qui est un pre-requis car le DHA est sensible à l'oxydation. Les lipases sont capables de discriminer entre les acides gras en fonction de la longueur de chaine et du degré d'insaturation. Les lipases agissent par résolution cinétique, en réagissant plus efficacement avec les acides gras saturés et mono-insaturés qu'avec les PUFAs résistants. Il reste toujours d'un grand intérêt de découvrir des enzymes spécifiques pour la purification du DHA. La lipase YLL2 de *Yarrowia lipolytica* apparait comme un bon candidat car elle est homologue à une des lipases les plus efficaces, la lipase de *Thermomyces lanuginosus*. En plus on a étudié les lipases Lip1 (CRL1), Lip3 (CRL3) et Lip4 (CRL4) de *Candida rugosa*. Ces lipases ont été étudiés en comparant leur capacité de concentrer DHA-EE et EPA-EE dans la fraction d'ester par hydrolyse d'un mélange d'éthyle ester d'huile de thon (FOEE). On considérera la discrimination entre PUFAS et particulièrement entre le EPA et DHA.

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2. Matériels et méthodes

Les matériels utilisés se trouvent dans la section 2.1. Également les techniques et procédures pour la production de lipases, l'activité enzymatique, la réaction d'hydrolyse et les hydrolyses successives se présentent dans les sections 2.2 à 2.6.

3. Résultats et discussion

Les principaux composants du mélange d'éthyle esters d'huile de thon (FOEE) représentant 89.6% mol du mélange et sont esters de DHA (23.6%), acide palmitic (21.4%), acide oléique (13.2%), acide palmitoléique (6.7%), acide stéarique (5.6%), EPA (5.2%), acide myristic (4.7%), acide alpha linolénique (2.2%), acide arachidonique (1.7%), acide linoléique (1.6%), acide gamma linolénique (1.2%), DPA (1.0%) et acide 11-eicosanoate (0.9%).

La principale lipase extracellulaire de la levure *Y. lipolytica* (YLL2), la lipase de *T. lanuginosus* (TLL) et les trois principales lipases de *C. rugosa* (CRL1, CRL3 et CRL4) ont été expresse dans la souche JMY1212 de *Y. lipolytica*, souche spécialisé sur l'expression d'enzyme et la comparaison des activités enzymatiques. Les activités ont été obtenu avec le test d'hydrolyse de p-nitro phényle butyrate et se présentent dans la Table 1.

Table 1. Caractéristiques des lipases.

Source	Lipase	Abréviation	Activité (U/ml)[a]
Yarrowia lipolytica	Lip2	YLL2	38.7
Thermomyces lanuginosus	-	TLL	26.2
	Lip1	CRL1	42.3
Candida rugosa	Lip3	CRL3	1.8
	Lip4	CRL4	11.3

[a] µmol de *p*-nitrophenol libéré per minute et ml d'enzyme.

L'hydrolyse a été effectué dans un système bi-phasique (FOEE en decane / enzyme dans eau, v/v) avec le cinq lipases. Pendant la réaction les lipases devraient hydrolyser les éthyle esters saturés et mono-, di- et tri-insaturés et laisser les PUFA dans la forme ester. Comme la concentration d'esters dans le mélange est très différente, la vitesse initiale n'est pas le paramètre approprié pour comparer l'efficacité d'enzyme contre les différents esters. On donne le coefficient d'efficacité, la vitesse initiale divisé par la concentration initiale d'ester, pour les couples d'enzyme/substrat. On peut aussi calculer le factor de compétition α pour évaluer la capacité de chaque enzyme pour discriminer entre les différents éthyles esters.

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Une α élevé indique un activité faible versus une éthyle ester spécifique, donc un haute discrimination (Table 2).

Table 2. Factor de compétition α pour les différentes lipases, obtenu après 6 heurs de réaction pour YLL2 and TLL et après 24 heurs pour les lipases de C. rugosa.

Enzyme	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	αC18:3	γC18:3	C20 :1	ARA	EPA	DHA	DPA
YLL2	2.5	2.9	1	3.7	0.9	1.7	2.5	13.5	3.2	10.3	9.1	29.4	3.9
TLL	1.0	1.2	1	1.3	1.1	1.4	1.5	5.9	1.3	4.1	3.6	10.5	1.4
CRL1	4.4	3.9	1	16.9	1.6	1.6	5.5	46.6	>100	>100	26.3	82.8	12.0
CRL3	5.1	3.7	1	14.1	2.5	2.8	9.6	47.0	22.5	48.5	39.4	36.7	>100
CRL4	3.3	1.4	1	2.6	1.0	2.2	5.6	27.6	78.0	>100	9.1	>100	32.1

Pour tous le esters YLL2 est l'enzyme plus efficace, étant 2 fois plus actif que TLL, la deuxième enzyme plus efficace. YLL2 présente une activité plus haute que TLL pour l'éthyle oleate, palmitoleate et linolenate (3.7, 3.0 et 2.7 fois plus actives respectivement), tandis que pour l'éthyle myristate, palmitate, stéarate, α linolenate, arachidonate et EPA, le ratio est inférieur à 2. Pour l'éthyle 11-eicosenoate et DHA, le deux enzymes présentent approximativement la même activité. Finalement le γ linolenate est moins reconnu par YLL2 que par TLL. Le trois lipases de *C. rugosa* sont moins actifs que YLL2. Étonnamment, CRL3, qui a présenté une activité d'hydrolyse p-NPB basse, est ici aussi efficace que ses deux enzymes homologues.

Pour YLL2 et TLL la discrimination contre des esters est fonction de la position de la double liaison la plus proche du groupe carboxylique. Si la double liaison plus proche au groupe d'ester est au moins à la position 7, la réactivité est haute avec un optimum pour les esters monoinsaturés. Au contraire, si la double liaison est aux positions 4, 5 et 6, l'activité est défavorable pour YLL2 et TLL. DHA le seul membre de la famille Δ4 est l'ester le plus résistant pour les deux enzymes. L'éthyle gamma linolenate, le seul membre de la famille Δ6, est plus résistante que les deux membres de la famille Δ5, éthyle ARA et éthyle EPA, pour les deux enzymes. Il a été précédemment rapporté que lipases montre une discrimination plus haute contre des acides gras avec leur premier double à un carbone avec un nombre pair (*cis*-4, *cis*-6) que le reste d'eux (*cis*-5, *cis*-9).

Avec les lipases YLL2 et TLL la concentration des éthyles ester mieux reconnus diminue rapidement et après elle reste constante. Le temps à lequel la réaction de une éthyle ester spécifique s'arrête dépend de sa reconnaissance par l'enzyme, ces observations nous font croire que chaque réaction individuelle s'arrête en raison d'un équilibre thermodynamique.

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Pour augmenter la pureté de DHA-EE dans le mélange, trois hydrolyses successives ont été exécutées avec des temps de réaction optimaux (5h pour YLL2 et TLL). Entre chaque phase de réaction, les acides gras ont été enlevés par saponification et enzyme fraîche a été ajoutée. Pendant ce processus, la plupart de l'éthanol a été aussi enlevé. Chaque hydrolyse a augmenté le pourcentage de DHA-EE (Figure 1). Après trois hydrolyse, la pureté plus haute du DHA-EE a été obtenue avec YLL2, 73 %, contre 65 % avec TLL. La récupération du DHA-EE était plus haute avec YLL2, 89 %, qu'avec TLL, 85 %.

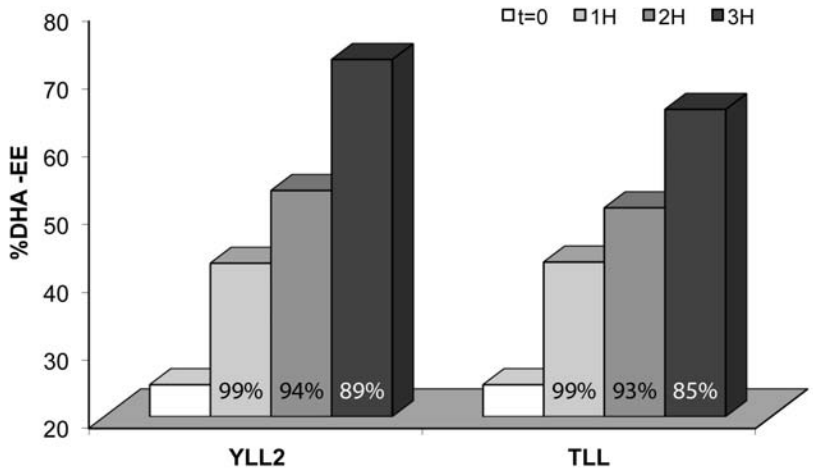

Figure 1. Pourcentage de DHA-EE après trois hydrolyses avec YLL2 et TLL; le pourcentage de récupération pour chaque hydrolyse se montre à la base de sa colonne. Temps de réaction 5h pour hydrolyse.

Même si efficace, un processus consistant de réactions successives, est complexe d'un point de vue industriel. Pour mieux comprendre les raisons qui causent l'arrêt de réaction, éthanol (50 mm) a été ajoutés au mélange de réaction initial. L'addition d'éthanol a diminué la conversion à l'équilibre dans 36%. Une expérience avec un réacteur ouvert a été testée pour favoriser l'évaporation d'éthanol. Avec cette méthode une pureté de 89.5% de ω3 esters et 77.1% de DHA ont été obtenus.

4. Conclusions

YLL2 a permis d'obtenir une discrimination très efficace. Les raisons de la sélectivité de l'enzyme ont été identifiées : il s'agit du positionnement de la double liaison la plus proche de la fonction carboxylique. La concentration en DHA la plus élevée a été obtenue avec YLL2 (77%) avec un pourcentage de récupération du DHA-EE de 89%. YLL2 est par conséquent l'enzyme décrite la plus efficace pour la purification du DHA.

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Publication 4: La mutagénèses dirigée améliorée la spécificité de Lip2 d'*Yarrowia lipolytica* vers la purification d'éthyle ester de DHA.

1. Introduction

L'intérêt pour les acides gras polyinsaturés (PUFAs) Oméga-3 (ω–3) a augmenté en raison de leurs effets positifs sur la santé. Particulièrement l'acide docosahexaenoic (DHA, C22:6) et l'acide eicosapentaeonic (EPA, C20:5) qui présentent propriétés anti-thrombotic et anti-inflammatoires.

La lipase Lip2 de *Yarrowia lipolytica* est capable de purifier le éthyle ester du DHA. La concentration en DHA la plus élevée obtenu avec YLL2 été de 77% avec un pourcentage de récupération du DHA-EE de 89%. Devant le grand intérêt de l'enzyme Lip2 de *Yarrowia lipolytica* pour la purification du DHA, la mutagénèse ciblée dans le site actif a été utilisée pour améliorer la sélectivité de cette enzyme. L'analyse de la structure 3D et les alignements avec des lipases homologues a permis de choisir les cibles de mutagénèse dirigée. Les acides aminés cibles ont été changés de manière à restreindre ou élargir le site actif.

2. Matériels et méthodes

Les matériels utilisés se trouvent dans la section 2.1. Également les techniques et procédures pour la construction de variantes de Lip2, production de lipases, l'activité enzymatique et la réaction d'hydrolyse se présentent dans les sections 2.2 à 2.5.

3. Résultats et discussion

Une structure 3D d'YLL2 avec le lid dans une position qui permet l'accessibilité à la serine catalytique n'est pas disponible. Le modèle tridimensionnel a été précédemment construit par techniques d'homologie en utilisant les structures de lipases de *Rhizomucor miehei* (4TGL) et *T. lanuginosa* (1GT6) comme des modèles (Bordes et al., 2009) (Figure 1). Les structures globales sont semblables, les différences plus significatives se trouvent dans la surface. Les trois résidus catalytiques (S162, D230 et H289) et les deux acides aminés impliqués dans le trou oxyanion (T88 et L163) sont parfaitement superposés dans ces enzymes. Le site de liaison de substrat apparaît comme une crevasse hydrophobe située à la surface de la protéine, avec la triade catalytique exposée au solvant.

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Figure 1. L'alignement multiple de Lip2 de Yarrowia lipolytica (Y.l.), Thermomyces lanuginosa (T.l.), Rhizomucor miehei (R.m.), Rhizopus niveus (R.n.), le feruloyl esterase d'Aspergillus niger (1USW) et un fragment de une putidaredoxin de Pseudomonas putida (1put). Le résidus des hélices α et feuilles β sont colores magenta et vert, respectivement. Le trois résidus catalytiques sont rouge, le deux résidus catalytique du trou oxyanion sont orange, les cystéines sont de couleur bleue et le lid est souligné.

Les positions sélectionnés pour mutagenèse dirigée sont T88, V94, D97,I98, R99, I100, F129, I231, V232, V235, D239, V285 et L290. T88 c'est un acide amide du trou oxynion, et les acides amines V94, D97, I98, R99 et I100 font partie du lid. En plus les acides amines I98, R99 et I100 forment un tour d'hélices α supplémentaire au C-terminus. Les variantes des positions V94 et V232 ont été construites précédemment pour changer la enantio-préférence de la lipase. Les variantes I98A, I98V, R99K, R99Q, I100A, I100L, F129I, V235A, V235F et V235L ont été construites pour ouvrir ou fermer le site actif. Les activités en *p*-nitrophenyl butyrate se trouvent sur la Table 1.

Pour chaque réaction d'hydrolyse deux facteurs ont été analysés: le coefficient d'efficacité et le facteur de compétition α. Une α élevé indique un activité faible versus une éthyle ester spécifique, donc un haute discrimination. Les variantes ont été classifiées en fonction de leurs cinétique et sélectivité en comparaison avec YLL2 sauvage.

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Table 1.Activité en p-Nitrophenol butyrate du WT de YLL2 et ses variantes.

Enzyme	Activité (U/mL)[a]	Enzyme	Activité (U/mL)[a]	Enzyme	Activité (U/mL)[a]
WT	38.7	**R99K**	13.9	**V235A**	67.0
T88S	12.2	**R99Q**	36.1	**V235F**	11.9
V94A	12.8	**I100A**	62.5	**D239E**	20.7
V94L	14.0	**I100L**	14.2	**D239K**	80.0
D97A	8.5	**F129I**	9.9	**V285A**	62.7
D97V	9.5	**I231F**	18.0	**V285L**	29.1
I98A	23.8	**I231V**	18.5	**L290A**	53.1
I98V	45.4	**V232A**	21.6	**L290F**	33.3
		V232F	47.6		

[a] µmol of *p*-nitrophenol liberated per minute and mL of enzyme.

Les variantes V94A et D239K avaient un comportement similaire à WT-YLL2. Les variantes avec un activité hydrolytique faible ont été D97V, D97A, L290F, V232A, V232C, V232F, V232L, V232S et V232T. Les variantes V232A, V232C, V232F, V232L et V232S ont un factor α plus faible que WT-YLL2 pour les éthyles esters polyinsaturés. La variante I100A a eu un coefficient d'efficacité faible et un α plus bas pour les esters saturés, di et tri insaturés. Les variantes T88S, I98A, R99K, I231F, I231V et D239E ont montré une préférence plus haute pour le C16:1 que pour le C18:1, qui est une sélectivité inversé que celui présenté par le WT-YLL2. Les variantes V290A, V285A, V285L, I98V, R99Q et I100L sont trouvées plus actives que WT-YLL2. La variante I100L été une des plus actives pour l'hydrolyse de ARA et EPA esters. Les variantes de la position V235 ont une sélectivité versus le DHA-EE avec facteurs de compétition plus faibles qu'il de WT-YLL2. De ce premier screening de variantes deux positions ont permis d'améliorer la spécificité de l'enzyme, les positions I100 et V235.

Le dernière factor analyse été la pureté de DHA-EE, EPA-EE et ω3 éthyle ester mélange. Les variantes des positions D97, V232 et I100A et V235F n'ont pas produit hautes concentrations de DHA-EE. Les puretés de DHA-EE plus haute ont été obtenues avec I100L (44.0%), L290A (43.9%), V235L (43.3%), D239K (43.0%) and V285L (43.0%) après 6 heures de réaction. Une récupération de DHA-EE plus haute de 88% a été obtenue avec ces cinq variantes. La hydrolyse plus grande de EP-EE a été trouvé avec I100L, R99Q, V235L, I98V, L290A et D239K.

4. Conclusions

La mutagenèse dirigée nous a permis d'étudier l'effet des positions spécifiques dans la sélectivité de longueur de chaîne d'YLL2. La sélectivité d'enzyme est principalement en raison du positionnement du double lien plus proche du groupe carboxylique. Des changements du profil de sélectivité des mutants et de la discrimination vers DHA-EE ont été obtenus.

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Publication 5: Optimisation de l'incorporation des acides gras à chaîne moyenne dans l'huile d'olive catalysée par Lip2 d'*Yarrowia lipolytica* immobilisé.

1. Introduction

Les lipides Structurés (SL) peuvent être définis comme triacylglycerols (TAG) qui ont été (i) restructurés pour changer la position des acides gras (FA) sur le glycérol, (ii) modifié par l'incorporation de nouveaux acides gras ou (iii) synthétisé de novo pour produire une nouvelle TAG, a partir de un procès chimique ou enzymatique (Iwasaki et al., 1999; Iwasaki and Yamane, 2000; Osborn and Akoh, 2002). Les MLM sont des SL avec acides gras à moyenne chaîne (M), contenant entre 6 et 10 carbones, dans les positions *sn-1* et *sn-3,* et des acides gras á longue chaîne (L), avec plus de 12 carbones, á la position *sn-2.* Ce type de SL évite des problèmes de santé liés avec les TAG á chaîne longue et ils ont des propriétés nutritionnelles, énergiques et pharmaceutiques.

Les lipases immobilisées commerciaux ont été utilisées pour la modification des différents huiles comme d'olive, cacahuète, carthame et soja pour la production de MLM (Shimada et al., 1996; Lee and Akoh, 1998; Xu, 2000; Fomuso and Akoh, 2002; Kim et al., 2002; Lai et al., 2005; Li et al., 2008; Nunes et al., 2011a). Les MLM plus intéressant ont acide caprylic (C8:0) ou capric (C10 :0) dans les positions *sn-1* et *sn-3* et un acide monoinsaturé (acide oléique) ou polyinsaturé dans la position *sn-2*.

L'objectif de cette étude était la production de lipides structurés (SL) par acidolysis enzymatique entre l'huile d'olive vierge et les acides caprylic ou capric utilisant la lipase Lip2 de *Yarrowia lipolytica* (YLL2) immobilisé. Le SL obtenu devrait être riche en acide oléique à la position *sn-2* tandis que les C8:0 et C10:0 devraient être principalement estérifiés aux positions *sn-1,3*. YLL2 immobilisé sur Accurel 1000 a été testé dans un système sans solvant. La réaction d'acidolysis d'huile d'olive avec C8:0 ou C10:0 catalysé par YLL2 immobilisé a été optimisée avec la méthodologie de surface de réponse (RSM).

2. Matériels et méthodes

Les matériels utilisés se trouvent dans la section 2.1. Également les techniques et procédures pour la production d'enzyme, l'activité enzymatique, l'immobilisation d'enzyme, la réaction de acidolysis, le design expérimental, l'analyse statistique, la validation du model et l'analyse des produits se présentent dans les sections 2.2 à 2.8.

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3. Résultats et discussion

Les réactions de screening ont été effectuées dans un système sans solvant pour 24h, à 40°C pour différents ratios molaires (2:1 à 8:1; FFA/TAG) et à ratio de molaire de 2:1, FFA/TAG pour différentes températures (30-50°C). Pour tous les ratios molaires les incorporations molaires étaient plus hautes pour C8:0 que pour C10:0. L'incorporation C8:0 a diminué de 20.3%mol à 15.9%mol du ratio 2:1 à 4:1 FFA/TAG. Dans la même gamme de ratios molaires, l'incorporation de C10:0 a resté constant (14.6%mol). Pour C8:0 et C10:0 l'incorporation a diminué avec hauts ratios molaires. La température n'a pas eu une grande influence sur le degré d'incorporation, particulièrement pour C10:0.

Les valeurs d'incorporation de C8:0 ou C10:0 dans l'huile d'olive vierge, par la réaction acidolysis, sans solvant, catalysé par YLL2 immobilisé, dans les conditions des designs expérimentaux suivis, sont présentées dans les Tables 1 et 2 de la section 3.3 de cet publication.

Les résultats montrent que pour l'acide caprylic la température n'a aucun effet significatif dans l'incorporation de cet acide gras. En plus, aucune interaction significative n'a pas été observée entre les facteurs. Le ratio molaire et le temps de réaction ont des effets linéaires significatifs sur l'incorporation C8:0 dans l'huile d'olive. L'incorporation d'acide carylic dans l'huile d'olive peut être adapté à une surface de réponse plate (Figure 1A), décrit par une équation de premier ordre (Table 1).

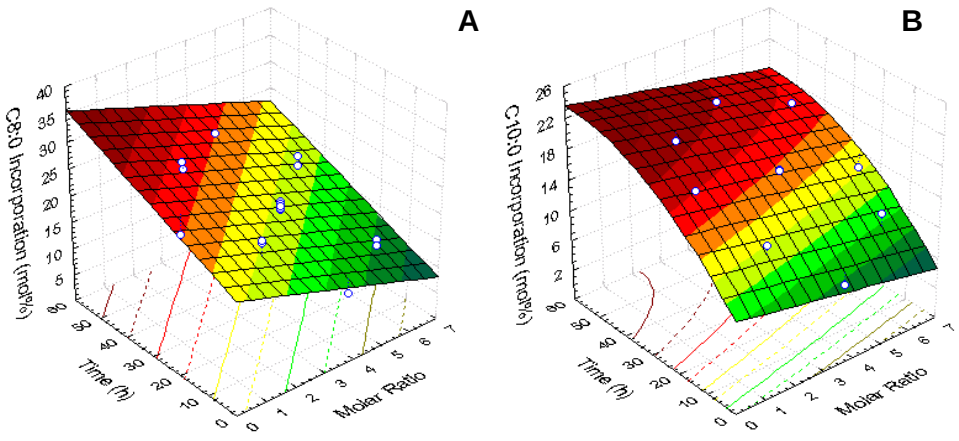

Figure 1. Surface de réponse adaptée à l'incorporation des acides caprylic (C8:0) ou capric (C10:0) dans huile d'olive vierge par acidolysis catalysé par YLL2 immobilisé, comme une fonction de temps de réaction et ratio molaire FFA/TAG.

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Dans l'incorporation acide capric on a observé une effet linéaire négatif de MR et un effet linéaire positif de temps de réaction. Ce système a été aussi affecté par un effet quadratique négatif de temps de réaction produisant une surface de réponse convexe (Figure 2B) qui peut être décrit par une équation de deuxième ordre (Table 1). Les hautes valeurs de R^2 et R^2_{adj} de ces modèles indiquent une bonne ajustement pour l'incorporation d'acide caprylic (R^2 = 0.89) et une parfaite ajustement pour l'incorporation acide capric (R^2=0.93) (Haaland, 1989).

Table 1. Équations modèles de la surface de réponse adaptée à l'acidolysis d'huile d'olive avec acide caprylic ou capric, catalysé par Lip2 immobilisé, comme une fonction de ratio molaire FFA/TAG (MR) et du temps de réaction (t, h).

System	Équation Model	R^2	R^2_{adj}
Huile d'olive + C8:0	C8:0 %mol incorporation = 17,41 - 2,23·MR + 0,289·t	0.89	0.88
Huile d'olive + C10:0	C10:0 %mol incorporation = 6,91 - 0,92·MR + 0,55·t -0,0047·t^2	0.93	0.90

A partir des surfaces de réponse on a identifié les régions correspondant aux incorporations plus hautes. Pour les deux systèmes, les incorporations plus hautes à l'intérieur du domaine expérimental devraient être atteintes à 40°C, avec un ratio de molaire de 2:1 FFA/TAG et un temps de réaction de 48ème. Le modèle a été validé dans ces conditions, et la composition du SL obtenu se montre dans la Table 2.

Table 2. Composition des acides gras (%mol) d'huile d'olive et SL obtenu a MR de 2:1 FFA/TAG, après 48h à 40°C.

Fatty acid	**Virgin olive oil**	**SL**	
		C8:0	**C10:0**
C8:0	-	25.6	-
C10:0	-	-	21.3
C16:0	12.7	8.1	9.8
C18:0	2.9	2.3	2.3
C18:1	7.7	58.6	60.7
C18:2	7.3	5.5	5.9

En plus on a suivi la cinétique de la réaction à 40ºC, pendant 48h, pour les ratios molaires de 1:1, 2:1 et 4:1, (Figure 3). L'incorporation d'acide caprylic après 48h sont été 26.2%mol, 25.6%mol et 16.6%mol pour MR de 1:1, 2:1 et 4:1, respectivement. Après 48h, les incorporations d'acide capric trouvées eté de 21.0%mol, 21.3%mol et 17.3%mol pour MR 1:1, 2:1 et 4:1, respectivement.

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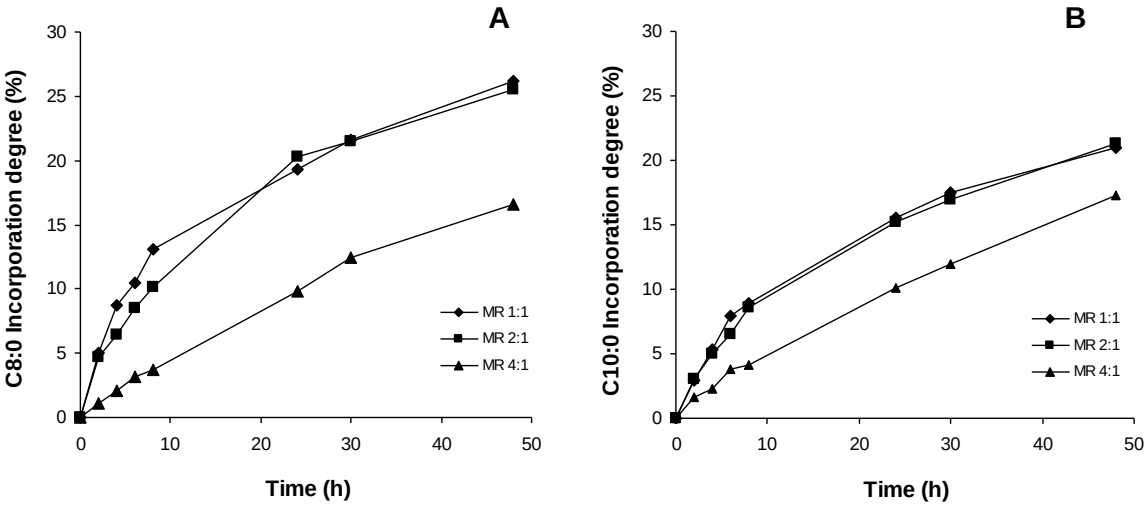

Figure 3. Cinétiques de la réaction de acidolysis entre l'huile d'olive et les acides caprylic (A) ou capric (B), pour différents MR, à 40°C. Molar ratio FFA/TAG, 1:1 (◆), 2:1 (■) and 4:1 (▲).

4. Conclusions

La productions de SL a partir d'huile d'olive et acides gras á chaine moyenne a été réussie avec YLL2 immobilisé. Les meilleures conditions de réaction pour la production SL avec les deux acides gras sont été: ratio molaire de 2:1 FFA/TAG, température de réaction de 40°C et temps de réaction de 48h. Dans ces conditions, le SL produit avait 25.6%mol de C8:0 et 21.3%mol de C10:0, qui a confirmé la validité du modèle.