UNIVERSITAT DE BARCELONA

Novel Lipases: Expression and Improvement for Applied Biocatalysis

Nuevas lipasas: expresión y mejoras para biocatálisis aplicada

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NUEVAS LIPASAS: EXPRESIÓN Y MEJORAS PARA BIOCATÁLISIS APLICADA

Memoria presentada por **Belén Infanzón Ramos** para optar al grado de Doctora por la Universitat de Barcelona

Programa de Doctorado:

Microbiologia Ambiental i Biotecnologia (2011 – 2017)

Tesis Doctoral llevada a cabo bajo la dirección de

Dra. **Pilar Diaz Lucea**

en el Departamento de Génetica, Microbiología y Estadística de la Universitat de Barcelona

VºBº de la Directora de Tesis

Dra. Pilar Diaz Lucea

Memoria presentada por

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Barcelona, Junio de 2017

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NOVEL LIPASES: EXPRESSION AND IMPROVEMENT FOR APPLIED BIOCATALYSIS

Thesis presented by **Belén Infanzón Ramos** to qualify for the
PhD Degree by the University of Barcelona

PhD Program: *Environmental microbiology and biotechnology*
(2011 – 2017)

PhD thesis performed under the supervision of
Dr. **Pilar Diaz Lucea** at the
Department of Genetics, Microbiology and Statistics, University of Barcelona

Dra. Pilar Díaz Lucea

Belén Infanzón Ramos

Barcelona, June 2017

"No es verdad que la gente pare de perseguir sus sueños porque sean mayores,

se hacen mayores porque dejan de perseguir sus sueños"

Gabriel Garcia Marquez

"Cuando quieres una cosa, todo el Universo conspira para ayudarte a conseguirla"

Paulo Coelho

Agradecimientos

Así como la tesis representa el gran trabajo realizado, los agradecimientos reflejan todas las personas que formaron parte de mi vida en todos estos años, porque es eso lo más preciado que me llevo, las grandes personalidades que conocí o que me acompañaron en alguna parte de este proceso. 7 años de vivencias únicas, qué mucho realmente viví en todo este tiempo, infinitas aventuras, memorables momentos, fuertes sentimientos. Y comienzo con un GRACIAS gigante, porque ustedes lo hicieron posible, ustedes son parte de esto. Esta tesis va dedicada a todos ustedes.

Seguí y conseguí lo que siempre quise, la oportunidad de vivir un intercambio cultural y de aprender a ser científica. Y hoy puedo decir que conseguí más cultura de la que jamás imaginé. Y si aprendí a ser científica? eso mejor lo dejo a consideración del lector... Fue así que llegué a la hermosa Barcelona. Gracias a mi primer grupo donde hice el máster, labo 7, un muy buen primer año. Maite, Markus, Nathalia, Yvannia, Rakel, Flor y los chiquillos que vinieron después. Almuerzos muy entretenidos, charlas ilimitadas y por supuesto, las tantas salidas sin fronteras (hasta Cornellà). También a todo el Departamento de Micro (Fase I y Fase II), compartimos equipos, autoclaves, almuerzos, cenas, algunos bailes y varias cerves. Gracias a la Secretaría del Departamento, a la Susana y a Manolo.

Gracias a los profes del Lab2, gracias por tantas enseñanzas, son excelentes maestros y excelentes personas. Gracias Javier por compartir los antojos de dulces y algún comentario o alguna enseñanza no enzimática en la siesta (pero parece que me hago PhD sin haber probado tu paella...). Jose, me llevo los mejores recuerdos de los almuerzos en tu casa de Collbató. Y por supuesto, muchas gracias a mi tutora, gracias Pilar por tantísima comprensión, por aceptar mis decisiones, por apoyarme cuando tiré la toalla y dejé el doctorado, y más aún por recibirme nuevamente a la distancia y por permitirme terminar los últimos años de esta forma. Definitivamente vos lo hiciste posible. Y como no olvidar los tantos viernes por las tarde quemando neuronas en tu despacho, donde las horas pasaban como si fueran minutos.

Las históricas del Lab2, Susana, Amanda y Mónica, con las que más compartí todos estos años, muchas enseñanzas, varios tips para los ensayos, intercambios de ideas y comentarios científicos. Pero más aún compartimos fuera del lab. Puedo decir que son las mejores amigas que me deja Barcelona, y que espero seguir visitándoles en los siguientes años. También Silvia, con quien no solo compartimos lipasas y papers, también compartimos las salidas con las chicas y hasta un viaje a Canarias con sus amigas. Los más históricos del Lab2, con quienes ya no coincidimos en el lab pero sí en varias cenas, en especial Arnau y Pere. También me quedan muchos buenos recuerdos de Lily, de los que estuvieron de paso (Mai, Itacio, Diego, Ma. Ángela, Fikret y sus geles) y de todos los chiquillos que fueron pasando todos estos años por el lab, y siempre con la mejor onda a pesar de que de pronto un día les aparecía una intrusa de Paraguay que no conocían y que vaciaba todas las cajas de puntas, se adueñaba de todos los equipos y en estos últimos meses de la computadora.

Sí, efectivamente existe ese lugar en donde uno puede trabajar tranquilo, aprender, intercambiar ideas, compartir equipos y reactivos (y galletas y chocolates), compartir risas, recibir explicaciones y ayuda, sin roces, sin enojos, sin discusiones o peleas. Yo viví ese ambiente todos estos años en ese lab. Un mundito ideal el Lab2 (cuando no aparece el fantasma nocturno que hace que no haya expresión de nuestras enzimas).

Un gran agradecimiento al Dr. Medhi y toda la gente de la RWTH Aachen con quienes también estoy aprendiendo un montón en esta estancia en Alemania. Nuevamente conociendo excelentes personas. También gracias Paco Valero y gracias a los chicos de la autónoma por los ensayos que hice con ustedes, en tan cortito tiempo me hicieron sentir parte del lab.

Gracias Su por tanto, por preocuparte donde voy a vivir cada vez que vengo, por tantas ayudas durante todos estos años, vos y Gabriel son mi familia en Barcelona. Tengo un hogar en el ático de Lepant.

Compartir piso es algo que aprendí en esta ciudad, y que dejaron distintas experiencias y que quizás condicionaron ciertas cosas. Pero sobre todo compartí con personas con quienes aprendí y con quienes disfruté. Sí hoy conozco cada hueco de Barcelona, una buena parte te lo debo a vos Diego, gracias por mostrarme tu versión de la ciudad.

La paraguayada en el viejo continente. Gracias mis amis paraguayas en Barcelona, por ser como mis hermanas (So y Pachi) y hermanitas, Pame y Sil, en estos últimos años, por prenderse siempre a cada gran idea o a esas súper actividades que casi nadie quiere acompañarme (Pame como cuando subimos a Montserrat y corrimos por todo Coballtó para no perder el último bus de vuelta, y Sil la única que logré que me acompañe en una de mis idas en bici a Collserola y a volver en bus desde Le Havre para visitarle un finde a los franceses paraguayizados) (me estaba olvidando, la Su también subió a los bunker en bici!). Gracias Gabriel por cada uno de nuestros encuentros, por las charlas, y por la gran ayuda que me diste hace unos años. Gracias Car, estuviste desde el inicio, compañera de viajes, cada uno de nuestros encuentros en Córdoba, en Barcelona o en Asunción, fueron más que necesarios. También gracias Cynthia por las vistas y viajes. Y gracias Mat por las constantes visitas, fue muy bueno tener al menos un familiar en este continente (un RR), de haberme ensañado a tragar pastillas tirándome de los pelos cuando éramos chicos, terminamos compartiendo vinos en Milano y playita en Barcelona.

De Paraguay estoy muy agradecida con Pablo y su grupo de Biotec; gracias Pablo por permitirme soñar nuevamente con que terminar la tesis podía ser posible y a los chicos del Lab porque en los meses que estuve ahí me hicieron sentir como en el Lab2 (extiéndase el cumplido al grupo de fito). Gracias al IICS por los permisos para poder terminar esta tesis, y en especial gracias Ale por la grandísima ayuda cada vez que vengo y no estoy en Paraguay, hacés que todo sea más simple.

Gracias las chicas y las lujuriosas! Mis amigas de siempre, de toda la vida. Disfruté de cada una de sus visitas, fue lo más de lo más llevarles a mi lugares preferidos de Barcelona. Y son una parte muy importante de mí sustento en Paraguay (y por mensajes en España, en Alemania o donde esté), son la desconexión del estrés del trabajo, la desconexión de la Ciencia, en quienes puedo confiar plenamente, son lo máximo. Gracias Nati Ba por poder contar contigo (la única que me acompaña en salidas deportivas), hasta te ocupaste de todo y me llegaste a hacer una de las matrículas del doctorado cuando tuve un inconveniente y no pude estando en Argentina. Gracias ALi y Lauri por escribirme constantemente cuando estoy en Barcelona y por todas las cenitas en Asunción. A todas, les quiero y espero que sigan siendo parte de mi vida por siempre.

Y por supuesto, infinitas gracias a mi familia. Los que hacen que todo sea posible. Gracias pá, má y mi sis, mis tres personas favoritas en este mundo. Gracias por apoyarme siempre en cada una de mis locuras, en cada una de mis decisiones, por no cortarme las alas, por nunca limitarme a nada. Gracias má por aprender a poder vivir tranquila a pesar de la preocupación que te debe generar cuando estoy muy lejos (dentro del algún bosque o en cualquier parte del globo) y sabes poco o casi nada de mí. Pero sobre todo les agradezco por enseñarme ese amor incondicional. Uno de mis mayores logros de estar acá fue que ustedes puedan conocer estas hermosas ciudades, el haber compartido esto con ustedes (logré que Julieto por primera vez en su vida se tomé vacaciones extra y que se convierta en viajero!)

Después de todo lo vivido durante el doctorado, puedo decir que soy otra persona, traté y sigo tratando de incorporar la mejor partecita de cada uno de ustedes. Porque la vida me puso y me sigue poniendo personas increíbles en el camino. Sólo hay que animarse. Y por eso hoy me siento orgullosa de mí misma, por haberme animado a empezar esta aventura hace 7 años.

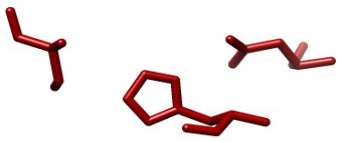
Termino con esta frase de Ruiz Zafón que resume muchos sentimientos de mi experiencia en Barcelona:

“Esta ciudad es bruja, sabe Usted, Daniel? Se le mete a uno en la piel y le roba el alma sin que uno se dé ni cuenta.

NOVEL LIPASES: EXPRESSION AND IMPROVEMENT FOR APPLIED BIOCATALYSIS

I N D E X

Introduction



1. Lipases

Lipases (acylglycerol hydrolases, EC 3.1.1.-) are enzymes that catalyze the hydrolysis, trans- and inter-esterifications of ester bonds. Esterases and lipases differ in the characteristics of their substrate-binding pockets, like size, shape and deepness of the hydrophobic interaction area, as well as in their physico-chemical properties. Thus, lipases accept esters of short (C4) to long chain (C16) fatty acids, while esterases accept only short acetyl moieties (Pleiss et al., 1998), being selective with respect to the length and the level of saturation of the fatty acid chains. The reaction mechanism of lipases is assumed to be analogous to that of serine proteases, with the catalytic triad consisted by the amino acid residues Asp, His, and Ser, the latter being responsible for the nucleophilic attack (Arpigny & Jaeger 1999).

Lipases have received increasing attention mainly due to their capability of catalyzing a wide variety of reactions, which allows a widespread range of applications in industry (Rosenau & Jaeger, 2000). The commercial use of lipases in biotechnology was a business of more than 1.5 bilion U.S. dollars in year 2000 (Houde et al., 2004). In a recent publication the existence of 671 documents of patents directed to kinetic resolution, 456 to detergent production, 165 to biodiesel production, and 544 to food and feed production with lipases are reported. And regarding scientific publications, 1352, 117, 439, and 315 documents respectively for each application of lipases have been found (Daiha et al., 2015).

1.1 Lipase Structure

Lipases belong to the "α/β-hydrolase fold" family; therefore they display the characteristic α/β-fold, containing a definite order of α-helices and β-sheets (Bornscheuer et al., 2002a). The canonical α/β-hydrolase fold consists of a central, mostly parallel β-sheet of eight strands with the second strand antiparallel **(Fig. I1)**. The parallel strands β3 to β8 are connected by helices, which pack on either side of the central β-sheet (Jaeger et al., 1999). This canonical fold is also found in haloperoxidases and epoxide hydrolases (Bornscheuer et al., 2002a). The curvature of the β-sheet may differ significantly among the various lipases reported, as well as the spatial position of the α-helices. Moreover, the binding sub-domains of the α/β-hydrolase fold proteins differ significantly in length and architecture, in agreement with the large substrate diversity of these enzymes (Jaeger et al., 1999).

As mentioned above, the active site of lipases is constituted by a catalytic triad consisting of a nucleophilic serine, a catalytic acid (aspartate or glutamate) and a histidine residue, always in this order in the amino acid sequence. The nucleophilic serine residue is located at the C-terminal end of strand β5, in a highly conserved pentapeptide Gly-X-Ser-X-Gly (where X can be any amino acid) (Arpigny & Jaeger 1999).

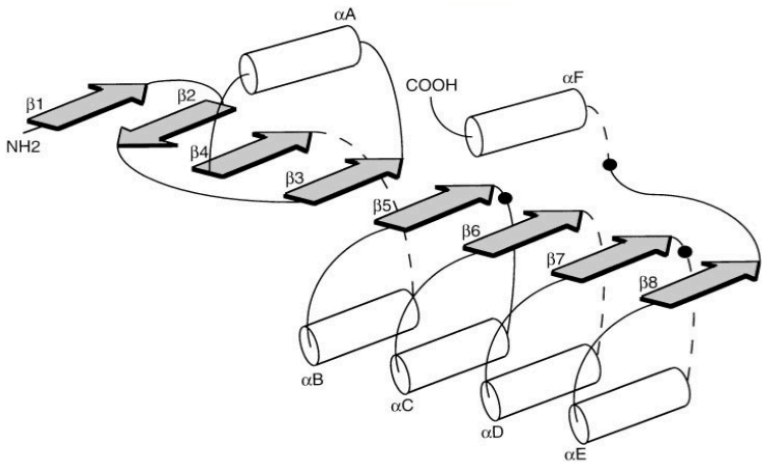


Figure I1. Structure of α/β-hydrolases. α-Helices are indicated by cylinders, and β-strands are indicated by shaded arrows. The topological position of the active-site residues is shown by a solid circle; the nucleophile is the residue after β-strand 5, the Asp/Glu residue is after β-strand 7, and the histidine residue is in the loop between β8 and αF (Jaeger et al., 1999).

The active site of lipases contains also other structures that facilitate the catalytic process such as the "scissile fatty acid binding pocket", which is a variable cavity, located inside the substrate-binding pocket, responsible for the accommodation of the acyl chain of the ester linkage that will be hydrolyzed by the enzyme. Esterases have a small acyl-binding pocket, which optimally fits to the acyl moiety of their short substrates. Lipases or "true lipases" instead have a long, hydrophobic scissile fatty acid-binding site located inside the binding pocket (in a crevice, at the wall of a binding funnel or in a tunnel) (Pleiss et al., 1999). Length and hydrophobicity of this binding site should correlate with fatty acid chain length profile of the respective lipase. Additional binding pockets for the other acyl chains of substrates such as triacylglycerols (TAGs) are also frequent, which contribute to attachment of the substrate to the active site of the enzyme during catalysis (Pleiss et al., 1998).

1.1.1 The lid

Most lipases have a remarkable amphiphilic structure covering the active site of the enzyme involving a single α-helix, two helices or a loop region, designated as "lid", which is an important entity for exposing a hydrophobic patch in the presence of a substrate. Thus, the lid-like structure constitutes a novel structural approach to distinguish lipases or "true lipases", from esterases (Fojan et al., 2000). This structure exhibits a variable position depending on the physicochemical environment of the enzyme (Grochulski et al., 1994; Schrag et al., 1997), that is, the lid is covering the active site of the enzyme while an interface is absent but when the enzyme contacts with a lipid-water interface, a structural rearrangement occurs and the lid is displaced, allowing free accession of the substrate to the active site of the enzyme. Consequently, the chain length specificity of a defined lipase might be predicted from the structure of the lipase, as far as the chain length of the substrate under investigation is shorter than the scissile fatty acid binding site inside the binding pocket (Pleiss et al., 1998).

Moreover, the structural rearrangements of the lipase active site region involving the lid leads to the phenomenon of "interfacial activation", a curious characteristic shown by lipases when their kinetic parameters are studied. Interfacial activation consists on a drastic increase of activity when lipases act at the lipid-water interface of micellar or emulsified substrates (Chapus et al., 1976). When the enzyme contacts the hydrophobic components of the lipid-water interface, the lid suffers a conformational change, leaving the catalytic residues more accessible to the substrate, which explains the resulting increased activity or interfacial activation of the enzyme. At the same time, a large hydrophobic surface is exposed, which is thought to facilitate the attachment of lipases to the interface. Lipases that do not have lid do not show interfacial activation (Jaeger et al., 1999), and a general observation that lipase activity is increased in the presence of a lipid interface was also demonstrated (Peters et al., 1997).

Comparison of open and closed conformations of *Candida rugosa* lipase indicates that activation of the lipase requires the movement and refolding, including a *cis* to *trans* isomerization of a proline residue, of a single surface loop to expose a large hydrophobic surface which likely interacts with the lipid interface (Grochulski et al., 1994). Hence, the lid in *Candida rugosa* lipase has been localized after the open and closed forms of the lipase have been crystallized.

1.1.2 The Oxyanion hole

After binding of the ester substrate, a first tetrahedral intermediate is formed by nucleophilic attack of the catalytic serine; the complete stabilization of this anionic carbonyl oxygen atom of the tetrahedral intermediate occurring during lipase catalysis is achieved by the collaboration of the "oxyanion hole", by using two hydrogen bonds, which are provided by two amide groups of the protein backbone (Henke et al. 2002). The ester bond is cleaved and the alcohol moiety leaves the enzyme. In a last step, the acyl enzyme is hydrolyzed **(Fig. I2)**. Lipases require water to hydrolyze the acyl ester bond. The nucleophilic attack by the catalytic serine is mediated by the catalytic histidine and aspartic (or glutamic) acid (Pleiss et al., 1998). The mechanism for ester hydrolysis or formation is essentially the same for lipases and esterases (Bornscheuer et al., 2002a; Jaeger et al., 1999).

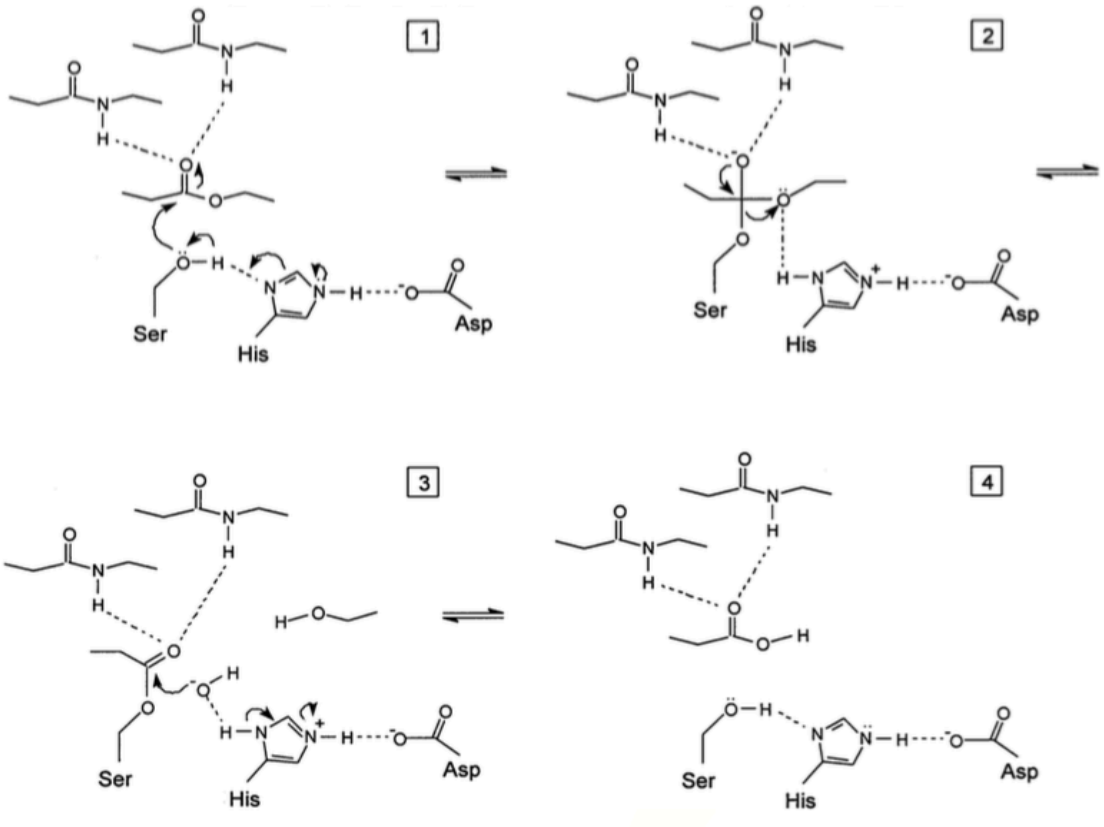


Figure I2. Reaction mechanism of lipases (Jaeger et al., 1999)**.** The reaction proceeds via formation of a tetrahedral intermediate (2), and a deacylation resulting from a nucleophilic attack on the acyl-enzyme by a water molecule (3).

Several studies were performed in order to predict the oxyanion hole backbone atoms and the distances of the residues forming the hydrogen bonds of different crystalized enzymes. However the resolution of the X-ray crystal structures are not always sufficient for describing the exact stereochemistry of the oxyanion hole, being necessary to perform site-directed mutagenesis studies for this purpose (Derewenda and Sharp, 1993).

Candida antartica Lipase A (CalA) was crystalized and the oxyanion hole composition was determined based on structure analysis. Tyr93 was identified as the oxyanion hole forming amino acid. Therefore, CalA was classified as a Y-class lipase, being the only lipase already crystalized of the *Candida antartica* Lipase A-like superfamily (Widmann and Pleiss, 2010). Also Asp95 has been proposed as a component of the oxyanion hole (Ericsson et al., 2008). Saturation mutagenesis of the corresponding CalA _D95 did not allow production of any functional variant and confirmed the crucial role of this residue for the catalytic activity of CalA (Sandström et al., 2009).

1.2 Lipase Classification

As mentioned, lipases belong to the class of α/β-hydrolases, which also contains esterases, acetylcholinesterases, cutinases, carboxylesterases and epoxide hydrolases, sharing a conserved active site consisting of the catalytic triad of S-D(E)-H, the GXSXG (seldom AXSXG) and GXDXG oxyanion hole motifs (Fischer and Pleiss, 2003; Pleiss et al., 2000).

Comparison of the amino acid sequences of lipases can provide a clear picture about the evolutionary relationship between enzymes of different origin, as well as the prediction of important structural features such as the catalytic site residues, type of secretion mechanism and requirements for a lipase-specific foldase, and the potential relationship to other enzyme families (Bornscheuer et al., 2002b).

Bacterial lipolytic enzymes were initially classified into eight families (Arpigny and Jaeger, 1999). This classification is based on amino acid sequence similarity and the presence of conserved motifs, like the GDSL-pattern of family II lipases. Other classification reporting three classes of lipases, which is not only for bacterial lipases, was constructed according to the amino acids involved in forming the oxyanion hole (Pleiss et al., 2000). Both classifications are described in the following sections.

1.2.1 Bacterial lipase classification on the basis of conserved sequence motifs and biological properties

Sequences of 53 bacterial lipases and esterases were compared and classified by grouping them into eight families on the basis of conserved sequence motifs and the biological properties of these enzymes (Arpigny and Jaeger, 1999).

Family I is the largest group, including most "true lipases", thus interfacial activation and presence of a lid is characteristic of this family, and is subdivided into 7 subfamilies (Jaeger and Eggert, 2002). Lipases of this family share the Gly-X-Ser-X-Gly consensus sequence. Subfamilies I.1 and I.2 include lipases encoded in an operon together with their cognate foldase (Lif), being secreted via the type II secretion pathway (Jaeger et al., 1994). Subfamily I.3 includes lipases that do not require intramolecular chaperones and are secreted via the type I secretion system. Subfamily I.4 groups the *Bacillus* lipases with the smallest molecular mass (≈20kDa). Subfamilies I.5 and I.6 contain *Geobacillus* and *Staphyloccus* lipases, respectively, and finally subfamily I.7 groups lipases from *Propionibacterium acnes* and *Streptomyces cinnamoneus* (Arpigny and Jaeger, 1999).

The other families described by Arpigny and Jaeger have less members than family I but display remarkable characteristics.

Family II, also called the GDSL family, groups enzymes that do not exhibit the conventional pentapeptide (Gly-X- Ser-X-Gly), but display a common motif made up of Gly-Asp-Ser-Leu that contains the active-site serine residue in a location close to the N-terminal end of the protein. GDSL enzymes are known to have flexible active sites that are able to modify its structure in the presence of specific substrates, thus increasing its substrate specificity range (Akoh et al., 2004). An interesting member of family II is the *Streptomyces scabies* lipase, which has a catalytic Ser-His dyad instead of the common Ser-Asp-His triad.

Family III includes lipases from *Streptomyces* and *Moraxella* spp., showing 20% amino acid sequence identity with intracellular and plasma isoforms of the human platelet-activating factor-acetylhydrolase (PAF-AH). Enzymes possess the conserved consensus sequence GXSXG.

Lipases that display amino acid sequence similarity to the mammalian hormone-sensitive lipase (HSL) are assigned to family IV. This family has 2 highly conserved consensus

motifs, the common GXSXG and His-Gly-Gly (HGG), which play a relevant role in the oxyanion hole formation (Ramnath et al., 2017).

Members of family V share significant homology to non-lipolytic enzymes, such as epoxide hydrolases, dehalogenases and haloperoxidases, which also possess the typical α/β-hydrolase fold and a catalytic triad including the GXSXG pentapeptide.

Quite small (23-26 kDa) enzymes are found in family VI, with some members displaying 40% sequence similarity to eukaryotic lysophospholipases. Some characterized enzymes have demonstrated to hydrolyze small ester-containing compounds with a broad specificity while they display no activity towards long-chain triglycerides (Hong et al., 1991).

In contrast, esterases from family VII are rather large (aprox. 55kDa), and share significant similarity to eukaryotic acethylcholine esterases and gut or liver carboxyl esterases.

Finally, family VIII includes enzymes which show high homology to a class of β-lactamases (Arpigny and Jaeger, 1999). This family consists of three characterized members (Ramnath et al., 2017).

Several new families of bacterial and fungal lipases/esterases have been proposed by different authors on the basis of phylogenetic criteria and other conserved sequence motifs. The original Arpigny & Jaeger classification included only eight families. Hence, this classification has been revised several times and currently there are fifteen families that are a part of ESTHER database (Lenfant et al., 2013). One of the new families of bacterial lipases, family X, has been reported to display an unusual oxyanion hole, never found before in bacterial lipases (Bassegoda et al. 2012a). Furthermore, new bacterial lipase families XVI and XVII were proposed more recently (Castilla et al., 2017; Li et al., 2016).

1.2.2 Lipase classification according to sequence and structure of the oxyanion hole

Depending on the amino acids involved in the oxyanion hole, lipases can be classified into three classes, the GGGX-, GX-, and Y-class, and further divided into superfamilies on the basis of their conserved pentapeptide **(Fig. I3)** (Pleiss et al., 2000).

All these superfamilies are annotated in the Lipase Engineering Database (LED), a resource of fully and consistently annotated superfamilies and homologous families of α/β-hydrolases including multisequence alignments of all families (Fischer and Pleiss, 2003). LED is a useful tool to identify functionally relevant motifs apart from the active site

residues, and to design mutants with desired substrate specificity. Release 3.0 of the LED contains 18585 proteins with 24783 sequences and 656 structure entries, of which about 14000 protein and 489 structure entries are new. The new superfamily ("*Candida antarctica* lipase A like" superfamily) was added to the LED database in a recent update process (Widmann and Pleiss, 2010).

The GX class comprises mainly bacterial and fungal lipases, eukaryotic lipases (hepatic, lipoprotein, pancreatic, gastric, and lysosomal acid lipases), cutinases, phospholipases and non-heme peroxidases (Fischer and Pleiss, 2003). None of the GX class enzymes was active towards esters of tertiary alcohol substrates (Fischer and Pleiss, 2003). The residue X is part of a functionally relevant network which stabilizes the local geometry of the oxyanion hole, although it does not indeed contact the substrate (Pleiss et al., 2000). "Cytosolic Hydrolases" and "Microsomal Hydrolases" are the largest GX class superfamilies (Widmann and Pleiss, 2010). *Candida rugosa* lipase like is another important family of lipases belonging to this class.

The GGGX class comprises bacterial esterases, α-esterases, eukaryotic carboxylesterases, bile-salt activated lipases, juvenile hormone esterases, hormone sensitive lipases, acetylcholinesterases, and thioesterases, as well as gliotactin, glutactin, neurotactin, neuroligin, and thyroglobulin. . In this case the residue X is mostly a hydrophobic amino acid that might interact with the substrate (Pleiss et al., 2000). Numerous esterases and lipases of the GGGX class were screened for activity towards esters of tertiary alcohols, and most of them showed activity and even enantioselectivity. "Carboxylesterases" and "*Moraxella* lipase 2 like" superfamilies are the largest in the GGGX class (Widmann and Pleiss, 2010), which includes four superfamilies of lipases and two esterase families on the basis of conserved pentapeptide sequence. Among these lipase superfamilies are the *Y. lipolytica* lipase like, *C. rugosa* lipase like, *Moraxella* lipase 2 like and hormone sensitive lipases, bearing the conserved pentapeptides GHSLG, GESAG, GDSAG and GASAG, respectively (Gupta et al., 2015).

The Y class of enzymes consists of five superfamilies with only one superfamily of lipases, the *C. antarctica* lipase A-like superfamily with a GASHG pentapeptide. The "*Candida antarctica* lipase A like" superfamily contains only one crystal structure (that of CalA; pdbs *2VEO* and *3GUU*), and 39 sequences, assigned to 32 proteins; all of them are from organisms belonging to the subkingdom Dikarya of the kingdom Fungi (Widmann and

Pleiss, 2010). Based on the structure of the oxyanion hole, CalA can be classified as a Y-class lipase, with Tyr93 identified as the oxyanion hole forming amino acid.

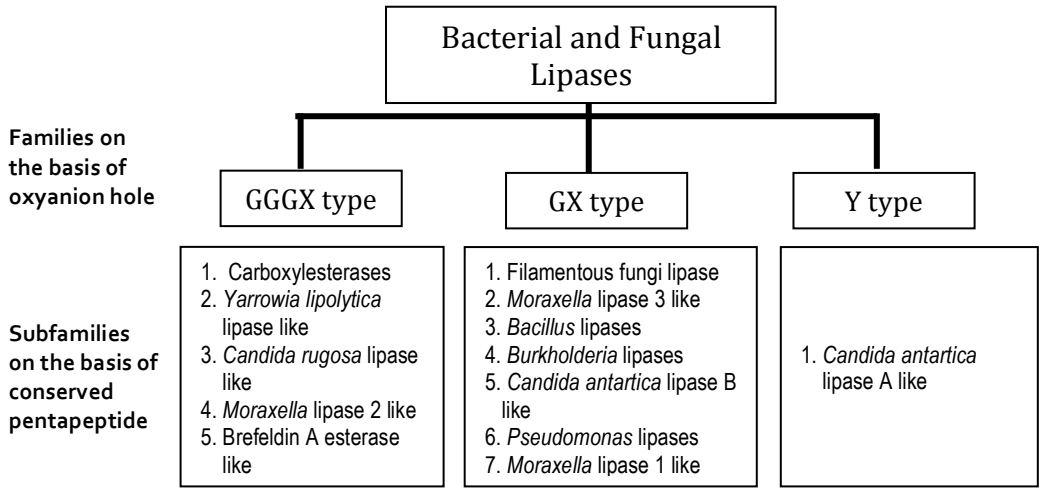


Figure I3. Classification of bacterial and fungal lipases based on lipase engineering database LED (Gupta et al., 2015).

1.3 Lipase Discovery

Lipases are ubiquitous in nature and are produced by plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry (Gupta et al., 2004). The great versatility of fungal lipases derive from genera such as *Candida*, *Geotrichum*, *Rhizopus* and *Thermomyces*, while among bacterial lipases the attention has usually pointed on particular classes of enzymes such as the lipases from the genus *Pseudomonas* and *Bacillus* (Arpigny and Jaeger, 1999). The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier and they do not require cofactors. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains for lipase production (Gupta et al., 2004).

Although there is an increased knowledge of the superfamily of α/β-hydrolase fold enzymes, novel lipases are required today to convert biomass into second and third-generation biofuels, new biomaterials or high added value chemicals (Bornscheuer

et al., 2012). Furthermore, to produce cost effective green technology-based processes, special emphasis should be given to the development of reusable and stable enzymes under harsh conditions (Gupta et al., 2015).

Lipolytic microorganisms are found in a variety of habitats including soils contaminated with oils, wastes of vegetable oils, dairy waste and deteriorated food (Gupta et al., 2015). Lipases have been isolated and purified from fungi, yeasts, bacteria, plant and animal sources (Snellmanet et. al., 2002) for application in more sustainable productive processes.

1.3.1 Screening for new biocatalysts

Microbial diversity is a major resource for biotechnological isolation of added value products and green process implementation. The biosphere is dominated by microorganisms, yet most microbes in nature have not been studied (Gupta et al., 2004). Moreover, the vast majority of microbes present in a single environmental niche are not cultivable in the laboratory and it is estimated that, on average, less than 1% have ever been identified (Lorenz et al., 2002).

The creation of "metagenome-libraries" is a promising strategy regarding the screening for new suitable biocatalysts. The possibility of searching in the entire DNA present in a defined habitat (metagenome) for desired activities, allows access to a pool of new enzymes which couldn't be found using typical culture-based methods. The main limitation of "metagenome-library" to actually screen all the metagenome are the heterologous expression systems used in the construction: it could happen that many genes are not correctly expressed by the heterologous host and will thus not be detected (Lorenz and Eck, 2005).

The enormous progress in sequencing technology in combination with metagenome libraries has led to an exponential increase in the number of sequence data (currently approx. 20 million sequences) in the databases. However, annotation of the putative protein function is performed automatically and consequently can lead to mistaken interpretations (Davids et al., 2013). Accordingly, an additional source for novel enzymes is the Brookhaven protein structure database (PDB), which contains numerous proteins, where the 3D-structure has been deposited, but where the proteins have not necessarily been biochemically characterized (Davids et al.,

2013).

Another strategy that has received considerable attention relies on activity-based searches. For example, a new research with solvent-tolerant microbes was performed allowing isolation of a microorganism that can survive in the presence of a range of organic solvents and logically, their enzymes are stable and efficient catalysts for functioning in such solvent media (Gupta and Khare, 2009).

1.4 Novel Bacterial Lipases in this work

1.4.1 *Paenibacillus barcinonensis* new esterase Est23

Paenibacillus barcinonensis is a soil bacterium bearing a complex set of enzymes for degradation of plant polysaccharides and lipids. This microorganism was isolated and identified from a rice field soil at the delta of river Ebro (Spain) (Blanco and Pastor, 1993; Sánchez et al., 2005). *P. barcinonensis* is a Gram-positive, endospore-forming bacterium, rod-shaped and facultative anaerobic.

P. barcinonensis has shown to be a very good source of hydrolytic enzymes. Up to date four cellulases (Blanco et al., 1998; Pastor et al., 2001; Sanchez et al., 2003), six xylanases, (Gallardo et al., 2003, 2010; Sainz-Polo et al., 2014; Valenzuela et al., 2010, 2014, 2016), and two pectinases (Soriano et al., 2000) have already been isolated, cloned and biochemically characterized. Moreover, some of them display interesting biotechnological applications (Bassegoda et al., 2010; Cadena et al., 2010; Fillat et al., 2015; Valenzuela et al., 2014).

Presence of hydrolysis haloes when growing on tributyrin-supplemented plates indicated also the presence of lipolytic activity. Zymogram analysis revealed the presence of at least two enzymes displaying activity on muf-butyrate. A cell-bound type B carboxylesterase was previously isolated, cloned and characterized (Prim et al., 2000), showing activity on short chain length fatty acid substrates, which was further improved for resolution of tertiary alcohols (Bassegoda et al., 2010). The second enzyme showing lipolytic activity was isolated and characterized in the present work.

1.4.2 *Rhodococcus* sp. strain CR-53 lipase LipR

Rhodococci are highly adaptable bacteria with respect to environment and substrate, displaying the ability to degrade a large number of organic compounds, including some of the most difficult compounds with regard to recalcitrance and toxicity. Rhodococci are

described as high G+C content, aerobic, Gram-positive, non-motile, mycolate-containing, nocardioform actinomycetes (Bell et al., 1998).

The wide range of chemicals transformed or degraded by *Rhodococcus* species makes them actually or potentially useful in environmental and industrial biotechnology, as does their ability to synthesize several products such as surfactants, flocculants, amides and polymers (Bell et al., 1998).

In the last years, the significance of the genus *Rhodococcus* in bioremediation, synthesis of useful products and virulence has motivated the development of different genome projects that have contributed to increase the available genomic information about this genus (McLeod et al., 2006).

Rhodococcus sp. strain CR-53 was previously isolated in our laboratory from a subtropical soil sample (Ruiz et al., 2005). Physiological tests plus analysis of the 16S rRNA gene of the strain revealed a high level of similarity (99%) to other strains described as *Rhodococcus erythropolis*, although a perfect match was not obtained (Falcocchio 2005). A remarkable trait of the strain was its high lipolytic activity, thus being a good candidate for lipase prospection.

LipR was isolated from *Rhodococcus* sp. strain CR-53 and characterized in a previous work (Bassegoda et al., 2012a). Different enzymes from rhodococci with biotechnological applications have been cloned, but no information concerning the lipases of the genus *Rhodococcus* was available before the report of LipR (Bassegoda et al. 2012a). LipR was proposed as the first member of the new family X of the Aspigny-Jaeger classification. Moreover, LipR displays the same oxyanion hole as that of CalA, classified as a Y-type oxyanion hole, and has sequence identity with some secretory lipases from *C. albicans*, also classified as members of the *C. albicans* lipase-like family. Interestingly, none of the lipases used to define the different bacterial lipase families has a Y-type oxyanion hole, therefore this fact again provides evidence of the differences existing between LipR and all members of the bacterial lipase families described to date.

Analysis of the amino acid sequence of LipR revealed a high content of nonpolar amino acids (59.3%) and the presence of a signal peptide, indicating an extracellular location. A LipR 3D homology model constructed using *Candida antarctica* lipase A (CalA) as a template allowed to detect the presence of a disulfide bond between cysteines Cys388 and Cys432.

LipR was described as a mesophilic enzyme showing preference for medium-chain-length acyl groups without showing interfacial activation, and displayed good long term stability (Bassegoda et al. 2012a).

The expression vector pGaston (Henke et al., 2003) with a rhamnose-inducible promoter was chosen for expression of LipR and expressed in *E. coli* strain Origami™ (Bassegoda et al. 2012a). A previous study showed the efficient expression of CalA using strain Origami™ as a host (Pfeffer et al., 2006). LipR only demonstrated activity when cloned into pGaston with its own signal peptide, despite the fact that absence of a signal peptide can help to avoid expression problems, since *E. coli* does not secrete overexpressed proteins. The Origami™ host strains are *E. coli* K-12 derivatives that have mutations in both, the thioredoxin reductase (*trx*B) and glutathione reductase (*gor*) genes, which greatly enhance disulfide bond formation in the cytoplasm and consequently increase the protein folding efficiency (Bessette et al., 1999), as happened for LipR expression.

1.4.3 *Pseudomonas* sp. 42A2 lipases LipA and LipC

Pseudomonas sp. 42A2 (NCIMB 40045) was isolated from an oil contaminated water sample as a highly polyhydroxyalkanoate producer and on the basis of its ability to act on lipid substrates. *Pseudomonas* sp. 42A2 was also selected due to its capacity to accumulate a variety of compounds when grown on mineral medium with oleic acid as the sole carbon source (De Andrés et al., 1994).

The strain was characterized and further identified as a member of the genus *Pseudomonas*, being closely related to *Pseudomonas aeruginosa* species, according to the 16S rDNA sequencing results. However, the reference strains, reported in several environments, were not able to accumulate poly-(3-hydroxyalkanoic acid; PHA) (Fernández et al., 2005) as observed for *Pseudomonas* sp. 42A2. This fact shows again the significant differences between bacterial strains, even though they show 100 % similarity in their 16S rRNA gene sequence. In general, bacterial isolates from different environments show divergences because they customize their genome to fit the needs for survival in a specific ecological niche (Mathee et al., 2008).

In general, active expression of lipases from *Pseudomonas* and *Burkholderia* requires the presence of a chaperone, protein known as the lipase-specific foldase (Lif), for precise folding of the lipase (Ramnath et al., 2017). A significant increase in extracellular activity of

the engineered strain was achieved when each lipase-coding gene was over-expressed, thus allowing purification and characterization of the two corresponding lipases for their evaluation and characterization. The newly isolated lipases were designated LipA and LipC in agreement with their corresponding counterparts in *P. aeruginosa* PAO1 (Bofill et al., 2010).

The isolated *lipA* and *lipC* genes were cloned with their specific foldase (LipH) in pBBR1MCS vector (Kovach et al., 1994) and transformed into the homologous host *Pseudomonas* PABST7.1 for over-expression and characterization (Bofill et al., 2010). Both lipases showed preference for medium fatty acid chain-length substrates. However, significant differences could be detected between LipA and LipC in terms of enzyme kinetics and behaviour pattern. Accordingly, LipA showed maximum activity at moderate temperatures, and displayed a typical Michaelis-Menten kinetics. On the contrary, LipC was more active at low temperatures and displayed partial interfacial activation, showing a shift in substrate specificity when assayed at different temperatures. Further protein engineering assays performed on LipC allowed isolation of LipCmut, a thermostable LipC variant maintaining its psychrophilic properties (Cesarini et al., 2012).

1.5 Lipase Expression

Lipases from bacteria and fungi are the most commonly used biocatalysts for inter- or transesterification reactions, and optimal parameters for the use of a specific lipase depend on the origin as well as on the formulation of the lipase (Fjerbaek et al., 2009).

Currently, a considerable number of lipase genes discovered have been cloned and functionally expressed (Gupta et al., 2004). Production of lipases is increasingly achieved by expression in recombinant organisms. The use of suitable expression systems, which often enable secretion of mature lipases into the culture medium, has made possible the effective production of large amounts of active lipase (Bornscheuer et al., 2002b).

There are several important points that should be considered before designing the heterologous expression system of a lipase. Many lipases are extracellular, and therefore must be translocated through the bacterial envelopes to reach their final destination (Jaeger and Eggert, 2002). Some lipases are folded by themselves in the cytoplasm or during the secretion process, but certain lipases require chaperones for a correct folding. These foldases help the newly synthesized enzymes to overcome an energetic barrier on the

folding-secretion pathway that allows completion of the active configuration (Rosenau et al., 2004). However, in a study of LipC from *Pseudomonas* sp 42A2 little lipase activity was observed without the presence of its cognate foldase, suggesting that a low number of LipC molecules could be properly folded and secreted in the absence of the foldase (Bofill et al., 2010). In addition, chaperones may also function as temporary competitive inhibitors of their cognate enzymes, thus avoiding undesirable activities of the enzyme on cell lipids. A crucial step in the synthesis of an enzymatically active biocatalyst is the correct folding of the polypeptide chain. It has recently been reported that a correct folding is generally needed to ensure proper secretion (Bofill et al., 2010; Jaeger et al., 1999).

Prokaryotic and eukaryotic systems are the two general categories of expression systems for protein over-expression. Prokaryotic systems are generally easier to handle and are satisfactory for most purposes. However, there is no universal expression system for heterologous proteins. Choosing the best one requires evaluating the options from yield to glycosylation or proper folding, to economics of scaling-up (Rai and Padh, 2001).

E. coli is by far the most widely employed host. Its popularity is due to the vast body of knowledge about its genetics, physiology and complete genomic sequence that greatly facilitates gene cloning and cultivation, and is usually selected provided that post-translational modifications of the product are not essential (Ramnath et al., 2017). Yeasts are the favored alternative hosts for expression of foreign proteins for research, industrial or medical use (Rai and Padh, 2001). *Saccharomyces cerevisiae* and *Pichia pastoris* have successfully used for lipase expression (Schmitt et al., 2002; Valero, 2012). Other expression hosts, such as *Bacillus* species, have also been explored (Sanchez et al., 2002).

Regarding purification of lipases, most of the commercial applications of enzymes do not always require homogeneous preparations of the enzyme. However, a certain degree of purity is needed, depending on the final application in industries such as fine chemicals, pharmaceuticals or cosmetics. Besides, purification of the enzyme is a must for understanding the 3-D structure and the structure–function relationships of the lipase, as well as characterizing the properties of the newly discovered enzymes. For industrial purposes, the purification strategies employed should be inexpensive, rapid, high-yielding and amenable to large-scale operations (Gupta et al., 2004).

1.6 Lipase immobilization

To produce cost effective technology based on enzymatic catalysis, special emphasis should be given to the development of reusable and stable enzymes under harsh conditions. Immobilization facilitates enzyme reuse and product purification, thus dramatically improving process economy (Gupta et al. 2015).

Therefore, biocatalysts can be improved by physico-chemical methods. Immobilization often stabilizes a biocatalyst and facilitates downstream processing by an easy separation of the enzyme. Besides, immobilization commonly implies the advantage of a catalytic activity increase compared to the non-immobilized enzyme (Adlercreutz et al., 2013).

Various immobilization techniques such as adsorption, covalent bonding, entrapment, encapsulation or cross-linking **(Fig. I4)** have been employed on lipases and have been used for biodiesel production (Tan et al., 2010).

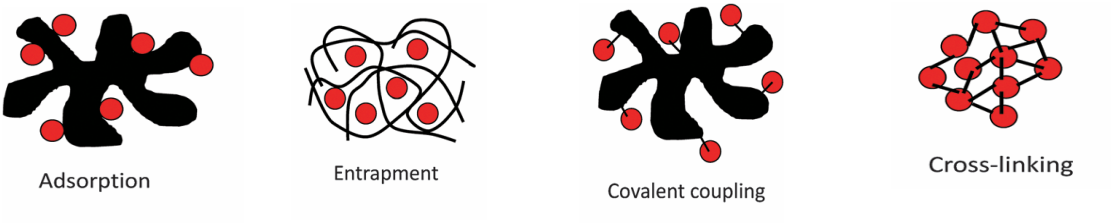


Figure I4. Schematic representation of enzyme immobilization methods. Enzyme molecules are represented by red circles (Adlercreutz et al., 2013).

Adsorption is the attachment of the enzyme on the surface of the carrier by weak forces, such as van der Walls, hydrophobic interactions or dispersion forces. The carriers used include acrylic resins, textile membranes, polypropylene, celite or diatomaceous earth (Tan et al., 2010). Physical adsorption is perhaps the simplest method for enzyme immobilization. An enzyme solution, usually aqueous, is simply brought into contact with a supporting material and, provided the enzyme–support interactions are strong enough, adsorption will occur. In the case of lipases, hydrophobic interactions are the most common, but ionic interactions with ion-exchange materials can also be useful.

Concerning the carrier pore size, diameters around 100 nm seem to be advantageous, although only a few direct studies have been carried out on the importance of this

parameter. Most mesoporous silicas are thus likely to have somewhat too narrow pores, while some of the porous organic polymers used for lipase immobilization have a considerable fraction of their pore volume in large enough pores, such as the polypropene Accurel EP-100, and Accurel MP-1004 (Adlercreutz et al., 2013). Accurel MP, a very hydrophobic macroporous polymer of propylene, was found not to adsorb glycerol, an important advantage during biodiesel synthesis by lipases (Séverac et al., 2011). Therefore, this method can be used for simultaneous purification and immobilization of lipases (Adlercreutz et al., 2013).

Candida antartica lipase B (CalB) immobilized on an acrylic resin, which is known by its commercial name Novozym® 435, is one of the most used lipases in industry. Novozym® 435 lipase shows excellent catalytic properties. Nerveless, this acrylic support can be source of polar compound adsorption leading to a loss of lipase activity during numerous applications. Thus CalB was also immobilized on the very hydrophobic support Accurel MP, enabling to avoid glycerol (and other polar compounds) adsorption, thus permitting long-lasting continuous transesterification reactions (Séverac et al., 2011).

Besides adsorption, there are other several systems for lipase immobilization. For example, enzyme entrapment in silica-based materials, a method based on a sol–gel methodology (Adlercreutz et al., 2013). Also, cross-linked enzyme aggregates (CLEAs) and protein-coated microcrystals (PCMCs) are promising immobilization methods. Both have been tested with a *P. cepacia* lipase for biodiesel production, resulting in better reactions than those with the same lipase immobilized on Accurel (Kumari et al., 2007). On the contrary, the traditional immobilization on an inert carrier dilutes the enzyme activity giving lower volumetric and space–time productivity, and contributes to increase the enzyme costs. However, several lipases have been successfully stabilized with this technique (Fjerbaek et al., 2009).

1.7 Lipase Modification by Protein Engineering

In order to increase the number of applications of enzymes, there are many challenges to solve. Low catalytic performance or stability under extreme reaction conditions are important points to improve. Protein engineering has found appropriate solutions in the last decade to overcome these problems. It is an efficient tool for the design of enzymes with broader substrate ranges, higher thermostabilities or enhanced or even inverted enantioselectivities (Jochens et al., 2011).

Lipases are well known enzymes, whose reaction mechanism is well understood, being often stable enzymes and easy to handle in the laboratory. Consequently, protein engineering of α/β-hydrolases not only has been used to solve problems in applied biocatalysis, but also has been used for proof-of-principle studies to establish new protein engineering methods (Jochens et al., 2011).

1.7.1 Random and site directed mutagenesis

The first approaches of protein engineering through directed mutagenesis involved iterative cycles of random amino acid changes in a protein, followed by selection or screening of the resulting libraries for variants with improved enzyme stability, substrate specificity and enantioselectivity. Changes in substrate specificity or other enzyme properties may be monitored by high-throughput methods, such as fluorescence-activated cell sorting, or catalysis-resulting color production, which can screen tens of millions of variants in a short amount of time. More recent developments have focused on improving the efficiency of directed evolution to create "smarter" libraries obtained by rational design **(Fig. I5)** (Bornscheuer et al., 2012). The simplest solution is a more efficient screening. Another approach is to limit the location of changes to the active site and to restrict the type of changes to those known from sequence comparisons to occur often at these sites in similar enzymes (Bornscheuer et al., 2012). This concept combines structural information with protein sequences, and is based on the "consensus approach" that means that most abundant amino acids at each position in a group of homologous enzymes contribute more than average to protein performance than the non-consensus amino acids; thus, comparison of sequences within large enzyme families can help to identify conserved amino acids as mutation hot residues, and accordingly, a semi-rational mutagenesis can be designed (Davids et al., 2013). In this context, the design of mutants with the desired substrate specificity or property can be performed by identifying functionally relevant residues apart from the active site residues. For lipases, this can be achieved using for example the Lipase Engineering Database (LED) navigation tool (Fischer and Pleiss, 2003). The advances in sequence based protein engineering and structure-guided approaches have benefited from a rapid increase in protein structure coordinates deposited in the RCSB Protein Data Bank. This facilitates both, rational protein design and directed evolution, because structural alignment of related proteins helps to identify distinct similarities and differences, guiding the more reliable design of mutant libraries (Bornscheuer et al., 2012).

A strategy that takes advantage of several predefined regions considered as being crucial for improving a given catalytic property is the iterative saturation mutagenesis. With the systematic application of saturation mutagenesis solely to "hot sites", as suggested by rational considerations resulting from structural information, only defined parts of an enzyme are considered. The iterative process then allows for high evolutionary pressure in confined regions of the protein sequence space, which increases the probability of success while reducing costs, time and human effort (Reetz and Carballeira, 2007). Regarding the methodology of iterative saturation mutagenesis, there is a worksheet that allows calculation of the screening effort resulting from each degenerate codon, named CASTER (Reetz and Carballeira, 2007). It is a tool that helps in the design of the more adequate degenerated primers for the creation of focused libraries, and permits to choose them according to codon usage. Thus, the NNK degeneracy (N: Ade/ Cyt/Gua/Thy; K: Gua/Thy) involves 32 codons and all the 20 possible amino acids as enzyme building blocks. And on the basis of statistical analyses, the number of clones that should be screened for 95% coverage in the case of randomization at one and two positions are 94 and 3066 clones respectively (Reetz et al., 2005). Of course, full coverage is not necessarily mandatory. Nevertheless, the higher the coverage, the greater the probability of finding improved variants (Reetz and Carballeira, 2007).

Nevertheless, the question for the optimal route of choice out of the many different possible pathways for obtaining enzyme variants remains still unanswered, and when a complete mutation scheme is considered, random or rational approaches can be systematically explored for improvement of a desired property. However, when there is a good knowledge of the structure/function, the possibilities of success to get a variant with the desired properties increase notably and most pathways can provide better mutants with enhanced characteristics (Bassegoda et al., 2012b).

1.7.2 Computational design and *in silico* predictions

In recent years, computational protein design is getting more and more attention as a novel strategy to predict the effects of mutations on protein structure, function or stability of libraries of enzyme variants generated by means of *in silico* approaches (Bassegoda et al., 2012b; Dalby, 2007).

The principle of focused directed evolution was extended with the development of the Combinatorial Active Site Saturation Test (CASSTing) (Sandström et al., 2012). It uses the

information derived from structural/functional data to identify amino acids in the substrate binding pocket in order to usually improve activity and selectivity of targeted enzymes; then the identified residues for randomization are mutated in an iterative manner (Cheng et al., 2015; Reetz et al., 2006b). To demonstrate the power of this approach, α/β-hydrolase enzymes were used (Jochens et al., 2011).

The work by Juhl and co-workers showed the potential of *in silico* approaches to predict mutant variants, and thus reducing the time-consuming high throughput screening assays (Juhl et al., 2010). Thus, variants accepting esters with branched and sterically demanding acids of CalB were generated after *in silico* predictions. A library of 2400 CalB variants was built and screened *in silico* by substrate-imprinted docking. From the virtual screening, nine variants with single amino acid exchanges and increased activity were predicted and generated by site directed mutagenesis. Among the nine predicted variants only one displayed higher activity than the wild type against branched acids but not against sterically demanding acids (Bassegoda et al., 2012b; Juhl et al., 2010). The success of a directed evolution method depends upon the quality of the mutant library, and hence the challenge is to generate functionally diverse libraries. Computational tools can also assist directed evolution in these steps by *in silico* analysis and screening of expected protein sequence space sampled by generated libraries (Verma et al., 2014).

Therefore some computational tools for directed evolution are aimed at overcoming the limitations of random mutagenesis methods for introducing defined mutations due to the organization of the genetic code. The statistical analysis shows that the organization of the genetic code compromises the generation of diverse amino acid substitution patterns, especially for transition mutations (Wong et al., 2007a). Transition mutations, in contrast to transversions, often lead to chemically similar amino acids or identical amino acids. The whole field of directed protein evolution would therefore benefit from random mutagenesis methods with a transversion bias (Wong et al., 2007b). Thus, Sequence Saturation Mutagenesis (SeSaM), a statistical analysis of random mutagenesis methods (Wong et al., 2006), is a promising development to overcome hurdles introduced by the organization of the genetic code, and to explore sequence space efficiently in directed evolution experiments. Individually, as well as in combination with existing methods, these new tools take the field one step closer to generating high-quality libraries, smaller in size and rich in beneficial mutations.

Another example of computational tools that can assist to design better-directed evolution strategies by providing the statistical analysis of random mutagenesis methods at protein level is MAP (Mutagenesis Assistant Program). It was developed to estimate the diversity at protein level in a library generated by random mutagenesis and the MAP extended MAP$^{2.0}$3D, being a sequence/structure based server for protein engineering (Verma et al., 2012). It predicts the residue mutability resulting from the mutational bias of random mutagenesis methods, and correlates the generated amino acid substitution patterns with the structural information of the target protein. In this way, the server offers the possibility to analyze at sequence and structural level the effects of the limited mutational preferences of random mutagenesis methods (Verma et al., 2014).

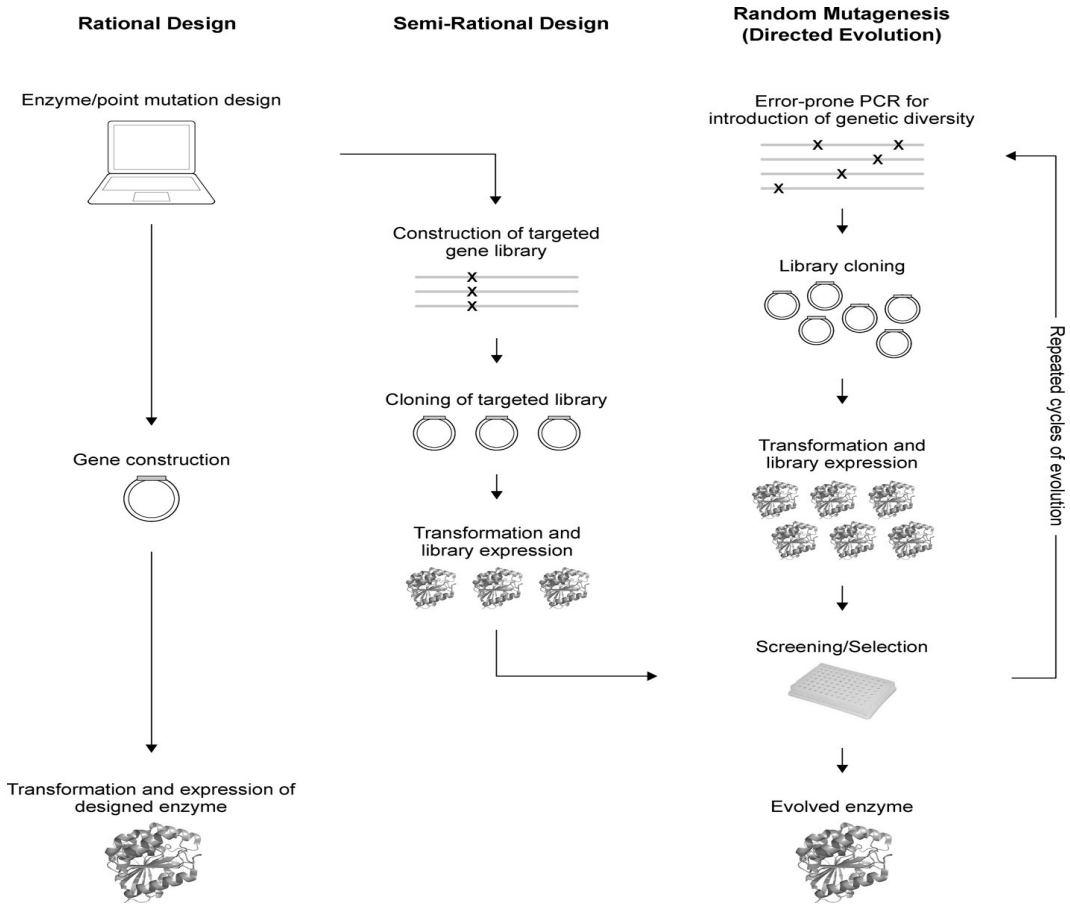


Figure I5. Strategies for the design or directed evolution of enzymes (Porter et al., 2016).

The ultimate goal of protein engineering is to understand how enzymes function mechanistically and what determines properties such as stability, specificity and activity. Such a knowledge should lead to a deeper understanding of catalysis and will allow the creation of customized enzymes for pharmaceutical science, medicine and synthetic chemistry (Jochens et al., 2011).

2. Lipases in Applied Biocatalysis

2.1 Triglyceride Transesterification by Lipases

Lipases are active in both, the presence and absence of water, thus reverse reactions of ester hydrolysis, esterification or transesterificationare possible. Therefore the range of reactions to be catalyzed by lipases is considerably broadened (Kourist et al., 2010). The major application of lipases is mainly based on the interest regarding their high activity and stability in non-aqueous systems (organic solvents, ionic liquids), a very broad substrate range, and high stereoselectivity (Bornscheuer, 2002a). There is a considerable industrial potential for exploitation of esterification and transesterification reactions, which are being used for the production of various types of flavor esters, cocoa butter equivalent, the human milk fat substitute "Betapol", structured lipids, and biodiesel (Gupta and Khare, 2009).

In mono-alkyl ester or biodiesel production the transesterification reaction occurs between oil (triacylglycerols, which may be vegetable oil, animal fat or grease, and an alcohol in the presence of a catalyst, either chemical (acid or base) or enzymatic (Fukuda et al., 2001). It is a sequence of three reversible reactions in which triacylglycerols (TAGs) are converted into diacylglycerols and then diacylglycerols are converted to monoacylglycerols followed by the conversion of monoacylglycerols to glycerol **(Fig. I6)**. The transesterification of triacylglycerols with methanol produces fatty acid methyl esters (FAMEs) along with glycerol (Narwal and Gupta, 2013).

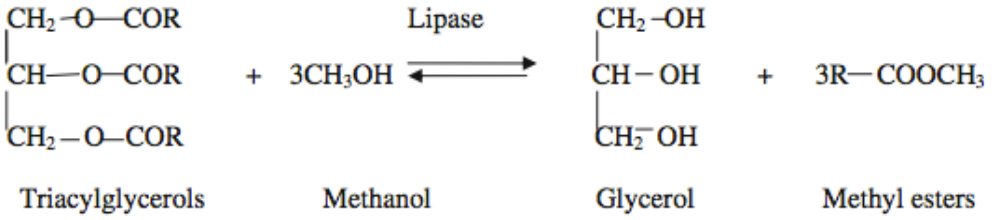


Figure I6. Lipase-catalyzed transesterification reaction between triacylglycerols and alcohol, yielding methyl esters and glycerol (Narwal and Gupta, 2013).

Transesterification of vegetable oils is the most popular method for producing this mixture of mono-alkyl esters of higher fatty acids or biodiesel. The high viscosity component, glycerol, is removed and hence the product has low viscosity like that of fossil fuels **(Fig.**

I7). The mixture of these mono-alkyl esters can hence be used as a substitute for such fuels (Ranganathan et al., 2008).

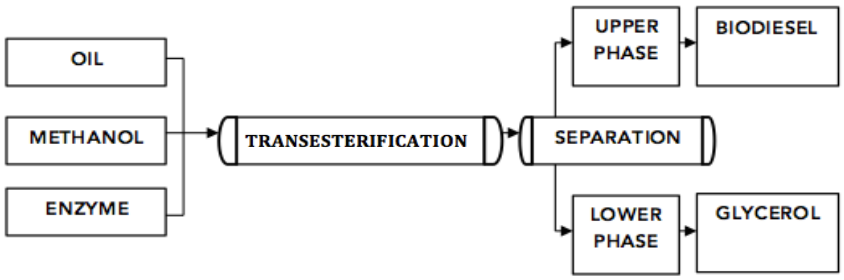


Figure I7. General process of biodiesel production (Narwal and Gupta, 2013).

Production of biodiesel is mainly carried out by alkali processes in an industrial scale, where raw materials with a high water or free fatty acid (FFA) content makes necessary a pretreatment with an acidic catalyst in order to remove the gums (Cowan and Nielsen, 2009; Lv et al., 2010) prior to esterification of the FFAs to the alcohol (Kumar Tiwari et al., 2007). But major problems arise in the downstream operations, including separation of the catalyst, the unreacted methanol and the acidic pretreatment necessary to reduce gums and soap formation during the reaction. In addition, the extensive handling for separation of biodiesel and glycerol together with removal of catalyst and alkaline wastewater become a challenge (Fjerbaek et al., 2009).

Contrarily to alkaline catalysts, enzymes do not form soaps and can esterify both FFAs and TAGs in one step without the need for a subsequent washing step. Thus enzymes are an interesting prospect for industrial-scale production and for reduction of production costs. This is especially interesting when using feedstocks with high contents in FFAs (Fjerbaek et al., 2009; Kumari et al., 2007). Lipases from various microorganisms like *C. antarctica*, *Pseudomonas cepacia* etc., have shown to be suitable for biodiesel formation, as far as used in proper conditions on refined oils (Narwal and Gupta, 2013). However, the costs generated by the use of refined oils are too high; therefore crude, unrefined raw materials are a preference for next-generation biodiesel production.

Enzymes show higher yields and have longer lifetimes than chemical catalysts when used on oils rich in FFAs, as is the case for many crude raw materials (Cesarini et al., 2013). But crude oils usually have also a high content in phospholipids (gums) that can inhibit lipases during biodiesel production (Fjerbaek et al., 2009; Shimada et al., 2002). The phospholipids are the main components removed during degumming and were identified in extracts from

immobilized lipases used for methanolysis of the crude oils, causing adsorption and enzyme inhibition (Chen et al., 2009; Lv et al., 2010). An option to avoid such inhibition problems is to perform simultaneous enzymatic degumming and transesterification in a single step process (Cesarini et al., 2014b; Shimada et al., 2002).

2.1.1 Raw materials

Every country develops biodiesel feedstock according to its national conditions. The United States mainly uses genetically modified soybean oil as raw material, while the European Union and Canada use rapeseed oil to produce biodiesel. Some Southeastern Asian countries such as Malaysia and Indonesia are abundant in palm oil, so these countries develop biodiesel from palm oil. Although China is a large agricultural country, the food supply system is still a big problem facing the whole country. In order to follow the principle of never competing with grain land, China's recent biodiesel development is based on waste cooking oil and *Jatropha* oil (Tan et al., 2010). *Jatropha* tree can grow on waste land with a minimum water and fertilizer demand, and the oil is non-edible because of the presence of some antinutritional factors such as toxic phorbol esters (Jegannathan et al., 2008; Shah et al., 2004). Following the most recent recommendations, the actual interest is to use non-food competing "waste carbon sources" as alternative feedstocks for second-generation biofuel production (Tan et al., 2010).

In this context, micro-algae oil and microbial oil have also significant potential, as they have short production cycles and they can be produced by fermentation using inexpensive sources, such as CO_2 or waste water (Tan et al., 2010). There are also many interesting studies about conversion of waste frying oils by transesterification into biodiesel (Azócar et al., 2010; Sabudak and Yildiz, 2010).

The problem of using these alternative raw materials, is mainly that many of the oils have been described with a relatively high FFAs and water contents (Ramadhas et al., 2005). The enzymatic catalysis with lipases takes advantage of the contents in FFAs, that can equally be esterified to synthetize FAMEs. Consequently, lipases admit the use of low cost and low quality feedstocks to convert them into high value biodiesel (Kumari et al., 2007; Shah et al., 2004).

2.1.2 Enzyme-catalyzed biodiesel production process

The production of biodiesel by alkali process in an industrial scale is cost effective and highly efficient (Sotoft et al., 2010). However, the feedstock accounts for more than 70% of the total production cost. In the future, *Jatropha* oil, microbial oil, micro-algae oil and waste oil will become the main feedstock for biodiesel production (Tan et al., 2010). A way to decrease the cost of biodiesel is to use waste products instead of refined oils. As stated above, these often have a high content of FFAs and gums, which is favorable to the use of enzymes. Moreover, the use of alternative feedstoks is also commendable as waste is turned into a resource, thus reducing the pressure on farmland otherwise used for food production. Unfortunately, waste oils are much more complicated and expensive to transform into biodiesel with chemical catalysts (Fjerbaek et al., 2009; Sotoft et al., 2010). Therefore, the use of enzymes such as lipases is a good alternative for solving these problems.

One common drawback with the use of enzyme-based processes is the high cost of the enzyme. Immobilization of enzymes has generally been used to obtain reusable enzyme derivatives. This enables recycling of the biocatalyst and hence lowers the cost. In the case of biocatalysts in non-aqueous media, immobilization is also reported to result in better activity (Shah et al., 2004). Thus, many transesterification processes employing lipases have used an immobilized form of the enzyme (Shah et al., 2004). New immobilization techniques with higher activity and stability at low cost still need to be explored and developed. If the cost of the catalyst lipase is reduced, the industrial production of biodiesel using enzymatic methods will soon have a bright future (Tan et al., 2010).

The cost price of enzymes has to be brought down if enzymes are to compete on the commodity chemical production market. As free enzymes are cheaper than immobilized, due to the process of immobilization, it is worthwhile to investigate whether enzymes for biodiesel production need immobilization. Thus, the price of immobilization can be lowered using other techniques different from the traditional immobilization with an inert carrier or finding new lipases stable and active in transesterification reactions used in soluble form (Cesarini et al., 2013; Fjerbaek et al., 2009).

Another way to decrease the operational enzyme cost is to increase the operational stability of the biocatalyst. This is crucial for its industrial applicability because it will directly

influence the enzyme cost per kilogram of product (Séverac et al., 2011). As stated above, enzyme engineering can help to reach this requirement.

Additional factors are important for the large-scale application of lipases in biodiesel production. The cost of supporting materials and other costs associated with the preparation of the lipase must be considered more critically than in small-scale work. In addition, the immobilized preparations must be suitable for use in the intended reactor, which may be a stirred-tank reactor, a packed-bed reactor or some other type. The particle size and shape, as well as the resistance to shear, compression, etc., should also be taken into consideration. Furthermore, the operational stability is important since it determines how much product can be produced per unit weight of enzyme. Existing industrial applications have shown that lipases can remain active for months of continuous use which, of course, is beneficial for process economy (Adlercreutz et al., 2013).

2.2 Tertiary Alcohol Ester Resolution by Lipases

In recent years, research on synthesis of optically pure compounds using lipases as biocatalysts has increased considerably. For instance, more than 1000 different substances have now been efficiently resolved using lipases (Bornscheuer, 2002b). Lipases are very useful biocatalysts in the kinetic resolution of racemates **(Fig. I8)**. However, alcohols have received most attention in the context of lipase-catalyzed kinetic resolution and mixtures of primary, secondary and tertiary alcohols, which have been resolved using lipases (Ghanem, 2007). Production of enantiopure tertiary alcohols, considered as building blocks containing quaternary carbon centres, is highly relevant for various applications in fields ranging from natural product generation to pharmaceuticals synthesis. Nevertheless, the availability of well-established and standardized methods for the production of large amounts of enantiopure tertiary alcohols with high yields and great purity is still an important challenge in synthetic organic chemistry (Kourist et al., 2008).

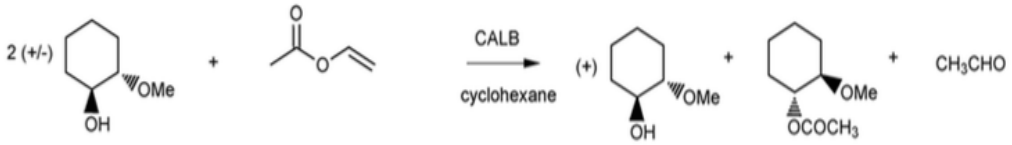
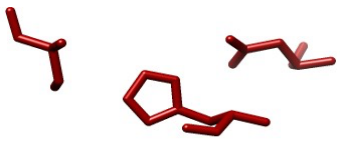


Figure I8. Example of lipase-catalysed reactions for the production of chiral building blocks. CalB: *Candida antartica* lipase B (Adlercreutz et al., 2013).

The use of true lipases and esterases for the kinetic resolution of tertiary alcohols is a promising alternative route to produce optically pure tertiary alcohols, and the biocatalysis processes for such purpose are more environmentally-friendly. However, tertiary alcohols are not accepted by all true lipases and esterases. The majority of lipases and esterases found to be active towards the sterically demanding tertiary alcohols (or their esters) bear the GGG(A)X motif in their oxyanion hole, close to the active site (Henke et al. 2002). Some attempts to create activity towards tertiary alcohols in the GX-type esterases from *P. fluorescens* and *B. stearothermophilus* by directed evolution failed (Henke et al. 2002). In view of the difficulty of inducing activity towards tertiary alcohol esters by random mutagenesis, this results seem to confirm the importance of the GGG(A)X pattern in the active site. However, it appears that activity towards tertiary alcohols is not restricted to the GGG(A)X-type α/β-hydrolases, as only very few examples bearing such an activity have been described (Fillat et al., 2014, 2015). For instance, the G(X)-type lipase from *R. miehei* was reported to be active towards cyclic tertiary alcohol esters (Kourist *et al.* 2008). Moreover, it has been observed that CalA (Y-type oxyanion hole lipase) accepts tertiary and sterically hindered alcohols (Kirk & Christensen 2002); however CalA is a very unique enzyme which shows very low sequence identity with all α/β-hydrolases even with *C.antarctica* lipase B (Kirk & Christensen 2002; Ericsson *et al.* 2008).

Therefore, further investigation is required to increase our knowledge about the oxyanion hole, how it determines the activity of the lipases and esterases against these substrates and how it determines the enantioselectivity of these enzymes in the kinetic resolution of tertiary alcohols.

Objectives



II- OBJECTIVES

The group of microbial enzymes for industrial application works on the identification and design of enzymes for biotechnological application. The research group is working on the biochemical characterization and genetic manipulation of lipases, esterases, cellulases, xylanases, expansins and lytic polysaccharide monooxygenases, from the early stages of sampling, cloning and purification up to studies of structure-function relationship and enzyme engineering, using conventional and *in silico* approaches.

The importance of lipases is increasing in several industries. However, the commercial use of lipases is still a drawback in the economics of the lipase-based industrial applications. There are many tools for improving and adapting the enzyme properties to the desired requirements of a process that could lead lipase catalysis through a cost-effective process. In this context, the main objective of this work was: "To characterize, express and to improve novel bacterial lipases for sustainable industrial processes".

SPECIFICS OBJECTIVES

1. To explore and to characterize a new esterase from *P. barcinonensis*.

This objective, confined in chapter I, defines the isolation from *P. barcinonensis* of the gene corresponding to the second esterase of the strain, and its cloning in a proper vector to perform expression and purification for biochemical characterization, as well as testing for tertiary alcohol ester resolution. It also includes *in silico* analysis of the 3D model structure and phylogeny.

2. To improve LipR activity by protein engineering

In order to improve LipR activity on long-chain substrates, chapter II proposes several enzyme engineering approaches to change the amino acids constituting the rare oxyanion hole of LipR for further industrial application. Hydrolytic activity over short, mid and long-chain substrates was assayed with the variants obtained.

3. To study the role of the amino acids forming the oxyanion hole of LipR

Exploring the role of the amino acids forming the unusual Y-type oxyanion hole of LipR by changing it to a bacterial like oxyanion hole and substituting the two key amino acids was also a second objective of chapter II. Analysis of the 3D model and *in silico* docking studies were also included.

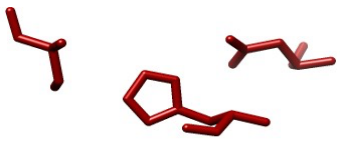
4. To improve *Pseudomonas* lipases stabilization by immobilization

In chapter III the immobilization of *Pseudomonas* lipases LipA, LipC and LipCmut was developed in order to stabilize these enzymes to be applied in transesterification reactions.

5. To test alternative feedstocks for triglyceride transesterification catalyzed by lipases

The objective of the last chapter was to apply the three immobilized lipase preparations and a commercial lipase in transesterification reactions, testing different oils as raw materials. Characterization of the tested raw materials in terms of FFAs, tri, di and monoglyceride contents measure was also of interest.

Publications



CHAPTER I: Exploring a new esterase: Biochemical characterization of *Paenibacillus barcinonensis* Est23

Carboxilesterasa descubierta en *Paenibacillus barcinonensis* BP-23 con un *oxyanion hole* inusual de tipo GGG(A)X

Belén Infanzón, Susana V. Valenzuela, Amanda Fillat, F. I. Javier Pastor, Pilar Diaz

Paenibacillus barcinonensis BP-23, aislada previamente del Río del delta del Ebro (España), posee un sistema hidrolítico complejo mostrando la presencia de al menos dos enzimas con actividad sobre sustratos lipídicos. EstA, una carboxilesterasa de tipo B fue previamente aislada de la cepa y fue caracterizada. El gen que codifica a una segunda putativa lipasa, localizado río arriba del gen que codifica a la celulosa Cel5A, fue obtenido por medio de la estrategia de paseo cromosómico y fue clonado en *Escherichia coli* para una caracterización. El clon recombinante obtenido demostró alta actividad en sustratos de ácidos grasos de cadena media/corta. La enzima, denominada Est23, fue purificada y caracterizada, demostrando máxima actividad sobre *p*NP-caprilato (C8:0) o sobre *muf*-heptanoato (C7:0) bajo condiciones moderadas de temperatura y pH. Además, Est23 contiene un *oxyanion hole* de tipo GGG(A)X, descrito como un motivo importante para la resolución de esteres de alcoholes terciarios. Sin embargo, no se detectó conversión ni resolución enantiomérica de alcoholes terciarios. El alineamiento de secuencia de aminoácidos de Est23 con las secuencias de familias de lipasas bacterianas conocidas y con relación proteica cercana, sugiere que la enzima clonada en este trabajo no pertenece a ninguna de las familias de lipasas bacterianas descritas. Mediante un árbol filogenético construido con la secuencia de aminoácidos de Est23 y de lipasas similares se demostró que la enzima pertenece a un *cluster* de secuencia diferenciado, por lo que probablemente constituya una nueva familia de enzimas bacterianas lipolíticas.

Biochimie (2014) 104:108-16

Biochimie xxx (2014) 1–9

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Unusual carboxylesterase bearing a GGG(A)X-type oxyanion hole discovered in *Paenibacillus barcinonensis* BP-23

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ARTICLE INFO

Article history:
Received 7 March 2014
Accepted 4 June 2014
Available online xxx

Keywords:
Carboxylesterases
Paenibacillus

ABSTRACT

Strain *Paenibacillus barcinonensis* BP-23, previously isolated from Ebro's river delta (Spain), bears a complex hydrolytic system showing the presence of at least two enzymes with activity on lipidic substrates. EstA, a cell-bound B-type carboxylesterase from the strain was previously isolated and characterized. The gene coding for a second putative lipase, located upstream cellulase Cel5A, was obtained using a genome walking strategy and cloned in *Escherichia coli* for further characterization. The recombinant clone obtained displayed high activity on medium/short-chain fatty acid-derivative substrates. The enzyme, named Est23, was purified and characterized, showing maximum activity on *p*NP-caprylate ($C_{8:0}$) or MUF-heptanoate ($C_{7:0}$) under conditions of moderate temperature and pH. Although Est23 displays a GGG(A)X-type oxyanion hole, described as an important motif for tertiary alcohol ester resolution, neither conversion nor enantiomeric resolution of tertiary alcohols could be detected. Amino acid sequence alignment of Est23 with those of known bacterial lipase families and with closely related proteins suggests that the cloned enzyme does not belong to any of the described bacterial lipase families. A phylogenetic tree including Est23 and similar amino acid sequences showed that the enzyme belongs to a differentiated sequence cluster which probably constitutes a new family of bacterial lipolytic enzymes.

1. Introduction

Esterases (EC 3.1.1.–), widely distributed in nature, are a diverse group of hydrolases catalysing the cleavage or formation of ester bonds [1]. Included under the general term of carboxylesterases, the utmost characterized lipolytic enzymes are lipases (EC 3.1.1.3, triacylaglycerol hydrolases) and esterases (EC 3.1.1.1, carboxyl ester hydrolases), which differ both in their kinetics and chain-length substrate preferences [1]. While esterases hydrolyse small ester-containing molecules partially soluble in water like short-chain acylglycerides, lipases have optimal activity towards long-chain triacylglycerides not soluble in aqueous environments [2]. Therefore, esterases display a typical Michaelis–Menten behaviour, whereas most lipases show an interfacial activation when lipids reach an equilibrium between the monomeric, micellar and emulsified states [2].

In recent years there has been a growing interest for carboxylesterases due to their broad array of substrate specificity and versatility in the reactions they catalyse [3–5]. Therefore, these enzymes are considered as powerful biocatalysts with diverse biotechnological applications such as food technology, detergent formulation or the synthesis of optically pure compounds, among other uses in chemical industry [2–6]. Conveniently, carboxylesterases do not usually require cofactors, are quite stable, and may be active in organic solvents [1,7,8]. Although the physiological function of many microbial carboxylesterases remains not clear, some of these enzymes are known to be involved in metabolic pathways that provide access to carbon sources, and some may also have a role in detoxification of biocides [9,10].

Both, lipases and esterases, hold the characteristic α/β-hydrolase fold in their tridimensional structure, consisting on a defined alternation of α-helices and β-sheets also found in other hydrolases [11]. These enzymes contain a catalytic triad constituted by a serine, an aspartic or glutamic acid, and a histidine, usually being the first residue embedded in the GXSXG consensus pentapeptide [12]. Bacterial lipolytic enzymes were initially classified into eight families [13], with successive revisions up to ten families described nowadays [12,14]. These classifications are based on amino acid sequence similarity and the presence of conserved motifs, like the GDSL-pattern of family II lipases [12,13].

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http://dx.doi.org/10.1016/j.biochi.2014.06.003
0300-9084/

Strain *Paenibacillus barcinonensis* BP-23, previously isolated from Ebro's river delta [15] and further classified [16], displays numerous hydrolytic activities. Three cellulases [17–19], five xylanases [20–25] plus two pectinases [26–28] have been isolated, cloned and characterized. Presence of hydrolysis haloes when growing on tributyrin-supplemented plates indicated also the presence of lipolytic activity. Zymogram analysis revealed the presence of at least two enzymes displaying activity on MUF-butyrate. A cell-bound type B carboxylesterase was previously isolated, cloned and characterized [29], showing activity on short-chain length fatty acid substrates, which was further improved for resolution of tertiary alcohols [30]. In this work we describe the identification, cloning and biochemical characterization of a second lipolytic enzyme of the strain, a carboxylesterase designated Est23. Assays on protease activity and tertiary alcohol resolution by Est23 are also reported.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

Wild-type strain *P. barcinonensis* BP-23 (CECT 7022) was grown in Luria–Bertani medium (LB) for 24 h at 30 °C, under aerobic conditions [16]. *Escherichia coli* BL21 Star (DE3) (Novagen®) was routinely cultured overnight at 37 °C in LB broth or on LB agar plates, and was used as the host strain for cloning and expression of lipase-encoding genes. Plasmid pET101/D-TOPO® (Invitrogen®) was used as expression vector.

2.2. DNA manipulation and cloning

DNA manipulations were carried out according to Sambrook [31]. Plasmid DNA was purified using commercial kits (Illustra PlasmidPrep, GE Healthcare, UK). Thermostable polymerases *taq* and *pfu* (Biotools, Spain) were used according to the manufacturers' instructions. PCR amplifications were performed in a GeneAMP PCR system 2400 (Perkin Elmer) using different cycling periods [31]. Genome walking for gene sequence identification was performed as previously described [14,24]. Specific primers FWEst23-Topo (5′-CACCATGCGAAAAAAGATAG), RVEst23-Topo (5′-TTAACTCGATCCGAAATATTCA), and RVEst23-Topo-His (5′-CGCACTCGATCCGAAATATTCA) were used for *est*23 ORF amplification and cloning either including or not a His tag at the C-terminus of the protein, using *P. barcinonensis* BP-23 genome as a template. Amplified DNA was purified through chromatography (Illustra™ *GFX*™), PCR DNA and Gel Band Purification kit (GE Healthcare). For ligation and expression, the pET101/D-TOPO® vector was used (Invitrogen®). To obtain the nucleotide sequences of DNA, PCR amplified fragments were analysed using the ABI PRISM® BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems), and the analytical system CEQ™ 8000 (Beckman–Coulter) available at the Centres Científics i Tecnològics of the Universitat de Barcelona. DNA samples were routinely analysed by agarose gel electrophoresis [31], and stained with GelRed™ 0.27% (v/v). Nucleic acid concentration and purity was measured using a Spectrophotometer ND-100 NanoDrop®.

2.3. Bioinformatics tools

Blast searches were routinely performed for DNA or protein sequence analysis [32]. Alignments were performed using the MAFFT (Multiple Alignment Fast Fourier Transform) server (http://mafft.cbrc.jp/alignment/server). BioEdit Sequence Alignment Editor v.7.0.1 [33] was used for restriction pattern determination. The webtool ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to identify the open reading frames, and the software CONTIG EXPRESS was used to assemble DNA sequences (Vector NTI version 8; Invitrogen, Carlsbad, CA). Identification of putative signal peptide [34] and transmembrane regions was performed through SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM tool (http://www.cbs.dtu.dk/services/TMHMM-2.0), respectively. Phylogenetic analysis was conducted using MEGA v5.2 software applying Maximum Likelihood (PALM) method (Tamura 3-parameter model). A bootstrap consensus tree was achieved after 1000 repeats and results were obtained with a cut off of 50% [35]. The DNA sequence of *est*23 was submitted to ENA databank at EBI and given the GenBank Accession number KF373032.1 (UniProt S5XG82).

2.4. Est23 purification

Exponential growth cultures (OD_{600nm} = 0.6) of recombinant clone *E. coli* BL21/pET101Est23 were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37 °C for 3 h. Cells were disrupted by using a French Press, as described, and cell extracts were concentrated using an ultrafiltration unit (Amicon® Ultra centrifugal filter devices) with a 30 kDa cut off membrane [36,37]. The recombinant His_6 tag-Est23 protein was purified from concentrated cell extracts by immobilized metal affinity chromatography (IMAC) using HisTrapHP columns of 1 ml (GE Healthcare) and eluted at a flow rate of 1 mL/min with 50 mM Tris buffer with a 0–300 mM imidazole (pH 7) gradient, on a fast protein liquid chromatography system (ÄKTA FPLC; GE Healthcare). Collected samples were desalted using an ultrafiltration unit with a 30 kDa cut off membrane. Purified esterase and crude cell extracts were analysed by SDS-PAGE [38] after a 2 min treatment at 100 °C. Zymogram analysis was performed as previously described [39]. The Bradford method was used for protein concentration determination [30,40].

2.5. Activity assays

Activity of free enzyme, crude cell extract and supernatant fractions was analysed by measuring the release of methylumbelliferone (MUF) from MUF-derivate fatty acid substrates (Sigma), using a spectrofluorometer (Variant, Spain), as previously described [41,42]. Alternatively, activity was analysed by measuring the release of *para*-nitrophenol (*p*NP) from *p*NP-derivative fatty acid substrates, as previously reported [37,43]. One unit of activity was defined as the amount of enzyme that released 1 mol of *p*NP or MUF per minute under the assay conditions used. Protease activity was assayed using skimmed-milk supplemented agar plates.

Optimum temperature of Est23 was determined by analysis of the activity over a range from 4 to 70 °C at pH 7, using MUF-heptanoate as substrate. Thermal stability of the enzyme was determined by incubating the purified enzyme at temperatures from −20 to 50 °C for 1 h or longer periods (1 month); residual activity was measured under standard conditions once samples reached room temperature. Optimum pH was established by analysing Est23 activity in a pH range from 3 to 10 using Britton–Robinson buffer adjusted to the different pHs [42,44]. To equal the different pH of each reaction, and therefore to avoid the effect of pH on the fluorometric determinations, 100 μL of 2 M Tris HCl (pH 7) were added to each reaction mixture before measuring MUF release [41]. Stability of the enzyme was also determined by measuring the residual activity on MUF-heptanoate after 1 h incubation at different pH. For inhibition studies, activity assays were performed on MUF-heptanoate in the presence of several metal ions, used at 1 mM and 10 mM concentration. Purified Est23 was also incubated in the presence of various concentrations of Triton X-100, SDS, urea or PMSF to measure the effect of such substances on enzyme activity [37,43]. Kinetic parameters (V_{max} and K_m) were

Please cite this article in press as: B. Infanzón, et al., Unusual carboxylesterase bearing a GGG(A)X-type oxyanion hole discovered in *Paenibacillus barcinonensis* BP-23, Biochimie (2014), http://dx.doi.org/10.1016/j.biochi.2014.06.003

Table 1
Summary of Est23-His purification procedure and yield, measured using *p*NP-caprylate.

Fraction	Activity (U/ml)	Fraction volume (ml)	Total activity (U)	Protein concentration (mg/ml)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude cell extract	0.143	50	7.15	70.1	0.002	1	100
Purified Est23	0.023	2	0.046	1.0	0.023	11.5	6.43

determined under optimal assay conditions by fitting hyperbolic Michaelis–Menten curves with SigmaPlot 4.0 (Jandel Scientific).

2.6. Kinetic resolution of tertiary alcohols

Linalyl and terpinyl acetates were purchased from Aldrich Biochemicals. Both tertiary alcohol acetates were dissolved in DMSO (6 mM) and mixed with 800 μL of enzyme solution (0.4 U of cell free enzyme solution in 20 mM Tris HCl pH 7) or with the same volume of buffer (20 mM Tris HCl pH 7) for autohydrolysis control. The reaction mixture was stirred in a thermoshaker (Eppendorf, Germany) at 37 °C, and 400 μL samples were taken after 2 and 4 h and extracted twice with 400 μL dichloromethane [30,45,46]. The combined organic layers were dried over anhydrous sodium sulphate, filtered to remove solid impurities, and the organic solvent removed under nitrogen and transferred to a GC vial. Samples were analysed by gas chromatography mass spectrometry (GCMS) on a chiral-phase GCMS Finnigan TRACE DSQ chromatographer (Thermo Electro Corporation) equipped with a BetaDEX™120 column of β-cyclodextrin (30 m × 0.25 mm × 0.25 μm film thickness) from SUPELCO. Retention times for the analysed products were: (±)-lynalyl acetate = 39.1 min, (*S*)-(+)-linalool = 27.6 min, (*R*)-(−)-linalool = 26.9 min, (±)-(α)-terpinyl acetate = 26.6 min, (*S*)-(−)-terpineol = 23.9 min, (*R*)-(+)-terpineol = 24.2 min. Enantioselectivity was calculated from the enantiomeric excess of the product (ee_p) values, according to Straathof and Jongejan [47].

3. Results and discussion

3.1. Est23 identification and cloning

Genome walking of *P. barcinonensis* BP-23 CECT 7022 [16] revealed the presence of an ORF with similarity to putative esterase/lipase proteins [32] contiguous to a gene coding for the previously described cellulase Cel5A [17]. The complete sequence of the newly discovered ORF was obtained and analysed showing poor identity to other DNA sequences in the databases except for the last 100 nucleotides, that were identical to the annotated upstream region of *cel*5A [17]. This confirmed that the two ORFs are contiguously located in the genome of *P. barcinonensis*, being transcribed from the same strand. No intergenic promoter or terminator motifs could be found between the two genes, suggesting that they might be expressed together. Such an architecture, with two consecutive hydrolytic enzymes being produced under the same induction signals would be of significance for a better use of organic matter, contributing to large substrate hydrolysis aimed at nutrient uptake [48].

The newly discovered gene, designated *est*23, was isolated from *P. barcinonensis* BP-23 using specific primers (FWEst23-topo and RVEst23-topo/RVEst23-Topo-His), and each amplified DNA fragment was ligated to pET101 vector. The resulting construction was transformed into *E. coli* BL21 Star (DE3), producing recombinant clone *E. coli* BL21/pET101Est23. Presence of activity in the recombinant clone was confirmed by detection of hydrolysis haloes after growth on tributyrin-supplemented plates or by fluorescence emission [39] produced by cell extracts after hydrolysis of MUF-butyrate (not shown). The corresponding cloned protein bearing lipolytic activity was designated Est23.

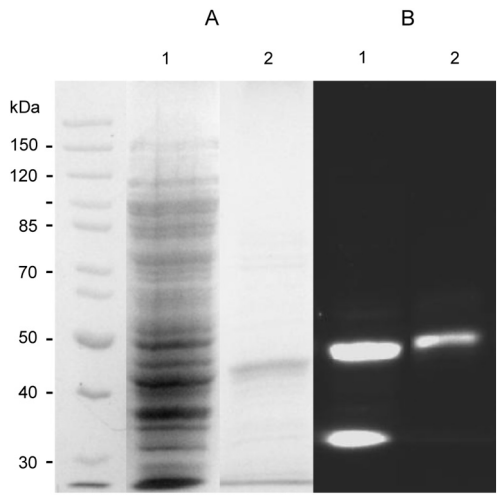

Fig. 1. SDS-PAGE and zymogram analysis of Est23. (A) Coomassie-stained SDS-PAGE of recombinant clone BL21/pET101Est23 cell extracts (1) and purified carboxylesterase Est23 (2). (B) Zymogram analysis on MUF-heptanoate of the same gel shown in (A). Position of molecular mass markers is indicated.

3.2. Est23 purification and characterization

For characterization purposes, cloned Est23 was purified by fast protein liquid chromatography from 50-fold concentrated crude cell extracts of recombinant *E. coli* BL21/pET101Est23-His. Presence of the histidine tag facilitated one-step purification [24]. The purification process rendered a yield of 6.43%, allowing isolation of 11.5-fold purified Est23 with a specific activity of 0.023 U/mg protein on *p*NP-caprylate (Table 1). The resulting purified enzyme was analysed by SDS-PAGE and zymogram [38,39], showing high purity and an estimated molecular size of 43 kDa (Fig. 1).

Table 2
Substrate profile of Est23.

*p*NP/MUF-derivative[a]	Specific activity (mU/mg protein)[b]	Standard deviation
*p*NP-butyrate ($C_{4:0}$)	14.2 (62)[c]	5.44
*p*NP-valerate ($C_{5:0}$)	18.2 (79)	6.58
*p*NP-caprylate ($C_{8:0}$)	23.0 (100)	0.00
*p*NP-caprate ($C_{10:0}$)	8.7 (38)	3.33
*p*NP-laurate ($C_{12:0}$)	4.0 (17)	5.33
*p*NP-palmitate ($C_{16:0}$)	3.4 (15)	1.19
MUF-butyrate ($C_{4:0}$)	0.33 (27)	0.19
MUF-heptanoate ($C_{7:0}$)	1.22 (100)	0.00
MUF-stearate ($C_{18:0}$)	0.004 (0.3)	0.17
MUF-oleate ($C_{18:1}$)	0.02 (1.6)	0.13

[a] Substrates were assayed at 1 mM (pNP-derivatives) and 0.2 mM (MUF-derivatives) concentrations for short chain-length (<12C) substrates, whereas half concentrations were used for longer chain-length substrates.
[b] Activity values are the mean of at least three independent assays.
[c] Data in parentheses correspond to the relative activity, considering activity on *p*NP-caprylate or MUF-heptanoate as 100%.

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Est23 substrate specificity was tested on several *p*NP and MUF-derivatives as previously described [14,37,42], showing preference for medium chain-length fatty acid substrates, and exhibiting the highest activity (23.0 mU/mg protein; 100%) on *p*NP-caprylate ($C_{8:0}$) (Table 2). The enzyme maintained almost 80% activity on *p*NP-valerate ($C_{5:0}$; 18.2 mU/mg protein) and 62% on *p*NP-butyrate ($C_{4:0}$; 14.2 mU/mg protein). However, a dramatic activity reduction was observed when long chain length substrates were used (Table 2). Similar results were observed when activity was measured using MUF-derivatives [41,42]; the highest activity in this case was found on MUF-heptonoate ($C_{7:0}$; 1.22 mU/mg protein), whereas a much lower activity was obtained (27%) when assayed on MUF-butyrate ($C_{4:0}$; 0.33 mU/mg protein), and almost no activity could be detected on long chain-length MUF-derivatives (Table 2).

The effect of temperature and pH on the activity of Est23 was also determined using MUF-heptanoate as a substrate (Fig. 2). Britton–Robinson buffer [44], adjusted at different pH (3–10) was used to determine the optimum pH of the enzyme. Est23 displayed optimum activity at 30 °C, showing 93% of the maximum activity at 37 °C (Fig. 2A). Activity decreased rapidly at higher temperatures, showing only 9% relative activity when assayed at 50 °C, indicating a low thermal tolerance of Est23. However, it is interesting to note that the enzyme displayed 66% and 47% of the maximum activity at 15 °C and 4 °C, respectively, showing a good behaviour under mesophilic environmental conditions, useful for certain biotechnological processes carried out at environmental temperature [1,50]. Regarding optimum pH, like most *Bacillus*-like esterases Est23 exhibited maximum activity at pH 7 and kept about 70% activity at pH between 6 and 9 (Fig. 2B), being thus adequate for processes requiring a moderate pH [29,51].

Thermal and pH stability of Est23 were determined after 1 h incubation over different temperatures (0 °C–50 °C) and pH (3–10). As shown in Fig. 2C, Est23 retained good activity when incubated for 1 h up to 30 °C, while a 60% activity decrease was observed at 37 °C, and no activity was found at 50 °C, confirming that the enzyme cannot tolerate high temperatures but on the contrary, it is well suited for low-temperature requiring processes [50,52]. Stability after 1 h incubation at different pH was good between pH 6 and 10 (Fig. 2D), whereas from the results obtained it is clear that the enzyme does not tolerate acidic pHs. Long-term thermostability was also tested to evaluate the possibilities of storage of the enzyme. For such purpose, purified Est23 was incubated for a 30-day period from −20 °C to 30 °C, and the residual activity after incubation determined as previously described [36,45]. The enzyme maintained almost full activity at −20 °C and 4 °C for the whole period, and more than 80% residual activity was found after 24 h incubation at 30 °C. Nevertheless, activity was almost lost after one-month incubation at 20 °C and 30 °C (not shown). These results indicate that stability improvements through either directed evolution or rational design should be performed for an eventual biotechnological application of Est23 requiring long-term stability [53].

Further characterization of Est23 involved study of the kinetic parameters of the enzyme on MUF-heptanoate, evaluated under optimum conditions. As for most esterases, the enzyme showed a typical Michaelis–Menten behaviour, with no interfacial activation [2]. The calculated apparent V_{max} and K_m values found for Est23 were 0.76 ± 0.07 U/mg protein and 63.49 ± 17.51 mM, respectively. From the substrate specificity results obtained above and considering the kinetic parameters displayed by Est23, showing lack of interfacial activation, we propose Est23 to be considered an esterase showing similar properties to those described for other *Bacillus*-related species carboxylesterases like *Bacillus subtilis* PnbA [54], *P. barcinonensis* BP-23 EstA [29], or *Bacillus* sp. BP-7 EstA1 [51].

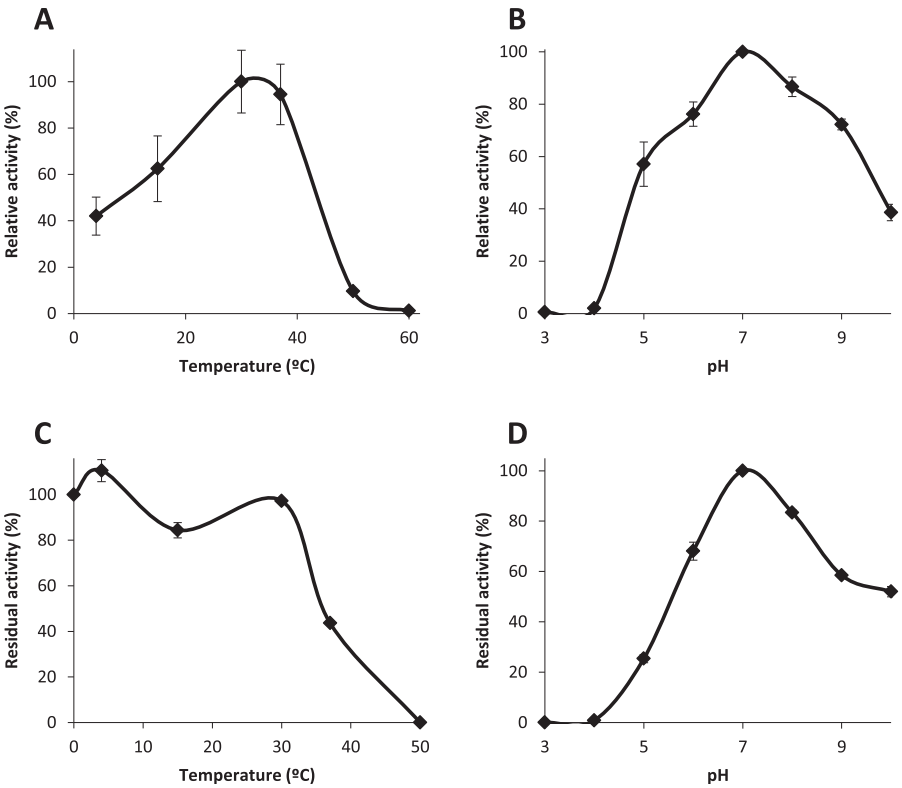

Fig. 2. Activity profile of Est23 assayed at different temperature and pH. (A) Optimum temperature; (B) optimum pH; (C) thermal stability after 1 h incubation; (D) pH stability after 1 h incubation at optimum temperature. Activity values are the mean of at least three independent determinations.

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The effect of different ions and chemicals on the activity of purified Est23 was also analysed using MUF-heptanoate as a substrate (Fig. 3). Activity of Est23 was severely reduced in presence of 1 mM Cu^{2+}, Zn^{2+}, Fe^{2+} and Ag^{+}, and it was almost completely lost when a concentration of 10 mM of these ions was used (Fig. 3A). However, no significant activity decrease was observed for the rest of ions tested at 1 mM, whereas only Ba^{2+}, Co^{2+} and Cs^{+} produced a pronounced (40–60%) activity decrease when assayed at 10 mM. Concerning inhibition by other substances, Est23 activity was lost in the presence of 2 M urea (Fig. 3B), a result that contrasts with those reported for other described lipases and esterases [37,42].

On the other hand, as expected for a serine-acting enzyme, activity of Est23 was greatly reduced by PMSF [29,55], showing only 50% residual activity when a 3 mM concentration of this serine-protease inhibitor was included in the reaction mixture (Fig. 3C). Lack of complete inhibition by excess PMSF might be due to anomalous fitting of this compound into the active site of the enzyme, a fact previously described for other carboxylesterases [37,55,56]. Activity of Est23 was also analysed in the presence of different commonly used detergents like SDS or Triton X-100. The enzyme was almost completely inhibited by low concentrations of SDS. Nevertheless, such inhibition was reversible by addition of 0.5–2% Triton X-100, which contributed to reactivate the denatured enzyme by refolding, a phenomenon previously described for other lipolytic enzymes [29,42,45], which can be used for activity recovery after enzyme aggregation. However, the activity of Est23 was strongly reduced when higher concentrations of Triton X-100 (over 2%) where used (not shown), as happened for other esterases [45].

It has been previously described that certain esterases containing a GGG(A)X-type motif at the oxyanion hole are capable to perform kinetic resolution of tertiary alcohol esters [30,45,57]. This suggested that Est23, bearing the motif GGPG might also be of interest for this purpose. The ability of Est23 for conversion and resolution of tertiary alcohols was evaluated using the esters linalyl and terpinyl acetate as substrates. Concentrated cell extracts with 0.5 U/mL hydrolytic activity on *p*NP-caprylate were used. However, presence of the motif GGG(A)X in the oxyanion hole is not a rule for tertiary alcohol racemates resolution, and in the case of Est23, no conversion was observed after 2 or 4 h incubation at 37 °C under the conditions described in Materials and Methods. Lack of activity on tertiary alcohols can sometimes be due to a non-fruitful interaction of the enzyme with the substrate [58], making it necessary to test other substrates [46] or to produce new improved enzyme variants [30,45,46].

3.3. Est23 sequence and structure analysis

Analysis of the nucleotide sequence of gene *est*23 showed an open reading frame of 1038 bp, encoding a protein of 345 amino acids with a predicted molecular mass and *p*I of 39.4 kDa and 5.5, respectively. A putative Shine-Dalgarno (GAAGG) sequence was found upstream the initiation codon [33], along with the −10 and −35 putative promoter regions. Two contiguous repeats (AGAGGTTT) were found downstream the stop codon but neither inverted repeats nor significant secondary structures that could act as transcription terminators appear at the intergenic region between Est23 and Cel5A [17,33], suggesting that common signals could serve for coordinated expression of both enzymes.

The typical carboxylesterase consensus pentapeptide –GXSXG– was found in the deduced amino acid sequence of the cloned

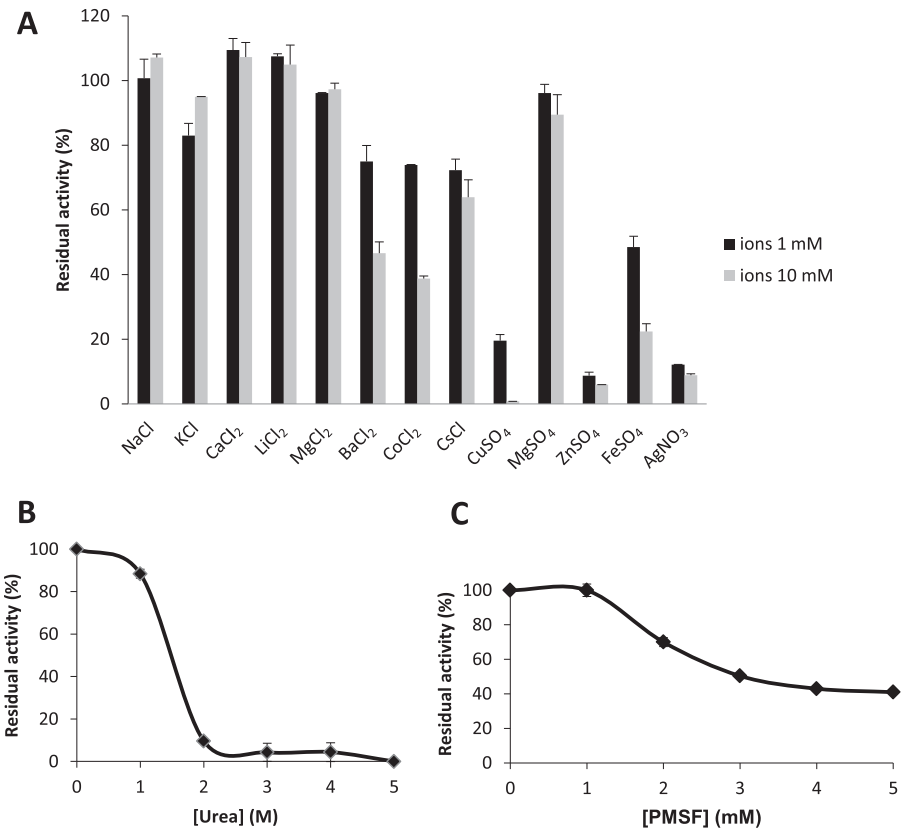

Fig. 3. Selected aspects of Est23 inhibition assayed on MUF-heptanoate. Effect of several ions (A), urea (B) and PMSF (C) on Est23 activity. Values are the mean of at least three independent determinations.

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enzyme, containing Ser[146] as a part of the predicted catalytic triad, together with residues Asp[294] and His[324], assigned by similarity. Using SignalP 3.0 [59], a predicted signal peptide (residues 1–35) containing a transmembrane region [60] was identified, suggesting that the cloned enzyme is a secreted protein [61]. According to previous data, secreted lipolytic enzymes are mainly "true" lipases [2], whereas finding secreted bacterial esterases like Est23 is not frequent in nature, a phenomenon being mostly associated with pathogens [62]. However, a more accurate analysis performed with SignalP 4.0 [34], designed to discriminate transmembrane regions from true signal peptides and to avoid false positives indicates that Est23 does not bear a true signal peptide, being most probably a

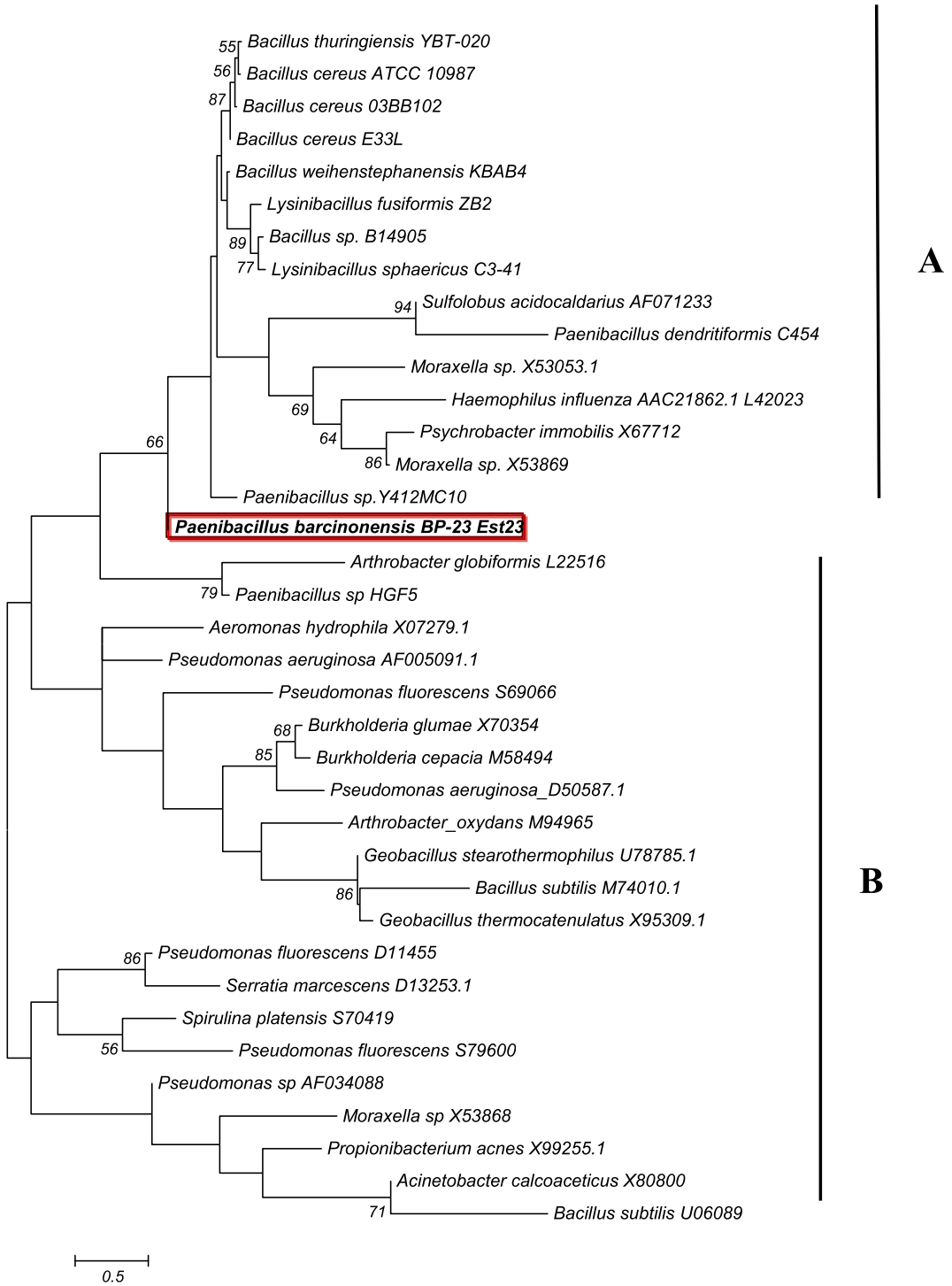

Fig. 4. Amino acid sequence analysis of Est23. A phylogenetic tree was obtained using the software MEGA_5 [35,69]. (A) Sequences of putative, uncharacterized α/β-fold hydrolases and other close putative proteins showing over 55% identity to Est23, obtained using BLAST. (B) Members of known lipase families I–VIII [13]. Central box corresponds to Est23, grouping in a different cluster but within the same root. The protein sequences for previously identified families of bacterial lipolytic enzymes were obtained from GenBank (http://www.ncbi.nlm.nih.gov).

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cell-bound esterase, as expected for an enzyme showing the properties of Est23 [29,36].

According to protein domain databases, Est23 is a single domain, globular protein belonging to the α/β-fold hydrolase family, showing the highest identity to non-described putative alfa-beta fold hydrolases (E-value: 1.03e-23) and to prolyl specific aminopeptidases (E-value: 2.31e-13; IPR002410) [49]. However, plate analysis of the cloned enzyme revealed that Est23 does not display activity on skimmed milk (not shown), being thus assigned to the carboxylesterase group of α/β-fold hydrolases_6 (PF12697), and identified as a lipolytic enzyme [63,64]. This assignment is supported by the high content of non-polar amino acids (77%) found in the protein sequence [65]. Moreover, secondary structure prediction (not shown) confirmed the typical α/β-fold of carboxylesterases, and allowed location of the conserved pentapeptide constituting the "nucleophilic elbow" between strand β5 and the following α helix [2,65]. A 3D model of Est23 was constructed with Phyre^2 using the closest structure (pdb *c1cr6*), corresponding to α/β-fold epoxide hydrolases, enzymes frequently shown to bear full esterase activity [55] and closely related to bacterial family V lipases [13,55]. From the 3D structure obtained (not shown), location of the catalytic triad Ser^{146}, Asp^{294} and His^{324}, and the oxyanion hole (G^{71}GPG) at the predicted positions was confirmed [66]. Analysis of the 2D and 3D structure of Est23 did not reveal the presence of a lid covering the active site, a fact that further supports the idea of Est23 being an esterase [2].

The increasing availability of protein sequences in the public databases allows comparison of a high number of amino acid sequences and may provide a better understanding of the evolutionary nature of related enzymes. Assignment of a defined carboxylesterase to a family is usually performed by inspecting the phylogenetic tree and thus deciding the membership of a protein by placing it into a cluster [67]. Classification of the newly isolated Est23 revealed that the enzyme has no significant similarities to any of the bacterial lipase sequences that define the bacterial lipase families described to date [12–14]. In fact, Est23 displays unique amino acid sequence motifs that clearly separate it from the other families of bacterial lipases [14,68]. However, BLAST searches revealed that Est23 has relevant sequence identity with other uncharacterized, putative α/β-fold hydrolases from Gram-positive bacteria, including a hypothetical protein from *Paenibacillus* sp. PAMC 26794 (67%), two α/β-fold hydrolase family proteins from *Paenibacillus* sp. FSL R5-192 and *Paenibacillus* sp. FSL H7-689 (67%), two α/β-fold hydrolases from *Bacillus vireti* ETI68348.1 and LMG 21834 (59%), two hypothetical proteins from *Paenibacillus daejeonensis* (61%) and *Paenibacillus* sp. OSY-SE (56%), or two α/β-fold hydrolase family proteins from *Paenibacillus alvei* EPY12907.1 (55%). Alignment of Est23 with the closest proteins highlighted the presence of partially conserved central regions (M^{55}IRG....HGGPGCSEIPYV^{81}; G^{103}KSYHFTEDYS^{113}; G^{144}H**S**FGTYIGMKAA…AYIGIGQ^{171}) along with a common sequence (S^{322}AHYPQFEE^{330}) located at the carboxy terminus of the protein. In all considered amino acid sequences, the catalytic triad is constituted by Ser–Asp–His, in the same order. The pentapeptide embedding the catalytic Ser, located in the most conserved central region, is constituted by the motif GH**S**FG. As a rule, the two other catalytic amino acids are mainly surrounded by hydrophobic amino acids, as expected for enzymes acting on hydrophobic substrates [65].

In order to make a classification trial, a multiple alignment and a derived phylogenetic tree (Fig. 4) was obtained. Est23 grouped far from any of the sequences that define each bacterial lipase family [13], making it difficult to assign it to any existing family and suggesting that it might be a member of a new bacterial lipase family not described yet (Fig. 4, segment B). Interestingly, the uncharacterized α/β-fold hydrolases showing significant similarity to Est23 grouped also in a different cluster (Fig. 4, segment A), also separated from Est23 but within the same root. This interesting positioning of Est23, separated by a significant distance from the other members of the tree, indicates that no family assignment of the newly isolated enzyme is possible for the moment due to lack of other members showing similarity to it and clustering at the same evolutionary position.

4. Conclusions

The novel esterase Est23 from *Paenabacillus barcinonensis* BP23 is the second lipolytic enzyme cloned, purified and characterized up to date from this microorganism. The enzyme is a carboxylesterase showing preference for medium chain-length substrates and acting under moderate temperature and pH conditions. Est23 can be inhibited by several compounds but in general it displays good stability. Presence of a GGG(A)X—type oxyanion hole suggests that Est23 could be a candidate for tertiary alcohol ester resolution. However, no conversion of linalyl and terpinyl acetates was obtained, indicating that other substrates should be tested and/or further enzyme improvement would be required for this purpose.

Amino acid and structure analysis revealed that Est23 contains conserved motifs of both, prolyl aminopeptidases and epoxyde hydrolases. However, the biochemical properties exhibited by the enzyme and the patterns found after analysis of the 2D and 3D structures of the enzyme revealed that it has the features of a lipolytic enzyme. Phylogenetic analysis of Est23 did not allow family assignment of the enzyme, which grouped apart from any of the sequences representing the known lipase families. Interestingly, Est23 does not cluster with other uncharacterized proteins showing significant similarity, suggesting that the newly discovered carboxylesterase might be a member of a new family of bacterial lipases whose members remain still undiscovered.

Conflict of interest

There are no conflicts of interest associated with this publication. The manuscript has been read and approved by all named authors for submitting to Biochimie.

Acknowledgements

This work was financed by the Scientific and Technological Research Council (MINECO, Spain), grant CTQ2010-21183-C02-02/PPQ, by the IV Pla de Recerca de Catalunya, grant 2009SGR-819, by PCI-AECID project A203563511, and by the Generalitat de Catalunya to the "Xarxa de Referència en Biotecnologia" (XRB). We thank Mónica Estupiñán for helpful assessment in phylogenetic map construction and the Centres Científics i Tecnològics of the University of Barcelona for technical support in GC/MS and sequencing. B. Infanzón acknowledges a doctoral fellowship from MAEC-AECID.

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CHAPTER II: Improving *Rhodococcus* sp. CR-53 lipase LipR by protein engineering

Explorando el inusual *oxyanion hole* de tipo Y de la lipasa LipR de *Rhodococcus* sp mediante evolución racional

Belén Infanzón, Pablo Sotelo, Josefina Martínez, Pilar Diaz

La lipasa LipR, aislada de *Rhodococcus* sp CR-53, fue el primer miembro caracterizado de la familia X de lipasas bacterianas. Interesantemente, LipR mostró cierta similitud con α/β-hydrolasas de la superfamilia de la lipasas similares a la lipasa A de *Candida antartica* (CalA) (abH38), teniendo un *oxyanion hole* de tipo Y, nunca encontrado anteriormente entre lipasas de origen bacteriano. Con el fin de entender el significado de este motivo inusual de tipo Y, y para mejorar la actividad de LipR sobre sustratos de cadena larga, se realizaron dos estrategias de modificación del *oxyanion hole* basadas en mutagénesis sitio dirigida o por saturación. Siendo GGG(X) y G(X) los motivos más comunes en el *oxyanion hole* de lipasas bacterianas, se diseñó una pequeña librería de mutantes para convertir el *oxyanion hole* de LipR (YDS) en uno de los encontrados en bacterias. Sin embargo, se ha perdido la actividad completamente en todos los mutantes obtenidos, comprobándose que es requerido el *oxyanion hole* de tipo Y en LipR para mantenerse de forma activa. Una segunda aproximación fue realizada para modificar los dos principales residuos Tyr^{110} y Asp^{111} del oxyanion hole, equivalentes a los previamente descrito en CalA como los aminoácidos más relevantes responsables de en la estabilización del intermedio tetraédrico. Se prepararon dos librerías por mutagénesis de saturación, una para cada residuo (Tyr^{110} y Asp^{111}), y fue ensayada la actividad sobre sustratos con cadenas de diferente longitud en las variantes resultantes. No se logró obtener ninguna variante funcional de LipR cuando el residuo Tyr^{110} fue sustituido por otros aminoácidos, indicando que tiene un papel crucial para la catálisis. Sin embargo, entre las variantes de Asp^{111} obtenidas, LipR D111G produjo una enzima funcional. Interesantemente, esta variante LipR-YGS mostró menos actividad que la lipasa salvaje LipR sobre sustratos con cadena de corta o media longitud, pero demostró un aumento de actividad de 5,6 veces sobre sustratos de cadena larga. El análisis del modelo 3D y el estudio *in silico* de *docking* de esta variante obtenida demostró que la sustitución de Asp por Gly produce un túnel de entrada más amplio que permitiría una mejor acomodación de sustratos más grandes.

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Manuscript Draft

Manuscript Number:

Title: Exploring the unusual Y-type oxyanion hole of Rhodococcus sp CR53 lipase LipR through rational evolution

Article Type: Research Paper

Keywords: Rhodococcus, lipase LipR, FamilyX, Oxyanion hole, Enzyme engineering

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Manuscript Region of Origin: SPAIN

Abstract: Rhodococcus sp CR-53 LipR was the first characterized member of bacterial lipase family X. Interestingly, LipR displays some similarity with α/β-hydrolases of the C. antartica lipase A (CAL-A)-like superfamily (abH38), bearing a Y-type oxyanion hole, never found before among bacterial lipases. In order to understand the significance of its unusual Y-type oxyanion hole, and to improve LipR activity on long-chain substrates, we performed two modification strategies of the oxyanion hole based on site directed or saturation mutagenesis. Being GGG(X) and G(X) the most common motifs of bacterial lipase oxyanion holes, we designed a small library of mutants to convert LipR Y-type oxyanion hole (YDS) into one closer to those found in bacteria. However, activity was completely lost in all mutants obtained, indicating that the Y-type oxyanion hole of LipR is required for activity. A second approach was addressed to modify the two main oxyanion hole residues Tyr110 and Asp111, previously described for CAL-A as the most relevant amino acids involved in stabilization of the tetrahedral intermediate. A saturation mutagenesis library was prepared for each residue (Tyr110 and Asp111), and activity of the resulting variants was assayed on different chain length substrates. No functional LipR variants could be obtained when Tyr110 was replaced by any other amino acids, indicating that this is a crucial residue for catalysis. However, among the Asp111 variants obtained, LipR D111G produced a functional enzyme. Interestingly, this LipR-YGS variant showed less activity than wild type LipR on short- or mid- chain substrates but displayed a 5.6-fold increased activity on long chain length substrates, as desired. Analysis of the 3D model and in silico docking studies of this enzyme variant demonstrated that substitution of Asp by a Gly produces a wider entrance tunnel that would allow for a better and tight accommodation of larger substrates.

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Cover Letter

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2nd May, 2017

Enzyme and Microbial Technology
Editor in Chief
Dear Sir,

Please find attached the manuscript entitled "**Exploring the unusual Y-type oxyanion hole of *Rhodococcus* sp CR53 lipase LipR through rational evolution**", signed by Belén Infanzón, Pablo H. Sotelo, Josefina Martínez and Pilar Diaz**,** to be submitted for publication in *Enzyme and Microbial Technology*. All authors have read and approved the manuscript, which has not previously been submitted to the journal.

The manuscript describes two enzyme engineering approaches to modify the rare, fungal-like oxyanion hole of the bacterial lipase LipR from *Rhodococcus* sp. CR53. From the results obtained after conversion of the Y-type oxyanion hole into the most common bacterial oxyanion hole motifs, we conclude that presence of a Tyr in the oxyanion hole is a strict requirement for LipR activity. Iterative saturation mutagenesis of the two representative amino acids of LipR oxyanion hole (Tyr^{110} and Asp^{111}) confirmed that $tyrosine^{110}$ is a crucial residue for catalysis, probably by stabilizing the tetrahedral intermediate. However, substitution of Asp^{111} by a Gly produced a functional enzyme with 5.6-fold increased activity on long chain length substrates. This was confirmed by *in silico* docking studies, where we observed that Gly produces a wider entrance tunnel that could allow for a better and tight accommodation of larger substrates.

Yours sincerely,

Pilar Diaz, PhD

Exploring the unusual Y-type oxyanion hole of *Rhodococcus* sp CR53 lipase LipR through rational evolution

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ABSTRACT

Rhodococcus sp CR-53 LipR was the first characterized member of bacterial lipase family X. Interestingly, LipR displays some similarity with α/β-hydrolases of the *C. antartica* lipase A (CAL-A)-like superfamily (abH38), bearing a Y-type oxyanion hole, never found before among bacterial lipases. In order to understand the significance of its unusual Y-type oxyanion hole, and to improve LipR activity on long-chain substrates, we performed two modification strategies of the oxyanion hole based on site directed or saturation mutagenesis. Being GGG(X) and G(X) the most common motifs of bacterial lipase oxyanion holes, we designed a small library of mutants to convert LipR Y-type oxyanion hole (YDS) into one closer to those found in bacteria. However, activity was completely lost in all mutants obtained, indicating that the Y-type oxyanion hole of LipR is required for activity. A second approach was addressed to modify the two main oxyanion hole residues Tyr^{110} and Asp^{111}, previously described for CAL-A as the most relevant amino acids involved in stabilization of the tetrahedral intermediate. A saturation mutagenesis library was prepared for each residue (Tyr^{110} and Asp^{111}), and activity of the resulting variants was assayed on different chain length substrates. No functional LipR variants could be obtained when Tyr^{110} was replaced by any other amino acids, indicating that this is a crucial residue for catalysis. However, among the Asp^{111} variants obtained, LipR D111G produced a functional enzyme. Interestingly, this LipR-YGS variant showed less activity than wild type LipR on short- or mid- chain substrates but displayed a 5.6-fold increased activity on long chain length substrates, as desired. Analysis of the 3D model and *in silico* docking studies of this enzyme variant demonstrated that substitution of Asp by a Gly produces a wider entrance tunnel that would allow for a better and tight accommodation of larger substrates.

Keywords: *Rhodococcus*, lipase LipR, FamilyX, Oxyanion hole, Enzyme engineering

1. INTRODUCTION

Lipases are carboxylic ester hydrolases that catalyse the hydrolysis or synthesis of ester bonds, mostly without cofactor requirement. In general, they are robust enzymes, capable of showing activity under a wide range of conditions and in all kind of solvents. Lipases are widely used biocatalysts, applied in oil and fat transformations, detergent industry, leather, textile, food/feed production, in fine chemistry, synthesis of pharmaceuticals, or in production of enantiopure compounds [1–6]. However, full biotechnological exploitation of lipases is still hampered by their mild activity, low stability and high costs. These drawbacks can be overcome by random directed evolution or by rational design approaches to obtain improved biocatalysts with the desired properties [4–9].

Lipases belong to the large family of α/β-fold hydrolases, where the catalytic domain is a central β-sheet of several strands interconnected by α-helices, with strand 2 running antiparallel to the rest. This structure positions the catalytic triad **S**-D(E)-H and the oxyanion hole on loops connecting the β-strands and α-helices [10–12]. The catalytic serine is embedded in the conserved pentapeptide G(A)-X-**S**-X-G, whereas the oxyanion hole can show variability among lipase families [13,14]. After extensive sequence–structure–function studies, the oxyanion hole was identified as a relevant site for lipase activity, being the structure that stabilizes the tetrahedral intermediate occurring during the transition states of catalysis [4–6,15]. It typically consists of backbone amides or positively charged residues, formed in most cases by hydrogen bonds from two main chain amides that bind the carbonyl oxygen and stabilize the tetrahedral intermediates [12,16–18]. In some cases like in *C. antarctica* lipase B (CAL-B), the side chain of the oxyanion hole residues (Thr^{40}) can provide an extra hydrogen bond donor, whereas in other cases the main chain N−H from the oxyanion hole is replaced with a side chain interaction like in *C. antarctica* lipase A (CAL-A), that uses the side chain of Asp^{95} [12,19–22]. Depending on the amino acids involved in the oxyanion hole, lipases are classified into three classes: GGG(X)-, G(X)-, and Y-class [12,16,22]. According to the general classification, the G(X) class consists of 11 superfamilies and 22 homologous families, which contain 376 protein entries with 600 sequences and 125 chains of known structure. This class comprises mainly bacterial and fungal lipases, eukaryotic lipases, cutinases, phospholipases and non-heme peroxidases. The GGG(X) class, consists of 5 superfamilies and 16 homologous families including 430 protein entries with 767 sequences

and 73 chains of known structure. It includes bacterial esterases, eukaryotic carboxylesterases, bile-salt activated lipases, juvenile hormone esterases, hormone sensitive lipases, acetylcholinesterases, and thioesterases, as well as gliotactin, glutactin, neurotactin, neuroligin, and thyroglobulin [23]. The "*Candida antarctica* lipase A like" superfamily, classified as Y-class, contains one crystal structure, CAL-A, and 39 sequences assigned to 32 proteins, being all of them from organisms belonging to the kingdom Fungi. 12 of these proteins are classified as either lipases or esterases in GenBank, while the other 20 are only described as putative or hypothetical proteins [20]. Structure-based studies of CAL-A identified and proposed Tyr^{93} as the oxyanion hole-forming residue [17,20], which was assigned to the Y-type class. Moreover, the role of Asp^{95} in stabilisation of CAL-A oxyanion hole through its side chain was also reported, showing that no mutants involving Asp^{95} were functional [19]. As expected for an α/β-fold hydrolase, both amino acids of the oxyanion hole, Tyr^{93} and Asp^{95}, are located in a loop in close proximity to the catalytic serine [21].

Lipase R (LipR) from *Rhodococcus* sp. CR-53 (CECT 7058) was purified and fully characterized in a previous work, showing preference for mid-chain triacylglycerides [24]. The enzyme was described as a new family X member according to the criteria established by Arpigny and Jaeger [25]. LipR was reported as a stable lipase that efficiently hydrolyses *p*-NP-decanoate, with maximum activity at 40 °C and pH 7, but unable to act on longer chain-length substrates [24]. The main trait of LipR was the finding of an uncommon Y-type oxyanion hole, a feature never described before in bacterial lipases [24,26]. This Y-type class of oxyanion hole is shared by all members of *C. antartica* lipase A-like superfamily (abH38), which includes *C. albicans* lipase-like family (abH38.2) [20,27]. It was confirmed that LipR oxyanion hole displays the consensus motif Tyr-Asp-Ser-Leu, having indeed the conserved tyrosine (Tyr^{110}) of the CAL-A lipase-like superfamily surrounding the active-site region and located in a loop near the catalytic serine [24,27].

Many approaches have been applied to identify the residues involved in activity of microbial lipases and to improve properties like thermal stability, catalytic efficiency, substrate specificity or chiral selectivity [4–6,22,28–30]. A smart way to engineer α/β-fold hydrolases is the use of available information of related proteins, in particular that concerning the active site and the oxyanion hole. Knowledge of previously described oxyanion hole motifs for the different lipase families and their specific mode of action, can help to obtain better lipase variants with improved stability or modified substrate specificity through a rational approach

[24,31,32]. Thus, protein engineering was used here to improve the catalytic efficiency of LipR versus longer chain-length substrates [12,22,33]. A rational approach to convert the fungal Y-type oxyanion hole into a more common bacterial GGG(X)-type, and the modification of specific residues to generate an enzyme variant with improved properties, including a shift towards longer chain-length fatty acid acyglycerol esters are described. During this process, additional information was acquired to unveil the role in activity and function of the residues involved in the unusual oxyanion hole of LipR.

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and growth conditions

Escherichia coli Origami™ was routinely cultured overnight at 37 °C in Luria Bertani (LB) broth or on LB agar plates and was used as the host strain for expression of *Rhodococcus* sp. CR-53 (CECT 7058) lipase LipR. Plasmid pGaston [34] was used as expression vector, and the previously obtained construct pGaston-LipR [24] was used as the wild type enzyme. Strains were grown at 37 °C in LB broth supplemented with ampicillin (100μg mL^{-1}). Lipase production was induced upon addition of 2% rhamnose (Sigma) and cultivation was continued overnight.

2.2 DNA manipulation

DNA manipulations were carried out according to Sambrook and Russell [35]. Plasmid DNA was purified using commercial kits (Thermoscientific). Restriction nucleases (Thermoscientific) and thermostable polymerases *taq* and *pfu* (Biotools, Spain) were used according to the manufacturer's instructions. PCR amplifications were performed in a GeneAMP PCR system 2400 (Perkin Elmer) using different cycling periods, seldom including a hot-start procedure [35]. Sequencing was performed using the ABI PRISM® BigDye® Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems) and the analytical system CEQTM 8000 (Beckman-Coulter) available at the Serveis Científics i Tecnològics of the University of Barcelona. DNA samples were analysed by agarose gel electrophoresis and stained with GelRed™ 0.27 % (v/v). Nucleic acid concentration and purity were measured using a Spectrophotometer ND-100 NanoDrop®.

2.3 Production of LipR variants

2.3.1 Site-directed mutagenesis. Five LipR variants with mutations at residues 110, 111, and 112 were obtained using the QuikChange® site-directed mutagenesis strategy after designing the appropriate primers (Table 1), and confirmed by sequencing. Primers were purchased from Sigma-Aldrich. The subsequent randomized PCR products were incubated for 3h at 37°C with *Dpn*I endonuclease in order to digest the template DNA, and the resulting libraries were transformed into *E. coli* Origami™.

2.3.2 Site-specific saturation mutagenesis. Two gene libraries encoding all possible amino acids at positions Y^{110} and D^{111} of LipR were constructed by replacing the target codon with

an NNK degenerate codon (N being A, T, G, or C, and K being G or T) by the QuikChange® site-directed mutagenesis strategy, using the appropriate primers (Table 1). 192 colonies of each library transformed into *E. coli* Origami^{TM} were isolated and inoculated on 96-well plates with 1ml LB supplemented with ampicillin (100μg mL^{-1}) per well, including the clone carrying the wild type LipR and *E. coli* Origami strain as positive and negative controls, respectively. Selected clones were cultivated in 5ml LB ampicillin for plasmid purification and sequencing.

Table 1: List of primers designed for LipR rational design mutagenesis and libraries of saturation mutagenesis (NNK degeneracy; [8])

Mutant	Primer	Sequence (5´- 3´)
LipR-GGG	LipRQC1 Fw	TCGGCC**GGCGGTGGA**CTGAACCCGGAAGAC
	LipRQC1 Rv	GGTTCAG**TCCACCGCC**GGCCGACTGGAACG
LipR-GGS	LipRQC2 Fw	TCGGCC **GGCGGT**TCA CTGAACCCGGAAGAC
	LipRQC2 Rv	GTTCAGTGAACCGCCGGCCGACTGGAACG
LipR-GGA	LipRQC3 Fw	TCGGCC **GGCGGTGCA** CTGAACCCGGAAGAC
	LipRQC3 Rv	GGTTCAG**TGCACCGCC**GGCCGACTGGAACG
LipR-AGG	LipRQC4 Fw	TCGGCC **GCCGGTGGA** CTGAACCCGGAAGAC
	LipRQC4 Rv	GGTTCAG**TCCACCGGC**GGCCGACTGGAACG
LipR-AGA	LipRQC5 Fw	TCGGCC **GCCGGTGCA** CTGAACCCGGAAGAC
	LipRQC5 Rv	GGTTCAG**TGCACCGGC**GGCCGACTGGAACG
LipR Y/NNK library	LipRY-NNK Fw	TCGGCC **NNKGATTCA** CTGAACCCGGAAGAC
	LipRY-NNK Rv	GGTTCAG**TGAATCMNN**GGCCGACTGGAACG
LipR D/NNK library	LipRD-NNK Fw	TCGGCC **TACNNKTCA** CTGAACCCGGAAGAC
	LipRD-NNK Rv	GGTTCAG**TGAMNNGTA**GGCCGACTGGAACG

2.4 Activity assays

Activity assays were carried out using cell-free enzyme solutions, obtained from disrupted cultured cells produced by sonication on ice for 1 min at 80% pulse intensity with an amplitude of 10 microns for five times in a sonicator SoniPrep150 (Sanyo-Gallen-Kamp, UK), as previously described [36,37].

Activity was determined by measuring the release of *para*-nitrophenol (*p*-NP) from *p*-NP-

derivative substrates, as previously reported [38]. Alternatively, activity was analysed by measuring the release of methyl-umbelliferone (MUF) from MUF-derivative fatty acid substrates (Sigma), using a spectrofluorometer (Variant, Spain), as previously described [39]. One unit of activity was defined as the amount of enzyme that released 1 mol of *p*-NP or MUF per minute under the assay conditions used. SDS-PAGE coupled to zymogram was used to detect lipase activity after protein separation, as previously described [40]. In order to detect hydrolysis activity on gels, MUF-butyrate was used as a substrate since better results were obtained than using other MUF-derivatives [40].

2.5 Sequence and structure analysis

Multiple sequence alignment was performed using ClustalW [41]. Homology models for LipR were built using the high-resolution crystal structure reported for CAL-A (pdb: *2VEO* and pdb: *3GUU*) as a template. Swiss Model Server and YASARA software (version 9.6.28, www.yasara.org) with default settings were used for model construction [42]. UCSF Chimera [43] and Pymol software (Schrödinger, LLC (http://www.pymol.org) molecular graphics systems were used for visualization of the 3D structures. Docking with *p*-NP-palmitate, *p*-NP-octanoate and *p*-NP-butyrate was performed using Patch Dock tool [44]. PDBs for the substrates were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/).

3. RESULTS

3.1 The unusual Y-class oxyanion hole of LipR

The most common oxyanion hole motifs found in bacteria are GGG(X)- and G(X)-, whereas the Y-type oxyanion hole has only been found in family X bacterial lipases, suggesting a close origin of these lipases and fungal CAL-A lipase-like superfamily, with subsequent evolutionary divergence [4,24,45]. Presence of such an uncommon motif in a bacterial lipase could be the cause for the lack of activity shown by LipR on long chain substrates [24]. Therefore, changing the atypical oxyanion hole of LipR was our goal towards improving its catalytic activity and to understand the role of the Y-type oxyanion hole residues. Two strategies were assayed for this purpose: site directed mutagenesis of selected residues of the oxyanion hole [4,28], and an iterative saturation mutagenesis approach [4,8,46] to randomly produce oxyanion hole-enzyme variants with improved properties or increased activity towards longer chain substrates.

Multiple sequence alignment of LipR with lipases of *C. antartica* lipase A-like superfamily (Lipase Engineering Database; LED) [16,20,23] confirmed that Tyr, which is the first oxyanion hole residue of *C. antartica* lipase A-like superfamily, is also found in one of the conserved blocks of family X bacterial lipases, as well as the second acidic residue Asp or Glu [24]. Despite the lack of a crystal structure or a high sequence identity with other templates, the 3D model constructed for LipR [24] using the *2VEO* template of *C. antarctica* CAL-A (29% sequence identity) was used here to design the libraries of mutants addressed at the residues of the oxyanion hole.

3.2 From Y-type to GGG(X)-class oxyanion hole

Site-directed mutagenesis was used to modify the fungal-like oxyanion hole residues of LipR by those more typical of bacteria, involving the replacement of Tyr and Asp by a GGG(X) bacterial oxyanion hole motif [4–6,46]. Therefore, five variants were constructed by site-directed mutagenesis of the Y-type oxyanion-hole YDS (Y^{110}, D^{111}, S^{112}) of LipR, whose residues were converted into the five more common oxyanion-hole motifs found in bacterial lipases: LipR-GGG, LipR-GGS, LipR-GGA, LipR-AGG and LipR-AGA. All these LipR variants were cloned in *E. coli* and expressed, as well as the wild type LipR, under optimum culture and induction conditions [4]. A 3D model structure was obtained for each variant to confirm that no significant changes in folding occurred (not shown). However, activity assays

and the SDS-PAGE plus zymogram analysis [40] performed to verify the hydrolytic activity of the cell extracts obtained from each recombinant clone resulted in a great loss of activity (Figure 1), with the highest activity values obtained for variants LipR-GGG and Lip-AGA (0.24 and 1.64 $mU \cdot ml^{-1}$, respectively) when measured on *p*-NP-decanoate, while activity of wild type LipR on this substrate was 560 $mU \cdot ml^{-1}$. These results indicate that although being a bacterial enzyme, LipR requires the Y-class fungal-like oxyanion hole sequence to stabilize the intermediates of the transition state, whereas the consensus motifs found in most bacterial lipases are not functional.

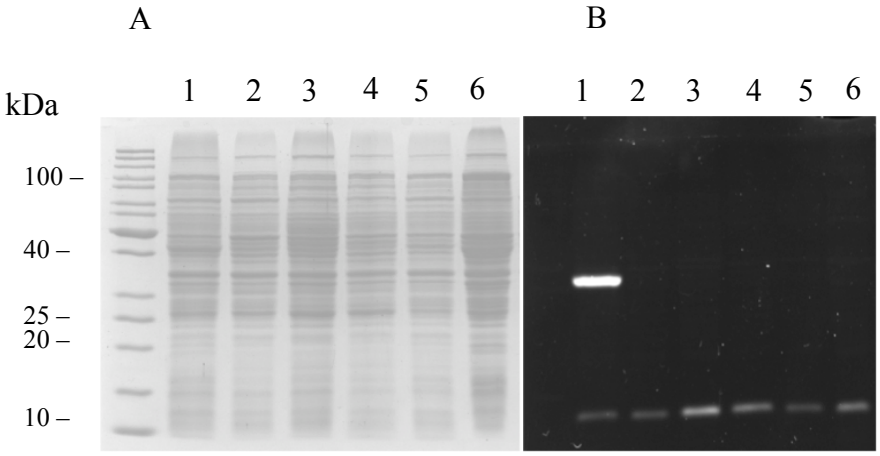

Figure 1: SDS-PAGE and zymogram analysis of LipR variants. (A) Coomassie-stained SDS-PAGE of cell extracts from recombinant clones Origami/pGastonLipR (1) wild type LipR, (2) LipR-GGG, (3) LipR-GGS, (4) LipR-GGA, (5) LipR-AGG, (6) LipR-AGA. (B) Zymogram analysis on *muf*-butyrate of the same gel shown in (A). Gel was loaded with the same protein amount [47]. A band under ca. 20 kDa visible in all lanes of the zymogram corresponds to a known intrinsic *E. coli* lipolytic enzyme.

3.3 Saturation mutagenesis of Y-class critical residues

In order to obtain new oxyanion hole variants of LipR, a saturation mutagenesis strategy was addressed [46,48] using a Quikchange® protocol to construct two NNK libraries for substitution of each Y-type fundamental residue (Y^{110}, D^{111}) of LipR by all possible codons [8,9]. Among the recombinant clones obtained, colonies were recovered for plasmid extraction and sequencing. The selected mutants (Table 2) plus wild type LipR were overexpressed in *E. coli* Origami at 37 °C with 2% rhamnose for expression induction and activity determination. The hydrolytic activity of crude cell extracts was determined using *p*-NP-decanoate, the substrate of preference for LipR [24]. Whereas wild type LipR showed normal levels of activity on this substrate, a dramatic activity loss was observed for all LipR

variants obtained. Similar results were obtained when activity was tested on MUF-butyrate or MUF-heptanoate (not shown). It is important to note that all mutants assayed (Table 2) displayed representative changes regarding the different amino acid properties, which were both, polar or hydrophobic, and covering all size ranges. However, despite these variations, no effective gains in activity on short or medium chain-length substrates were achieved.

Table 2: Mutants obtained from the libraries designed with NNK codon degeneracy [8]

Y^{110}-NNK library		**D^{111}-NNK library**	
Mutant	**Activity *muf*-oleate**	**Mutant**	**Activity *muf*-oleate**
*Tyr / Ser (**S**DS; tct)*	-	*Asp / Thr (Y**T**S; acg)*	±
*Tyr / Arg (**R**DS; cgt)*	-	*Asp / Val (Y**V**S; gtt)*	-
*Tyr / Pro (**P**DS; cct)*	-	*Asp / Pro (Y**P**S; cct)*	-
*Tyr / Ala (**A**DS; gct)*	-	*Asp / Gly (Y**G**S; ggg)*	+
*Tyr / His (**H**DS; cat)*	-	*Asp / His (Y**H**S; cat)*	-
*Tyr / Cys (**C**DS; tgt)*	-		
*Tyr / Glu (**D**DS; gag)*	-		
*Tyr / Phe (**F**DS; ttt)*	-		

Nevertheless, when activity of the mutants resulting from the directed evolution libraries was measured using MUF-oleate as a substrate, a significant increase of activity was observed for mutant LipR-D111G in comparison with the very low activity shown by wild type LipR when assayed on this long-chain substrate. After these preliminary results, wild type LipR (Y**D**S) and the two clones LipR-Y**G**S and LipR-Y**T**S still showing some, although low activity on *p*NP-decanoate, were chosen for further activity assays on different substrates in a larger scale production (Figure 2). An enzyme variant (LipR-Y**V**S), showing complete loss of activity was also assayed as a negative control. Again, when activity was measured on MUF-oleate, mutant LipR-Y**G**S displayed higher activity than the wild-type enzyme, showing an interesting 5.6-fold increase of activity over native LipR. Nevertheless, this enzyme variant revealed a loss of activity on MUF-butyrate and MUF-heptanoate (4.8% and 11.8%, respectively of the activity observed for wild type LipR). Therefore, substitution of the oxyanion hole Asp by a Gly generates a LipR variant showing a shift in substrate specificity, accepting longer chain fatty acid substrates but loosing activity on shorter substrates.

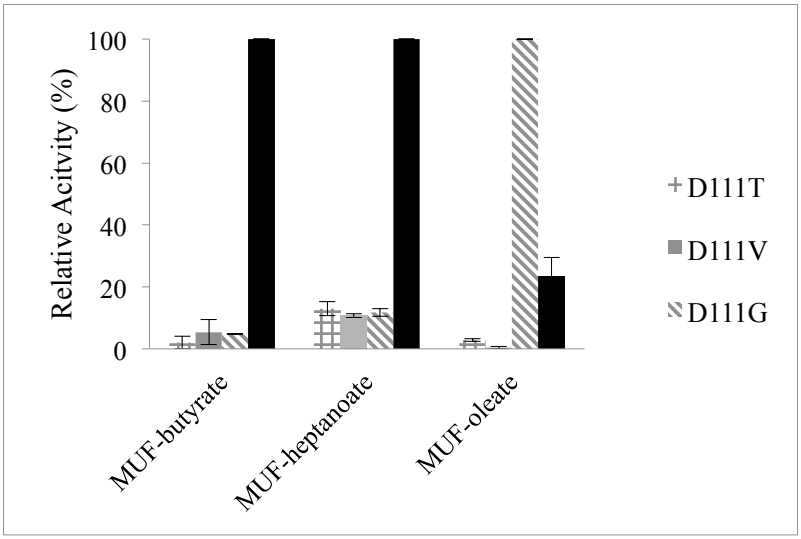

Figure 2: Activity profile of LipR variants assayed on different chain-length substrates: MUF-butyrate, MUF-hepatonate and MUF-oleate. Values are the mean of 6 replica assays.

3.4 Model structure of variant LipR-YGS

A 3D homology model of variant LipR-YGS was constructed, using the high-resolution crystal structures reported for CAL-A (PDB ID: *2VEO* and PDB ID: *3GUU*) as a template. The model obtained (100% confidence, 86% coverage) confirmed the presence of the catalytic triad Ser^{212}, embedded in the superfamily consensus sequence GYSGG, Asp^{372} and His^{404}, and allowed identification of the amino acids proposed as components of the oxyanion hole: Tyr^{110}-Gly^{111}-Ser^{112}.

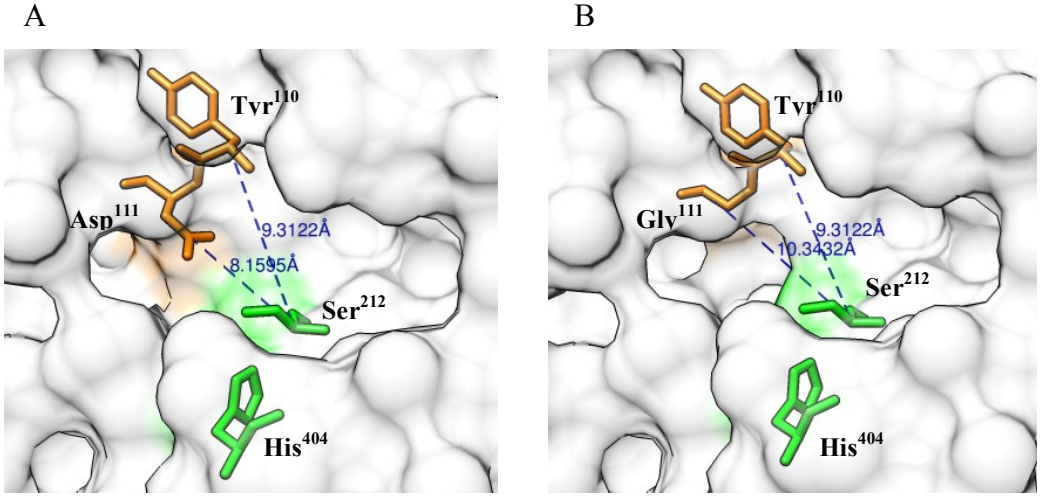

Figure 3: Homology model obtained for (A) wild type LipR, (B) LipR D111G variant. Detail of the amino acids of the oxyanion hole (orange) and the conserved amino acids of the catalytic triad (green). The 3D model was generated with the Swiss-Model server and obtained using pdb *2VEO*; UCSF

Chimera and PyMol were used as molecular visualization systems and to calculate the distances between amino acids (blue).

The model (Figure 3) permitted to appreciate differences in the distances between the catalytic residues and the amino acids of interest, both in wild-type and variant LipR-YGS. A detailed analysis of the two model structures shows that the substitution occurred in LipR-YGS (variant D111G) changes the distances between these residues and the catalytic serine, showing a shorter distance when Asp is part of the oxyanion hole and probably also causing more interactions during the transition state (Figure 3).

3.5 *In silico* docking analysis

To further understand the role of each oxyanion hole residue, a molecular docking within LipR and LipR-Y**G**S model structures was performed, using *p*-NP-butyrate, *p*-NP-octanoate and *p*-NP-palmitate, as representative substrates for short-, mid- and long-chain length fatty acids (Figure 4). It is important to mention here that several binding variations were acquired for each interaction, thus the models chosen for analysis were selected taking into consideration two criteria: (*i*) the substrate should be correctly positioned inside the pocket constituted by the catalytic triad and the oxyanion hole motif, and (*ii*) a feasible interaction between the catalytic serine (Ser^{212}) and the tested substrate should occur. From an average of ten docking models generated for each substrate, only those covering these criteria were selected and used for further analysis.

In all docking models considered, the role of Ser^{212} as the catalytic residue acting on the different substrates was clearly demonstrated. As can be observed in Figure 4, variant LipR-YGS would generate enough space in the vicinity of the active site to welcome *p*-NP-palmitate. Remarkably, the docking positioning with *p*NP-butyrate appears less favourable in both, the wild type and mutant models. In fact, the interaction cavity occurring in LipR-YGS might be too large to tightly accommodate shorter substrates, which could be easily released without being catalysed. These observations are in agreement with the experimental results obtained, showing poor or no activity of mutant LipR-YGS on short- mid- substrates.

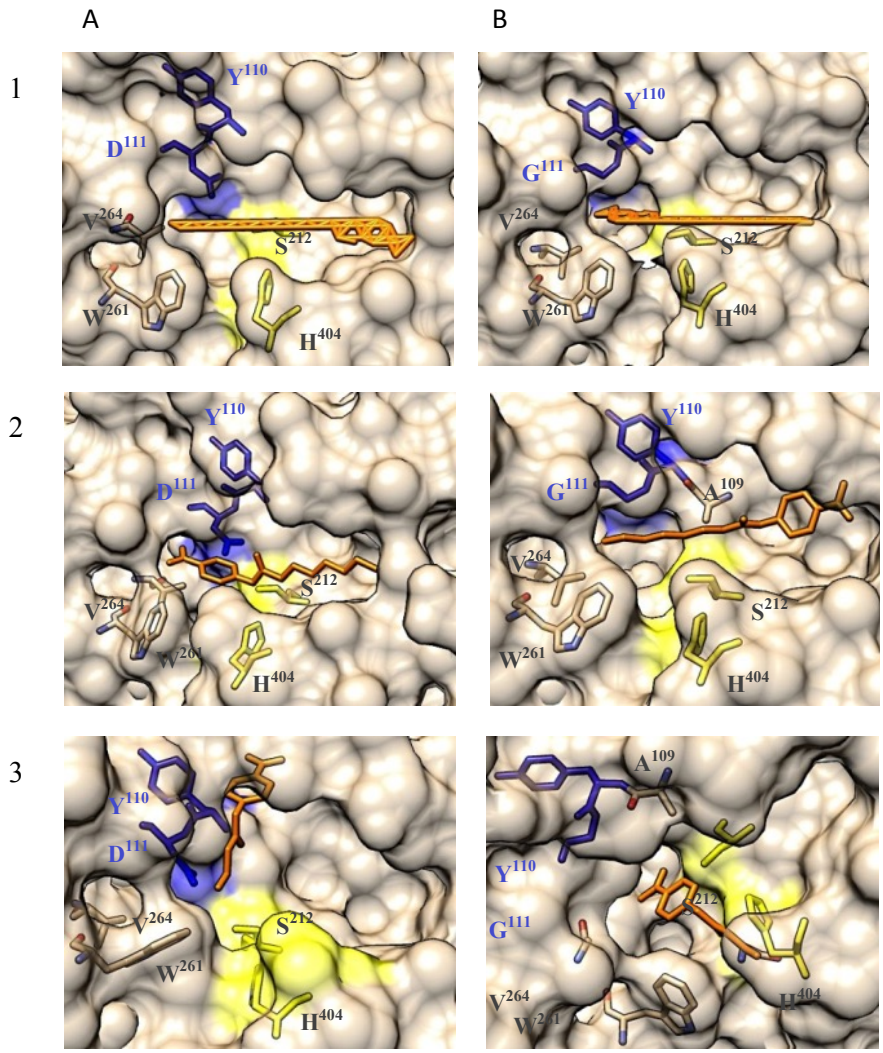

Figure 4: Docking analysis of (A) wild type LipR and (B) LipR-YGS mutant, representing the binding mode of *p*-NP-palmitate (1), *p*-NP-octanoate (2) and *p*-NP-butyrate (3) (substrates in orange) inside the cavity. Detail of the amino acids of the oxyanion hole (green) and the conserved amino acids of the catalytic triad (yellow) is shown. Putative Trp^{261}, Val^{264} and Ala^{109} extra amino acids involved are represented in brown.

Based on the *in silico* approach, residues Trp^{261} and Val^{264} caught our attention because of their proximity to LipR oxyanion hole and the possibility of producing additional interactions (Figure 4). These amino acids could even be additional residues of LipR oxyanion hole, not found in CAL-A. Further enzyme variants would be required to understand the role of these additional residues during the catalysis process. Moreover, Ala^{109} appears in the LipR-YGS docking models as a protruding residue that could interfere with short- mid- chain length substrate accommodation (Figure 4: 2B, 3B). Additional mutations of the surrounding

residues Trp^{261}, Val^{264} and Ala^{109} would therefore be helpful to get a wider knowledge of all the amino acids involved in the stabilization of the transition state intermediates and substrate accommodation to completely elucidate the oxyanion hole mode of action of LipR.

4. DISCUSSION

The most common oxyanion hole motifs found in bacteria are GGG(X)- and G(X)-, whereas the Y-type oxyanion hole has only been found up to date in *Rhodococcus* sp. CR-53 LipR, which constitutes the first characterised member of the new bacterial lipase family X according to the nomenclature established by Arpigny and Jaeger [25]. This finding suggests a common origin of family X lipases and fungal CAL-A lipase-like superfamily, with subsequent evolutionary divergence [4,24,45].

By changing the atypical Y-type oxyanion hole of LipR we intended to increase activity on long chain-length substrates and to get knowledge on the influence of defined amino acids in its performance. According to previously reported data, in some families of bacterial α/β-fold hydrolases like in *Bacillus*/*Paenibacillus* esterases capable to convert tertiary alcohols [4,5,49], the three glycines of the GGG(X)-motif in the consensus fold are highly conserved, although substitution of the first glycine by an alanine seldom provided also good results [4,5,49]. The glycine at the third position is the most variable, with an abundance of nearly 64 %, whereas those having an alanine represent 21 %, 10 % contain a serine, and less than 5 % have other amino acids as the third residue [4]. On the other hand, more than 95 % known esterase sequences bear a glycine at the central position of the oxyanion hole [20], suggesting that this residue plays an important role in biocatalysis. Nevertheless, earlier reports on *Yarrowia lipolytica* lipase Lip12 showed that substitution of the second amino acid of the oxyanion hole by those found in the other seven lipases of the same yeast clearly improved the enzyme behaviour and the catalytic efficiency on long chain substrates [50].

Taking this information into consideration, two mutagenesis strategies were addressed: site directed mutagenesis of the residues of the oxyanion hole [4,28,46], and iterative saturation mutagenesis [8] to randomly produce oxyanion hole-enzyme variants with improved properties. Despite the lack of a high sequence identity with other templates, knowledge of the structure-function of other bacterial lipases was enough to identify hot-point motifs to design a rational approach for modification of LipR oxyanion hole.

Mutants LipR-GGG, LipR-GGS, LipR-GGA, LipR-AGG and LipR-AGA, obtained by site directed mutagenesis for conversion of LipR Y-type oxyanion hole into a bacterial GGG(X) motif, did not display significant activity, indicating that the residues of the Y-type oxyanion hole are a requisite for LipR activity. On the other hand, saturation mutagenesis of residues Y^{110} and D^{111} produced enzyme variants with very low or no activity on short or medium

chain-length substrates. These results are in agreement with previous saturation mutagenesis assays performed on CAL-A, where the important role of residue D^{95} was shown: no functional protein variants could be obtained when this amino acid was changed [19]. Moreover, the importance of Y^{110}, included in the highly conserved motif of CAL-A superfamily oxyanion hole, was unambiguously evidenced, as no active LipR variants affecting this residue could be obtained. Nevertheless, site directed mutagenesis of *Candida parapsilosis* lipase, based on a structural model generated using CAL-A structure as a template, produced a functional enzyme variant ─D90N─ although it displayed loss of activity on ethyl oleate. Thus, the role of residue D^{90} in the oxyanion hole of this lipase still remains unclear [51] but it seems clear that Tyr is an essential amino acid for Y-type oxyanion hole lipases, including LipR.

For LipR, when activity of the mutants obtained by saturation mutagenesis was measured on MUF-oleate, mutant LipR-Y**G**S (D111G) displayed higher activity than the wild-type enzyme, showing a 5.6-fold increase of activity over native LipR on this substrate. Nevertheless, activity of variant LipR-Y**G**S on shorter substrates was clearly reduced. Therefore, substitution of Asp^{111} by a glycine induces a change in substrate specificity, probably due to conformational changes in the substrate entrance tunnel, thus allowing accommodation of larger substrates but preventing catalysis of shorter acyl-glycerides. In fact, CAL-A was described as a true lipase because it displayed a much higher conversion rate towards triglycerides than towards hydrophilic esters [52], and *Kurtzmanomyces* lipase, with a high sequence identity with CAL-A and the same YED oxyanion hole motif, was described to hydrolyse long-chain fatty acids more efficiently than short-chain substrates [53]. The substitution D111G occurred in mutant LipR-Y**G**S is therefore a promising result for application of such a LipR variant in triglyceride esterification/transesterification reactions [54,55] or in bulk reactions involving long-chain fatty acid esters.

In order to assess the hypothesised conformational changes, a 3D homology model of variant LipR-YGS was constructed and all significant motifs, including the active site and oxyanion hole, were identified. The substitution occurred in LipR-YGS produces changes in the possible contacts between the oxyanion hole residues and the catalytic serine, and generates a longer distance between these amino acids (Figure 3). The possibility of additional interactions appearing when Asp^{111} is present could limit a favourable interaction between the transition state intermediate and Tyr^{110}, thus preventing catalysis of bulky substrates. On the

contrary, when Gly is located at this position, a wider pocket is created, increasing the size of the substrate entrance tunnel in LipR-Y**G**S (Figure 3), a fact that would allow stabilization and catalysis of larger substrates. In this case, the existing interactions would not be enough to stabilize a short- or mid- chain length substrate, being only Tyr^{110} the main responsible residue for stabilization of the tetrahedral intermediate. These considerations contribute to justify the shift in substrate specificity of variant LipR-Y**G**S towards longer chain substrates, and reveals that residues at position 111 have a significant impact on LipR substrate specificity. Whereas Tyr^{110} is a crucial residue, required for activity during stabilization of the tetrahedral intermediate in Y-type oxyanion hole lipases, Asp^{111} would be more involved in substrate accommodation and tightening the enzyme-substrate interactions. The *in silico* docking studies performed confirmed this hypothesis (Figure 4), clearly showing that the glycine at position 111 produces conformational changes in the substrate-binding region that can justify the shift in substrate length specificity observed experimentally. In addition, lack of interaction of butyrate with Ser^{212} in wild type LipR (Figure 4: 3A) suggests that short substrates cannot properly interact with the catalytic residue, in agreement with the experimental results obtained here and previously [24].

5. CONCLUSIONS

We have proved here the importance of LipR Y-type oxyanion hole after changing its sequence from a fungal Y-type to a bacterial sequence motif, where no variants with activity were obtained. In addition, lack of activity of all LipR variants where Tyr^{110} was substituted by any other amino acid indicate that this residue is crucial for activity, playing an important role in stabilization of the tetrahedral intermediate. The shift in substrate specificity towards longer chain length substrates found for mutant LipR-D111G indicates that D^{111} would be more involved in substrate accommodation/stabilization than in intermediate stabilization. This was confirmed *in silico* after model structure analysis and molecular docking, showing the presence of a wider substrate binding pocket in variant LipR-YGS.

6. FUNDING

This work was financed by the Scientific and Technological Research Council (MINECO, Spain), grants CTQ2014-59632-R, CTQ2013-48995-C2-2-R and AC2015-00008-00-00, by the Pla de Recerca de Catalunya, grants 2009SGR-819 and 2014SGR-534 00327, by PCI-AECID project A203563511, and by the Generalitat de Catalunya to the "Xarxa de Referència en Biotecnologia" (XRB).

7. ACKNOWLEDGEMENTS

We thank the Serveis Cientifico-Tècnics of the University of Barcelona for technical support in sequencing. B. Infanzón acknowledges fellowships from CONACYT-Paraguay (14-VIN-014).

8. ETHICAL APROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

9. CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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Descripción de métodos rápidos y económicos para inmovilización de lipasas no comerciales de *Pseudomonas*

Silvia Cesarini, Belén Infanzón, F. I. Javier Pastor, Pilar Diaz

Antecedentes:

Existe un creciente interés en buscar nuevas preparaciones de enzimas para el desarrollo de nuevos productos derivados de bioprocesos para obtener materiales alternativos de tipo bio. En este contexto, cuatro lipasas no comerciales de especies de *Pseudomonas* fueron preparadas, inmovilizadas en diferentes soportes de bajo coste, y examinadas en cuanto a su potencial para aplicación biotecnológica.

Resultados:

Para reducir costes de una eventual producción a gran escala, las nuevas lipasas fueron obtenidas directamente del extracto del lisado celular o de los sobrenadantes de los cultivos, y fueron inmovilizadas por adsorción simple a los soportes Accurel EP100, Accurel MP1000 y Celite®545. Las enzimas evaluadas fueron LipA y LipC de *Pseudomonas* sp. 42A2, un mutante termoestable de LipC, y LipI.3 de *Pseudomonas* CR611, las cuales fueron producidas en huéspedes homólogos o heterólogos. Los mejores resultados de inmovilización fueron obtenidos por adsorción de LipA al soporte Accurel EP100, mientras que de LipC y la variable termoestable de LipC sobre el soperte Accurel MP1000. La lipasa LipI.3, la cual requiere un paso de *refolding*, fue muy pobremente inmovilizada en todos los soportes ensayados (siendo los mejores resultados sobre Accurel MP1000). Para comprobar el comportamiento, las lipasas inmovilizadas fueron ensayadas en tranesterificación de trioleina, en donde los mejores resultados fueron observados en las inmovilizaciones sobre Accureel MP1000 para todas las lipasas.

Conclusiones:

El protocolo sugerido no requiere purificación de proteínas, utilizando inmovilizaciones obtenidas con técnicas rápidas de adsorción sobre soportes de bajo coste con extracto crudo de enzimas, lo que hace al método adecuado para una producción a mayor escala con fines de aplicaciones biotecnológicas. Por tanto, fue estandarizado un método rápido,

simple y económico para la preparación de inmovilizaciones. El bajo precio de los soportes ensayados y la simplicidad del procedimiento, además de obviar los pasos tediosos y costosos de purificación, contribuirá a la reducción de costes en procesos biotecnológicos catalizados por lipasas.

BMC Biotechnology (2014) 14: 27.

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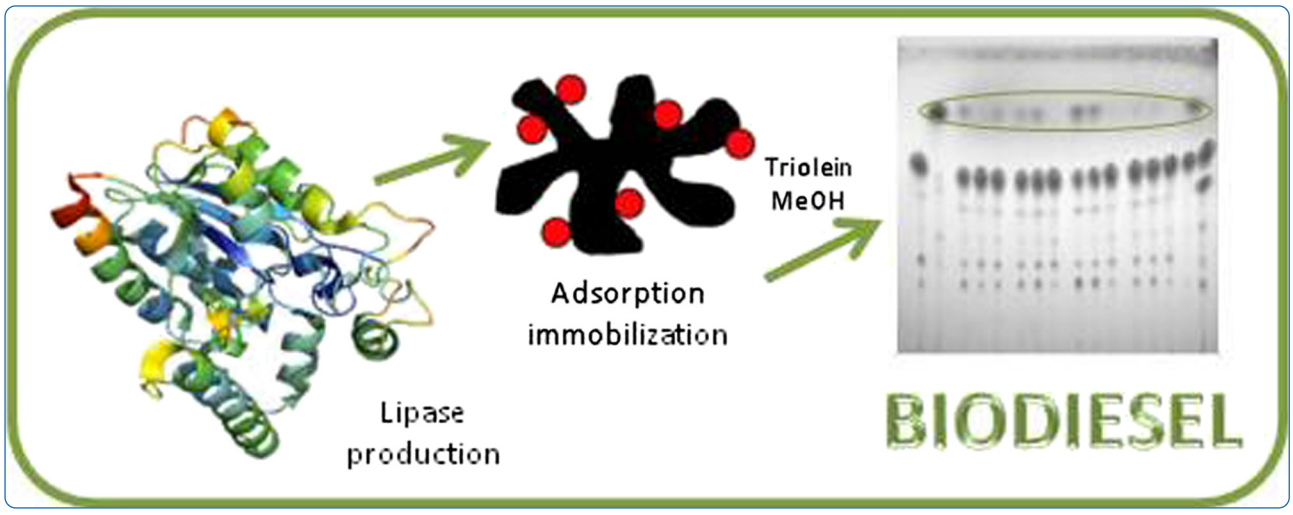

Fast and economic immobilization methods described for non-commercial *Pseudomonas* lipases

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Fast and economic immobilization methods described for non-commercial *Pseudomonas* lipases

Silvia Cesarini, Belén Infanzón, F I Javier Pastor and Pilar Diaz*

Abstract

Background: There is an increasing interest to seek new enzyme preparations for the development of new products derived from bioprocesses to obtain alternative bio-based materials. In this context, four non-commercial lipases from *Pseudomonas* species were prepared, immobilized on different low-cost supports, and examined for potential biotechnological applications.

Results: To reduce costs of eventual scaling-up, the new lipases were obtained directly from crude cell extracts or from growth culture supernatants, and immobilized by simple adsorption on Accurel EP100, Accurel MP1000 and Celite®545. The enzymes evaluated were LipA and LipC from *Pseudomonas* sp. 42A2, a thermostable mutant of LipC, and LipI.3 from *Pseudomonas* CR611, which were produced in either homologous or heterologous hosts. Best immobilization results were obtained on Accurel EP100 for LipA and on Accurel MP1000 for LipC and its thermostable variant. Lip I.3, requiring a refolding step, was poorly immobilized on all supports tested (best results for Accurel MP1000). To test the behavior of immobilized lipases, they were assayed in triolein transesterification, where the best results were observed for lipases immobilized on Accurel MP1000.

Conclusions: The suggested protocol does not require protein purification and uses crude enzymes immobilized by a fast adsorption technique on low-cost supports, which makes the method suitable for an eventual scaling up aimed at biotechnological applications. Therefore, a fast, simple and economic method for lipase preparation and immobilization has been set up. The low price of the supports tested and the simplicity of the procedure, skipping the tedious and expensive purification steps, will contribute to cost reduction in biotechnological lipase-catalyzed processes.

Keywords: *Pseudomonas*, Lipase, Immobilization, Accurel, Celite, FAMEs

Background

According to the Roadmap of the European Commission for Environmental and Sustainable Development (July 2013), "*it is now time to move towards an energy and resource efficient economy, based on the use of nature's toolbox*". Bio-inspired processes or enzymatic reactions have a low environmental impact, reduce the amount of waste material and minimize costs, thus serving the requirements to integrate environmental sustainability with economic growth and welfare (Roadmap of the European Commission for Environmental and Sustainable Development, July 2013). Therefore, there is an increasing interest to seek new enzyme preparations and to develop new products derived from bioprocesses to obtain alternative bio-based materials [1,2]. In this context, biotechnological production of biodiesel with lipases has received great consideration in recent years and is undergoing a fast development [3]. Employment of lipases as biocatalysts in the transesterification of triglycerides looks attractive because of the mild reaction conditions required and the easy recovery of glycerol, without an elaborate purification procedure involving production of chemical wastes. Moreover, the enzymatic process tolerates the water content of oil and increases the biodiesel yield by avoiding the typical soap formation caused by alkaline transesterification [4].

Enzyme immobilization is a well reported technology that allows application of enzymes in many biocatalyzed

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processes like in lipase-mediated biodiesel production [5]. In general, immobilization allows reuse of the biocatalyst, makes the product recovery easier, and frequently enhances the enzyme resistance against inactivation by different denaturants, providing more stable catalysts [6]. Efficiency and recyclability of immobilized enzymes depends not only on the procedure and support utilized but also on the specific enzyme used and the type of process where it is applied [7]. Various immobilization procedures like adsorption, cross-linking, encapsulation or entrapment have been employed on lipases used for biodiesel production [8]. However, most immobilization procedures use sophisticated protocols for lipase entrapment on expensive supports [9], not suitable for a real scale-up, causing an increase in the costs of industrial processes. Therefore, adsorption, used in this work, is a convenient system for lipase immobilization that could be successfully applied to large volume industrial processes, as in biodiesel production, because of its simplicity and low cost [10,11]. This type of immobilization occurs via binding of the lipase onto the surface of the support by weak forces, such as Van der Waals or hydrophobic interactions or through dispersion forces [8]. Adsorbed lipases can be prepared and used under mild conditions without significant activity loss, and the associated process is very simple, as the carrier can be easily recovered for repeated immobilization rounds [5]. Lipases can be adsorbed onto many different supports but generally, porous materials are the best option. These supports allow the effective dispersion of enzyme molecules on a large surface, thus allowing a higher number of enzyme molecules to deliver their catalytic potentiality [12]. In order to obtain stable, efficient and economic enzyme preparations suitable for potential biotechnological applications, in this work three hydrophobic and porous materials were tested as supports for lipase immobilization: two polypropylene matrices (Accurel EP100 and MP1000) and a diatomaceous silica (Celite®545).

Accurel EP100 and MP1000 are commercially available hydrophobic, macroporous, low-density polypropylene powders that display a large surface area for adsorption because of their very small particle size [13,14]. These preparations are extremely resistant towards organic solvents [14-16], a feature that increases their interest for lipase immobilization when aimed at oil transesterification [12,17]. Celite®545 consists of highly porous diatomaceous beads composed of silica (SiO_2), also containing some other inorganic oxides. Because of its chemical inertness and special interconnected pore structure, Celite®545 constitutes a very suitable support for physical adsorption [18] which has been extensively used in immobilized-lipase biodiesel production [11,19,20].

Previous works aimed at lipase-mediated biodiesel synthesis used mainly commercial enzyme preparations. As an alternative, in the present work we assayed four new lipases from *Pseudomonas* sp. 42A2 and *Pseudomonas* sp. CR-611 [21-23], previously isolated and characterized in our laboratory [24]. Classical enzyme immobilization generally uses purified lipases, a step that requires laboratory materials and equipment that considerably increases the preparation time, thus raising the price of enzyme preparations and the total costs of the process. Therefore, we tried to avoid purification by considering the high hydrophobic nature of lipases and probably of their selective adsorption on the hydrophobic and porous supports used. Accordingly, we describe here the preparation of four non-commercial lipases for potential biotechnological applications. A simple immobilization protocol by fast adsorption of crude enzymes onto three different, inexpensive supports was developed. As a possible application, the behavior of the immobilized lipase preparations was assayed in triolein transesterification for fatty acid methyl esters (FAMEs) production.

Results

Lipase production

Pseudomonas sp. 42A2 extracellular lipases LipA and LipC were purified and fully characterized in a previous work [21]. LipA is a robust, mesophilic enzyme whereas LipC is more thermolabile and shows a cold-adapted behavior [21,22]. LipCmut, instead, is a LipC thermostable variant previously obtained in our laboratory, still keeping the wild type cold-adapted behavior, thus being a good candidate for biotechnological applications [22,25]. For lipase production, genes coding for LipA, LipC and LipCmut were ligated to pBBR1MCS vector together with their cognate foldase gene *lip*H [21,22]. These constructions were cloned and expressed in the homologous host *Pseudomonas* PABST7.1, where lipases are naturally folded by LipH and further secreted to the culture medium in their fully active conformation [21,22]. Supernatants from each strain were obtained and directly used for activity determination and immobilization. The use of *Pseudomonas aeruginosa* PAO1 mutant strain deficient for chromosomal lipase production (PABST7.1), guarantees that all extracellular lipolytic activity found in supernatants from LipA, LipC or LipCmut recombinant clones corresponds indeed to each individually cloned lipase. Therefore, use of the selected strains as hosts for multicopy plasmid-encoded lipases allowed us to skip the purification steps for each enzyme, thus reducing time and costs of the entire enzyme production process.

Pseudomonas sp. CR611 lipase LipI.3 was previously cloned in *E. coli* 5 K, where it is expressed in an inactive form as inclusion bodies [23]. Nevertheless, enzymatic preparations of LipI.3 adequate for evaluation were successfully obtained from inclusion bodies by refolding in urea, as previously described [23]. However,

activity recovery from inclusion bodies makes the whole process longer and more expensive, indicating that further improvements of the expression system and enzyme preparation protocol are required to make LipI.3 a faster and more efficient enzyme system. Several attempts to increase Lip I.3 expression were conducted by means of using different vectors and hosts, but in all cases the enzyme was found in the form of inclusion bodies. Consequently, only low but sufficient amounts of active LipI.3 were obtained from the initial construction in order to test this lipase for immobilization on different supports with the same loading charge.

Effectiveness of lipase immobilization

Adsorption of lipases on hydrophobic polymeric supports has been reported previously [12,13]. However, previous works mainly assayed existing commercial lipases. In this work we wished to discover the behavior of four non-commercial lipases towards immobilization using different kinds of supports, for further biotechnological applications. Thus, direct supernatant samples of PABST7.1 bearing LipA, LipC or LipCmut constructions, and refolded crude cell extracts of *E. coli* LipI.3 were successfully immobilized on EP100, MP1000 and Celite®545. During adsorption on EP100 and MP1000, residual activity corresponding to unbound lipases was measured in supernatants of the immobilization batches as an indicator of the process yield. For this purpose, supernatant samples were obtained at different times, and the decrease of residual activity was plotted for immobilization assessment. As shown in Figure 1 (EP100) and Figure 2 (MP1000), all four lipases tested were efficiently adsorbed on the Accurel matrices used. A fast decrease of residual activity could be observed during the first 5 h incubation for all tested enzymes. For LipC and LipCmut, 90% immobilization was produced within the first hour of incubation, both on EP100 and MP100. Adsorption of LipA on polypropylene matrices proceeded slightly slower, but all lipolytic activity of the batch supernatant was lost after 24 h incubation, indicating a complete immobilization. Enzyme-loaded EP100 and MP1000 were recovered through vacuum filtration and lipase activity of the supports was measured. As shown in Table 1, in general, immobilization on MP1000 resulted in the most active enzyme preparations. Since immobilization on Celite®545 followed a different protocol, only the final activity adsorbed to the silica could be measured (Table 1). Celite®545 showed to be the best support for LipA, although this enzyme also displayed good activity when adsorbed to MP1000. LipC displayed the highest activity values after immobilization on EP100 and MP1000, whereas the catalytic potential of LipCmut and LipI.3 was better preserved on MP1000. Taking into consideration the great variation of activity values found among replica assays (Table 1), and trying to describe a common support for the four lipases tested, it could be concluded that Accurel MP1000 would be the support of choice for immobilization of LipC, LipCmut and LipI.3, whereas LipA would be better immobilized on Celite®545. In general, immobilized lipases displayed full activity over a long period, indicating a good stability of the biocatalysts [10].

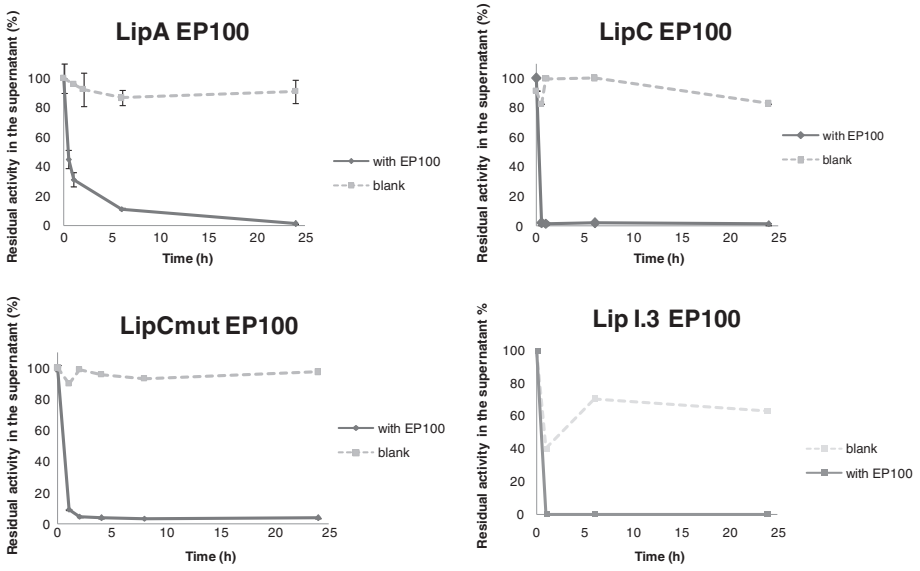

Figure 1 Immobilization on EP100. Continuous lines show the decrease of residual activity in immobilization batch supernatants when incubated with the support. As a control, dotted lines show the same supernatants incubated without any support: initial activity was maintained during the 24 h immobilization process, as shown by recovery of initial activity.

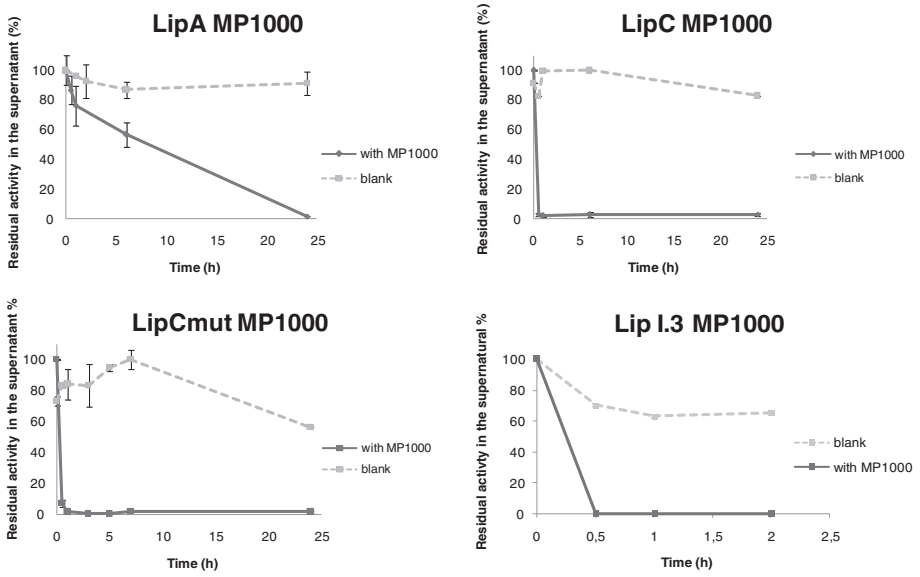

Figure 2 Immobilization on MP1000. Continuous lines show the decrease of residual activity in immobilization batch supernatants when incubated with the support. As a control, dotted lines show the same supernatants incubated without any support: initial activity was maintained during the 24 h immobilization process, as shown by almost full recovery of initial activity. Samples were taken mainly during the first 8 h of incubation to better define the rapid decrease of supernatant residual activity. The apparent activity decrease observed for LipCmut control sample was probably due to a different degree of substrate emulsion in samples assayed after 8 h incubation. High immobilization rates were obtained after 2 h incubation.

FAMEs production

As a possible biotechnological application, the four immobilized lipases were assayed in preliminary transesterification reactions for FAMEs production. The transesterification reaction conditions used were an adaptation of a previous protocol to the immobilized enzymes [26]. Impure triolein, (Sigma) containing also di- and monolein, was used as a substrate to simulate a natural vegetable oil, generally constituted by a mixture of glyceride forms. The reaction mixture contained the oil substrate, immobilized enzyme, and a defined amount of water and methanol, as described in materials and methods. The resulting products were analyzed through TLC (Figure 3), used as a fast and simple qualitative system for confirmation of FAMEs synthesis. A control sample showing the substrate impurity is visible in the first lane of Figure 3, where several spots appear, the most abundant corresponding to triolein. An industrially synthesized biodiesel sample, loaded in the second lane, was used as a control for FAMEs production. FAMEs appearance in course of the reaction with lipases LipA, LipC, LipCmut and LipI.3 can be identified in Figure 3 as spots migrating at the same position as the industrially synthesized biodiesel (lane 2). Although moderate, production of FAMEs by LipA, LipC and LipCmut preparations was detectable. Unfortunately, no FAMEs spots were revealed in the case of LipI.3-mediated transesterifications. Independently of the behavior of LipI.3, the highest yield in FAMEs production could be detected in reactions carried out with LipA-EP100, LipA-Celite®545 and with LipC and LipCmut immobilized on the two Accurel matrices, where discernible biodiesel spots appeared. Once demonstrated that new, non-commercial lipases can be efficiently immobilized on low-cost supports following a simple and fast protocol, and having shown that these lipases can perform transesterification reactions, further experiments are being conducted to increase the immobilized activity and to assess the re-usability of such preparations [10].

Table 1 Enzyme activity after immobilization on EP100, MP1000 and Celite®545

	LipA	LipC	LipCmut	LipI.3
EP100 (U g^{-1} support)	6.8 ± 0.8	42.7 ± 1.4	28.6 ± 0.1	34.3 ± 1.1
MP1000 (U g^{-1} support)	36.0 ± 1.7	39.4 ± 7.8	62.6 ± 0.1	86.2 ± 6.2
Celite®545 (U g^{-1} support)	48.3 ± 1.0	7.2 ± 3.0	21.7 ± 0.2	13.8 ± 1.9

Discussion

Lipases LipA, LipC, LipCmut and LipI.3 were efficiently immobilized on all assayed supports with no need for time-consuming and expensive purification protocols. Use of the selected strains as hosts for multicopy plasmid-encoded lipases allowed high enzyme production and permitted to skip the purification steps for each enzyme [27], thus reducing time and costs of the entire

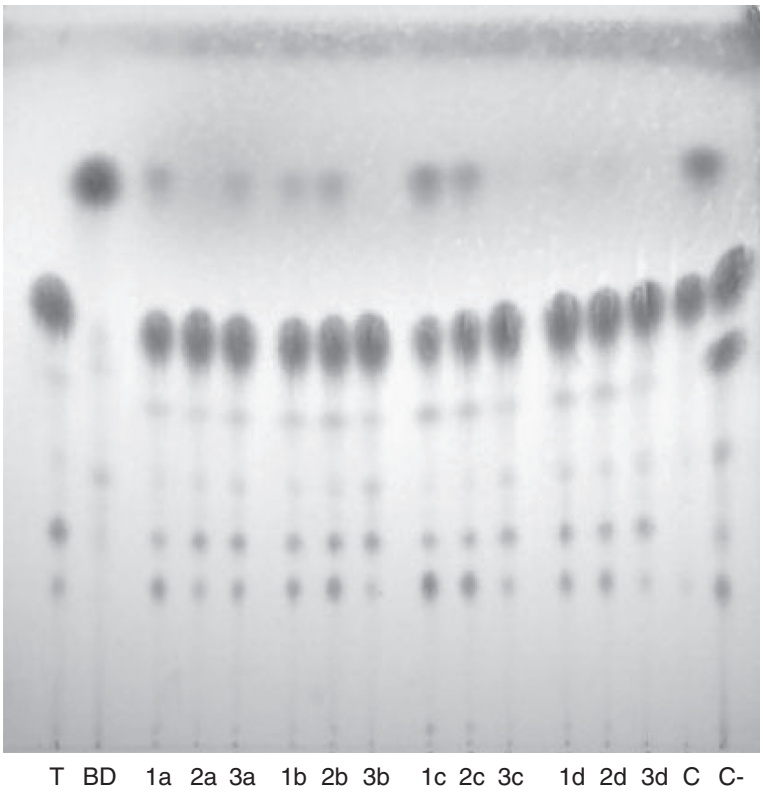

Figure 3 TLC showing the products resulting from transesterification reactions. T: triolein 65% (substrate); BD: industrial biodiesel (product control); C: transesterification reaction performed with Novozym 435, shown as a control; C-: negative control of a transesterification reaction run without alcohol. Enzymes immobilized on 1) EP100; 2) MP1000; 3) Celite®545. Reaction products of a) LipA; b) LipC; c) LipCmut; d) LipI.3. FAMEs production was clearly detected in lanes 1a, 3a, 1b, 2b, 1c, 2c and C, where spots at the same height of industrial biodiesel appeared.

enzyme isolation process. Moreover, supernatants from PABST7.1 cultures generally produce a very low amount of total protein (ca. 0.033 mg mL^{-1}), where each cloned lipase is the most abundant enzyme, as they are produced in higher copy number than any other secreted, chromosome-born protein. Therefore, the corresponding culture supernatants obtained can be considered as semi-purified enzyme preparations [10]. Cost and time savings in lipase isolation would be a great advantage in future scaling up of enzyme production for immobilization or biotechnological application. Although several attempts were performed to obtain higher expression yields of Lip I.3, low expression rates were obtained, indicating that new strategies should be developed for further lipase production improvement. Nevertheless, the amount of enzyme obtained by inclusion body refolding was sufficient for the immobilization assays performed.

In contrast to other immobilization procedures using sophisticated protocols and expensive supports [7,9], economic matrices were tested here to make the process industrially feasible. All four lipases tested were efficiently adsorbed on the Accurel matrices used, where a fast decrease of residual activity could be observed during the first 5 h incubation. For these supports, lipolytic activity of the batch supernatant was lost after 24 h incubation, indicating a complete immobilization. Immobilization on Celite®545 provided the best results for LipA. However, although the protocol for immobilization on Celite®545 is extremely fast, cheap and easy [9], the use of cold acetone in high proportions for enzyme precipitation could become a disadvantage during an eventual scaling up of the immobilization process. Therefore, we suggest polypropylene supports like Accurel MP1000 as the more appropriate carriers for lipases LipA, LipC, LipCmut and LipI.3, considering their possible role in biocatalysis.

It has been reported that adsorption occurs via hydrophobic interactions and is generally more effective for lipases than for other proteins [28]. Considering the high hydrophobicity shown by lipases we can assume that the highest proportion of proteins adsorbed on the supports assayed here were the lipases tested, with only minor adsorption of other proteins derived from supernatants or urea-dissolved cell extracts. It has also been reported that the success of hydrophobic materials in lipase adsorption depends on the structural features of "true" lipases, that is, those bearing a structural domain called

"lid" [29]. The "lid" contributes to provide an open and a closed configuration to the enzyme. In the closed conformation, the "lid" exposes the hydrophobic residues towards the catalytic site, thus preventing substrate binding and activity. But in the presence of a hydrophobic interface, the "lid" changes its conformation and exposes the catalytic site to the substrate (triglyceride), in a phenomenon known as "interfacial activation" [29]. LipA, LipC and LipCmut possess a "lid" that covers the active site and is related to the affinity of lipases towards hydrophobic matrices [30]. Although LipA bears a "lid", it does not show interfacial activation [21] a fact that could justify its different immobilization behavior with respect to LipC and LipCmut, which display interfacial activation and resulted here better immobilized on hydrophobic Accurel supports. Interestingly, LipI.3, belonging to a different lipase family, does not have a canonical "lid" but displayed the highest activity when immobilized on the hydrophobic support Accurel MP1000, probably due to a major exposure of its catalytic site. In the case of LipC and LipCmut, it is likely that during adsorption on polypropylene matrices, these lipases change their conformation in the presence of the hydrophobic support surface, being thus adsorbed in the open configuration and therefore being more active than when immobilized on Celite®545. To understand why immobilization of LipA on Accurel EP100 produced the lowest activity recovery, another important factor should be considered: the orientation of the adsorbed enzyme. Indeed, due to the hydrophobic nature of the support, it is possible that some lipase molecules interact with the support through the very hydrophobic surface surrounding the active site. This might hinder the substrate reaching of the active site [12], producing low activity values. It has also been shown that enzyme loading is directly correlated with the activity of the immobilized biocatalyst [31]. At this respect, it was reported that when *Thermomyces lanuginosus* lipase was immobilized on EP100 at low loading, part of the enzyme molecules were completely inactivated: with a loading of 6.6 mg of protein g^{-1} support, only 70% of the active sites were titrable compared to the 93% titrable active sites obtained at a loading of 55 mg g^{-1} support [15]. Moreover, immobilization might also lead to the inactivation of some lipase molecules by the distortion of the tertiary structure of the enzyme caused by the enzyme–support interaction. This would lead to structural rearrangements that could cause a decrease in lipase activity. All these effects may affect to a different extent each immobilized enzyme, thus making necessary to assay each individual lipase for immobilization efficiency on the desired support prior to application and scaling up for industrial purposes.

Immobilized lipases were evaluated for FAMEs production using triolein as a model substrate. LipA, LipC and LipCmut produced a visible FAMEs spot when analyzed by TLC, indicating that they are candidates for further study and process improvement. Enzyme or reaction engineering could contribute to increase the FAMEs yield obtained here. Lack of FAMEs formation with Lip I.3 could be explained in terms of substrate specificity, knowing that triolein is not a good substrate for this enzyme when used in hydrolysis reactions [23]. Alternatively, lack of transesterification activity could be due to a negative effect of immobilization on Lip I.3 when used in synthesis reactions, to a low enzyme concentration, or to inhibition by methanol, reasons that deserve further investigation.

The results obtained demonstrate that non-commercial lipases LipA, LipC, LipCmut and LipI.3 can efficiently be immobilized on all assayed supports, being polypropylene matrices (Accurel EP100, for LipA, and Accurel MP1000, for LipC and its variant) the best supports to be employed, especially in transesterification reactions, since they do not involve elaborate protocols, they are easy to recover and sufficiently cheap to be used in an eventual scaling up of the process. Immobilized LipA, LipC and LipCmut were capable to produce FAMEs from triolein under the conditions used here; therefore, they deserve further investigation.

Conclusions

From the results obtained it can be concluded that it is possible to expand the existing enzyme toolbox by testing new, non-commercial enzymes produced by easy and fast procedures, which can further be immobilized by simple adsorption on economic supports. These methodologies can contribute to introduce new enzymatic tools for biotechnological applications such as biodiesel production, as they do not involve any time or solvent consumption, they are easy to conduct and sufficiently economic to be used in an eventual scaling up.

Methods

Materials and reagents

All chemicals and solvents were obtained from Sigma Aldrich and Serviquimia (Spain) and, except when stated, had quality for analysis. Accurel EP100 (particle size 200–500 μm) was from Azko Nobel (Obernburg, Germany; kindly provided by Dr. F. Valero). Accurel MP1000 (particle size under 1500 μm) was purchased from Membrana GmbH (Wuppertal, Germany). Celite®545 (diatomaceous earth, 30–80 mesh) was obtained from Sigma-Aldrich.

Bacterial strains and enzyme production

Pseudomonas strains and the plasmids used in this work are listed in Table 2. *Escherichia coli* 5 K, used as recipient strain for recombinant plasmids, was grown in Luria–Bertani medium (LB, Panreac, Spain) at 37°C and

Table 2 Strains used

	Strain	Features	Reference
	Pseudomonas sp. 42A2	Wild type	[33]
	Pseudomonas sp. CR-611	Wild type	[24]
	P. aeruginosa PABST7.1	Δ*lip*A Δ*lip*H miniD-180 (*tet*A *tet*R *lac*Iq P*lac*UV5-T7 gene1)	[32] Kindly provided by Dr. Rosenau.
LipA	*P. aeruginosa* PABST7.1 lipAHpBB	Contains *lip*A and *lip*H 42A2 gene	[21]
LipC	*P. aeruginosa* PABST7.1 lipCHpBB	Contains *lip*C and *lip*H 42A2 gene	[22]
LipCmut	*P. aeruginosa* PABST7.1 lipCHpBB (variant D2_H8)	Contains mutated *lip*C and wild type *lip*H 42A2 gene	[22]
LipI.3	E. coli 5 K (pGEM-T Lip I.3)	Contains *lip*I.3 CR-611gene	[23]

supplemented with 50 μg chloramphenicol mL^{-1} when necessary. *Pseudomonas* strains were routinely grown in LB medium (supplemented with 400 μg mL^{-1} chloramphenicol and 50 μg mL^{-1} tetracycline, when applied) at 30°C on a reciprocal rotary shaker (180 rev min^{-1}). *Pseudomonas* PABST7.1 is a PAO1 mutant deficient for *lip*A and *lip*H genes, where LipC is inactive due to the lack of the specific foldase LipH [32].

Pseudomonas sp. 42A2 lipases, LipA and LipC [21,33], and the thermostable variant LipCmut [22] were previously cloned with their specific foldase (LipH) in pBBR1MCS vector [34] and transformed into homologous *Pseudomonas* PABST7.1. All of them are naturally secreted lipases [21] and the enzyme recovery was easily carried out by centrifugation of the culture broth (20 min at 8000 rpm).

LipI.3, isolated from *Pseudomonas* sp. CR-611 [35], was previously cloned in pGEMT and transformed into *E. coli* 5 k [23] where it is expressed as inclusion bodies in an inactive form. Inclusion bodies were obtained by French press cell-disruption and solubilised according to Kojima's method [36]. Using a refolding protocol with urea 8 M, active LipI.3 was obtained in phosphate buffer 50 mM pH 7, after urea removal by dialysis [23].

Enzyme immobilization

Direct culture supernatants were used for LipA, LipC and LipCmut immobilization, whereas LipI.3 was immobilized from a solubilised inclusion body solution, thus skipping the high costs and time-consuming steps of protein purification.

Adsorption on EP100 and MP1000

Supports were pretreated following an adaptation from Guillen and coworkers [37] where the polypropylene matrices were activated with ethanol (3 mL per gram of MP1000 or EP100), followed by alcohol removal by vacuum filtration. Immobilization was carried out by mixing the pretreated supports with 5 mL cell extract or supernatant samples, following a ratio of 1 mg protein per g of EP100 and 2 mg protein per g of MP1000. Orbital shaking of the mixture was carried out for 24 h at each enzyme's optimum temperature, being 4°C for LipC and LipCmut, and 30°C for LipA and LipI.3. Control samples were run without any support under the same conditions. Supernatant samples from immobilization mixtures were taken at different times and their residual lipase activity was assayed as described [38]. The enzyme-loaded supports, containing the immobilized lipases, were recovered by vacuum filtration, dried in a speed vacuum and stored at 4°C after lipase activity determination.

Adsorption on celite®545

Immobilization on Celite®545 did not require any pretreatment and was performed by mixing 5 mL of cell extracts or supernatants with the support, using a ratio of 0.5 mg protein per g Celite®545. The mixture was incubated for 30 min at 4°C and precipitation of the enzyme onto the support was carried out with 5 mL cold acetone. Immobilized enzymes were then collected by vacuum filtration and dried with a speed vacuum concentrator prior to enzyme activity determination and storage at 4°C [9].

Activity assays

Activity of free enzyme in cell extract and supernatant fractions, or in supernatants from the immobilization mixture was analyzed by measuring the release of *para*-nitrophenol (*p*NP) from *p*NP-derivative fatty acid substrates, as a result of enzymatic hydrolysis, measured at λ = 405 nm as previously described [21,38]. One unit of activity was defined as the amount of enzyme that released 1 mol of *p*NP per minute under the assay conditions used. Lipase activity was routinely assayed at the optimum temperature for each enzyme (30°C for LipA and LipI.3; 4°C for LipC and LipCmut), using the standard conditions: 20 mM Tris HCl buffer at optimum pH of each enzyme (pH 8 for LipA, LipC and LipCmut; pH 5.5 for LipI.3), and 0.6% Triton X-100®. $CaCl_2$, at a final concentration of 20 mM, was added to the reaction mixture for LipI.3 activity assays [23].

Activity of immobilized lipases was measured using 2 mg support carrying the immobilized enzyme adsorbed on EP100 and MP1000, and 10 mg enzyme-loaded support for Celite®545. The reaction mixture was incubated at each enzyme's optimum temperature and was stopped by 2 min centrifugation at 14,000 rpm. Activity determination was carried out in 400 μL Tris 100 mM plus 100 μL *p*NP-buffer, as described above. One unit of immobilized lipase activity was defined as the amount of enzyme that released 1 mol of *p*NP per minute and per g of support, under the assay conditions used [21,38]. Protein concentration of free and immobilized samples was determined as previously described [39]. When required, dilution of lipase solutions was done using buffer Tris 20 mM pH 8 for LipA, LipC and LipCmut, and pH 7 for LipI.3 [21,23].

Transesterification assays

FAMEs synthesis reactions were carried out in 2 ml glass vials for 24 h with maximum agitation in a horizontal vortex. LipC and LipCmut reactions were carried out at room temperature, according to the cold-adapted behavior of these enzymes [21,22]. A temperature of 30°C was used for LipA and LipI.3 [21,23]. The reaction mixtures contained 1 g triolein of 65% purity to simulate the composition of mixed vegetable oils. Methanol 15% w/w of oil was used as the second substrate, and was added in two steps (7.5% each) at 0 h and 7 h, to prevent possible enzyme inhibition. Water (10% w/w of oil) was added to the reaction mixture to increase enzyme efficiency [26] and, for LipI.3, 20 mM $CaCl_2$ was also added [23]. In order to compare the effectiveness of immobilized lipases in transesterification assays, all enzymatic preparations were loaded of 2U lipase, calculated from their hydrolytic activity when immobilized. As positive control, the same reaction conditions were applied to a commercial immobilized lipase (Novozym 435, Novozymes DK), whereas in negative controls, the same reaction was performed without methanol for detection of possible hydrolysis processes. After 24 h reaction, the products were evaporated in a speed vacuum concentrator at 60°C for 1½ h to remove excess methanol and the upper layer, obtained by centrifugation during the same evaporation process, was prepared for TLC analysis.

Thin layer chromatography

AluGram UV254 silica gel plates (Mecherey Nagel) were used as stationary phase for TLC analysis, and a mixture of hexane-diethyl ether-acetic acid (80:20:1.5 v/v/v) was used as the mobile phase. Commercial triolein (Sigma) and a sample of industrially synthesized biodiesel (BE- Biodiesel, Spain) were used as standards for glycerides and FAMEs, respectively. Enzymatic reaction products and standards were diluted 1:50 in hexane, and 8 μL samples were then applied to a 20 × 20 cm plate. 15% phosphomolibdic acid in ethanol was used as color developer through a fine spray. Spot visualization was completed by mildly heating the plate with an air dryer.

Competing interests

No interest or financial relationships that could influence the author's objectivity exist regarding the information provided in it. Neither patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker's fees from a company apply to the manuscript authors who are a Post-doctoral fellow, a PhD student, and two public University professors. Therefore, the authors declare that they have no competing interests.

Authors' contribution

SC participated in the design of the study, carried out most of the experiments, organized and interpreted the data, and drafted the manuscript. BI made some of the immobilization experiments, performed the TLC assays and contributed to the manuscript draft. FIJ P contributed to critical discussion, and revised the manuscript. PD coordinated the project and the design of the study, critically interpreted the data and corrected the manuscript. All authors read and approved the manuscript.

Acknowledgements

This work was financed by the Scientific and Technological Research Council (MINECO, Spain), grant CTQ2010-21183-C02-02/PPQ, by the IV Pla de Recerca de Catalunya, grant 2009SGR-819, by PCI-AECID project A203563511, and by the Generalitat de Catalunya to the "Xarxa de Referència en Biotecnologia" (XRB). S. Cesarini and B. Infanzón acknowledge doctoral fellowships from the Spanish Ministry of Science and Education (AP2008-04579) and from MAEC-AECID, respectively. We thank Dr. F. Valero for kindly providing Accurel EP100.

Received: 2 December 2013 Accepted: 9 April 2014
Published: 22 April 2014

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doi:10.1186/1472-6750-14-27
Cite this article as: Cesarini *et al.*: Fast and economic immobilization methods described for non-commercial *Pseudomonas* lipases. *BMC Biotechnology* 2014 **14**:27.

CHAPTER IV: Lipases for FAMEs production: transesterification assays using alternative feedstocks

Aceites ensayados como materias prima alternativas para la síntesis de FAMEs utilizando lipasas inmovilizadas de bajo costo

Belén Infanzón, Silvia Cesarini, Josefina Martínez, F. I. Javier Pastor, Pilar Diaz

Lipasas previamente aisladas de *Pseudomonas* y caracterizadas fueron inmovilizadas en un soporte de bajo costo, MP1000, mediante procedimientos de recargas permitiendo la obtención de una mayor actividad por mg del soporte. Las enzimas LipA, LipC y LipCmut inmovilizadas, así como la enzima comercial Novozym® 435 fueron ensayadas en reacciones de síntesis de FAMEs utilizando trioleína y aceite desgomado de soja como sustratos modelos, y adicionalmente fueron ensayados sobre aceite residual de fritos y aceite del hongo *Mucor circinelloides*, los cuales constituyen materias primas no alimentarias y de bajo costo. Los aceites fueron primeramente caracterizados en términos de contenido de FFA y de glicéridos. Como ensayo preliminar, se establecieron las mejores condiciones reacción en cuanto a concentraciones de metanol y agua, las cuales fueron luego utilizadas para la síntesis de FAMEs a partir de las cuatro materias primas seleccionadas y con las cuatro enzimas mencionadas. En la reacción catalizada por la enzima Novozym® 435 con el aceite de *M. circinelloides* como sustrato, se obtuvo una producción de 44% de FAMEs. Los resultados también revelaron, que a pesar de la producción de FAMEs, las lipasas de *Pseudomonas* inmovilizadas fueron incapaces esterificar los ácidos grasos libres, lo que indica que se require una adicional mejora de las condiciones de reacción. Sin embargo, cuando la enzima Novozym® 435 fue ensayada en condiciones óptimas reportadas previamente, se obtuvieron unas concentraciones prometedoras de FAMEs de 91 %, 85 % y 76 % utilizando como sustrato trioleína, aceite residual de fritos y aceite de *M. circinelloides* respectivamente, los cuales resultan en atractivas materias primas alternativas y no alimentarias para la producción de biodiesel.

Biotechnology Progress. Sometido

Biotechnology Progress

Alternative oils tested as feedstocks for enzymatic FAMEs synthesis using low cost immobilized lipases

Journal:	*Biotechnology Progress*
Manuscript ID	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Infanzón, Belén; University of Barcelona, Faculty of Biology, Dept. Genetics, Microbiology & Statistics Cesarini, Silvia ; University of Barcelona, Faculty of Biology, Dept. Genetics, Microbiology & Statistics Martínez, Josefina; University of Barcelona, Faculty of Biology, Dept. Genetics, Microbiology & Statistics Pastor, Francisco; University of Barcelona, Faculty of Biology, Dept. Genetics, Microbiology & Statistics Diaz, Pilar; University of Barcelona, Faculty of Biology, Dept. Genetics, Microbiology & Statistics
Keywords:	Lipases, FAMEs, Transesterification, Novozym® 435, Biodiesel

Alternative oils tested as feedstocks for enzymatic FAMEs synthesis using low cost immobilized lipases

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ABSTRACT

Previously isolated and characterized *Pseudomonas* lipases were immobilized in a low-cost MP-1000 support by a re-loading procedure that allowed a high activity rate per weight of support. The immobilized LipA, LipC and LipCmut lipases and commercial Novozym® 435 were tested for FAMEs synthesis using conventional and alternative feedstocks. Triolein and degummed soybean oils were used as model substrates, whereas waste cooking oil and *M. circinelloides* oil were assayed as alternative, low cost feedstocks, which were previously characterized in terms of FFA and acylglyceride contents. In a preliminary approach, the reaction conditions were established, setting up the best water and methanol concentrations for a proper conversion rate. These conditions were further applied to the study of FAMEs synthesis using the four selected feedstocks and the four lipases. A 44 % FAMEs production was obtained when *M. circinelloides* oil was used as a substrate in a reaction catalysed by Novozym® 435. The results also revealed that, although producing a good FAMEs rate, the immobilized *Pseudomonas* lipases were unable to use the FFAs for esterification, indicating that the reaction conditions should still be further improved. However, when Novozym® 435 was tested under previously described optimal conditions, promising values of 85 and 76 % FAMEs synthesis were obtained for waste cooking oil and *M. circinelloides* oil, respectively, which might result in promising, non-food, alternative feedstocks for enzymatic biodiesel production.

Keywords: Lipases, FAMEs, transesterification, Novozym® 435, biodiesel

INTRODUCTION

Biodiesel, a renewable product, is made mostly by alkali-catalyzed transesterification of triacylglycerols (TAGs) from fats and oils. It consists of monoalkyl esters of long-chain fatty acids with short chain alcohols, mainly methanol and ethanol, resulting in fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs) [1,2]. Usually, the most widely used raw materials to produce biofuels come from edible oil crops, like soybean, canola or palm oils [3–5]. In this context, finding TAG sources for the large-scale production of biodiesel without greatly affecting the supply and cost of food becomes a priority. Accordingly, several studies have been set up to acquire and to implement alternative sources as raw materials for biodiesel production [6].

The use of new feedstocks will allow production of second-generation biofuels, based on non-food competing "waste carbon sources" [6]. Up to date, several works have reported the use of novel oil sources containing triglycerides like the non-edible *Jatropha curcas* seed oil [7,8] or microalgae oils, which have recently been studied and used for biodiesel synthesis [9,10]. For this reason, many screening attempts are in progress to find new strains capable to produce high amounts of lipids useful for biodiesel synthesis [11]. Another innovative source of TAGs, but not yet extensively studied, are fungi oils; certain oleaginous yeasts for instance are able to store large quantities of TAGs in the form of lipid bodies inside their cells [12,13]. During the last years, strains of the genus *Mucor* have gained attention because of their high contents in lipids and the good growth rate of the strains. This positions the oils produced by *Mucor* yeasts as interesting potential biofuel feedstocks [14], and specifically *Mucor circinelloides* is already being studied for transesterification in biodiesel production [15,16]. Among other non-food, alternative oils for biofuel production are the waste cooking oils. The catalytic conversion by transesterification of these residual oils into biodiesel could result in a marked economic and environmental benefit, converting this waste material into an attractive biodiesel feedstock [17,18].

The major disadvantage of using some of these alternative TAG sources is the presence of relatively high amounts of free fatty acids (FFAs), and their water contents, which may lead to a significant decrease of the biofuel productivity [19]. Taking into consideration that the raw materials can represent about 80% of the total biodiesel production costs, finding a way of using such FFAs as feedstock would be desirable to avoid raw material loses. However, extra conversion processes are required for this purpose, especially when a basic catalysis process is used, to avoid deactivation of the

catalyst by the FFAs through saponification during the reaction. A two-step transesterification process has been proposed by several authors to solve this critical drawback, but it involves the use of more solvents and catalysts, thus raising the total costs and the hazardous substances required for the process [7,20,21].

Contrarily to alkaline or acidic transesterifications, the enzymatic catalysis with lipases allows taking advantage of the high FFA contents found in unconventional oils to get them either esterified into TAGs for further transesterification into FAMEs, or directly esterified to the alcohol for conversion into FAMEs [22]. Many reports with promising results have appeared in the last years aimed at lipase-mediated biodiesel synthesis using mainly commercial enzyme preparations and testing several feedstocks [23]. Although the price of the enzymes is still high, lipases admit the use of low cost and low quality feedstocks, which are converted into high value biodiesel by means of green technology approaches [24,25]. To further reduce the costs of the whole process, discovery of lower cost lipases in comparison to those commercially available would be of great interest to make the whole process not only more sustainable but also cost-efficient.

In a previous work, we reported enzyme immobilization by fast adsorption of crude enzymes on low cost supports, avoiding the use of purified lipases for the process, thus getting a better price of enzyme preparations for potential biotechnological applications [26]. The enzymes used in that study were new lipases from *Pseudomonas* sp. 42A2 (NCIMB 40045) and *Pseudomonas* sp. CR-611 (CECT 8156), previously isolated and characterized in our laboratory [27,28]. *Pseudomonas* sp. 42A2 extracellular lipases LipA and LipC were purified and fully characterized in a previous work [27]. LipA is a robust, mesophilic enzyme, whereas LipC is more thermolabile and shows a cold-adapted behaviour [27]. LipCmut, instead, is a LipC thermostable variant previously obtained in our laboratory, still keeping the wild type cold-adapted properties, thus being a good candidate for biotechnological applications [29]. These lipases were successfully immobilized [26] and tested for transesterification of conventional soybean oil [30]. To complement the previous works, we here describe the use of such newly described lipases for transesterification of alternative oils with different origin and distinct FFA and TAG profile, for FAMEs synthesis.

MATERIALS AND METHODS

Bacterials strains and lipase production

Pseudomonas sp. 42A2 (NCIMB 40045) lipases LipA and LipC [27], and the thermostable variant LipCmut [29] were previously cloned with their specific foldase (LipH) in pBBR1MCS vector [31] and transformed into homologous *Pseudomonas* PABST7.1 [32]. All of them are naturally secreted lipases in the host strain, and enzyme recovery was easily carried out by centrifugation of the culture broth (20 min at 8000 rpm) previously grown in LB medium (supplemented with 400 μg mL−1 chloramphenicol and 50 μg mL−1 tetracycline, when required) at 30 °C on a reciprocal rotary shaker (180 rev min). A commercial lipase from *Candida antartica* immobilized on acrylic resin (Novozym® 435) was also used as a control.

Enzyme immobilization

Supernatants obtained from each strain were used for immobilization in a hydrophobic and porous material, Accurel MP1000 (Membrana GmbH), previously activated with ethanol, following the ratio of 2 mg protein per g of support. Accurel MP1000 is a commercially available hydrophobic, macroporous, low-density polypropylene powder that displays a large surface area for adsorption because of the very small particle size [33,34]. A modification of the method for lipase immobilization set up in a previous work [26] was developed here in order to improve the retained activity in the support. The mixtures of concentrated bacterial growth cultures and the support were maintained for 3 h at 4 °C with gentle shaking. The enzyme-loaded matrices were recovered by vacuum filtration, and were further re-loaded with more culture supernatant, following the same process. Three total charges were carried out for each enzyme and support, and the final immobilized lipases were dried with a speed vacuum concentrator and stored at 4 °C.

Hydrolytic activity of the immobilized enzyme preparations was measured using 2 mg of immobilized lipase in 500 μL buffer Tris 100mM and 100 μL *p*NP-octanoate, as previously described [26]. The reaction mixture was incubated at each enzyme optimum temperature (30 °C for LipA, 4°C for LipC and LipCmut), and was stopped by centrifugation at 14,000 rpm for 2 min. Activity was analysed by measuring the release of *para*-nitrophenol (*p*NP), as previously described [35]. One unit of immobilized activity was defined as the amount of preparation that released 1 mol of *p*NP per minute per mg of support under the assay conditions used.

Feedstocks

Four oils were tested as transesterification substrates for FAMEs production. Waste cooking oil was collected from a restaurant of the University of Barcelona and used as a homogeneous sample in the reactions. *Mucor circinelloides* oil, obtained from strain MU241 derived from R7B, was kindly provided by Dr. Victoriano Garre from the University of Murcia. Soybean water-degummed oil, kindly supplied by Cargill (Spain), was selected to ensure conversion using the immobilized commercial enzyme Novozym® 435, which had previously been reported to efficiently convert degummed soybean oil into its corresponding FAMEs. On the contrary, this enzyme preparation could not undergo methanolysis when crude oil was used as a substrate [36]. Finally, triolein (Sigma, Spain) of 65 % purity was used as a model substrate to simulate the composition of mixed vegetable oils for evaluation of FAMEs production.

Free fatty acid titration

The free fatty acid contents is generally expressed as the acidic value of a sample, corresponding to the amount of NaOH (mg) required for neutralizing the fatty acids. Hence, for FFA determination, 100 mg of each oil or their evaporated samples were weighed into a 5-ml flask and dissolved in 5 mL 2-propanol. 0.5 mL of 1 % phenolphthalein in ethanol, used as indicator, were added and the solution titrated with 0.05 M aqueous solution of NaOH until getting a purple color. Percent FFAs (expressed as oleic acid) was calculated as c (NaOH) × V (NaOH) × MW (oleic acid)/m (sample) × 100 %. All determinations were made in duplicate.

GC-MS analysis of acylglycerides

For identification of the acylglycerides contained in the oils, an analysis by Gas chromatography–mass spectrometry (GC–MS) was performed on a Thermo Scientific TRACE™ Ultra chromatographer coupled to a Thermo Scientific Mass Spectrometer ITQ™ 900, fitted with a BD-ASTM D6554 column EN 14105 (15 m long × 0.32 mm i.d. × 0.10 μm). The oven temperature was programmed from 50 (1 min hold) to 180 °C at a heating rate of 15 °C min^{-1}, then from 180 °C to 230 °C at a rate of 7 °C min^{-1}, and finally from 230 °C up to 370 °C (15 min hold) at a rate of 10 ºC min^{-1}. Split/splitless injector with split injection mode was used at split flow 50 mL min^{-1}, and injection temperature 350 °C. Helium was used as carrier gas with constant pressure of 80 kPa, and mass spectrometry was carried out with electron impact ionization at 70 eV, in a

mass range of 50–900 amu, ion source temperature 200 °C, and interface temperature 350 °C. Stock solutions with 5 mg mL^{-1} of glycerol (99.5%), 1-mono[*cis*-9-octadecenoyl]-*rac*-glycerol (monoolein; 99 %), and 1,3-di(*cis*-9-octadecenoyl)glycerol (diolein; 99%), purchased at Sigma-Aldrich (Spain) were prepared and used for the standard mixtures preparation. 200 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and 200 µL pyridine were added to the mixtures for derivatisation of the standards. Like for the standards, after 20 min, 8 mL *n*-heptane were added to the samples. 100 mg of each, sample and standard, were weighed and 200 µl pyridine and 200 µl MSTFA were added for derivatization prior to injection of 1 µL in the GC-MS.

Transesterification assays

All chemicals and solvents used were obtained from Sigma-Aldrich and Serviquimia (Spain). Different conditions of methanol and water contents were assayed to determine the adequate conditions for each enzyme, using water-degummed soybean oil as a control substrate. The reaction mixtures contained 5 U of each immobilized enzyme, 1 g of oil, 0-15 % methanol (w/w of oil), and 0-10 % water (w/w of oil). Transesterification reactions were carried out in 2 ml glass vials for 24 h with maximum agitation in a horizontal vortex at 30 °C. As positive control, the same reaction conditions were applied to the Novozyme® 435 commercial enzyme. Optimum MetOH and H_2O reaction conditions were also established for each enzyme and for the rest of feedstocks under evaluation. In addition, transesterification reactions using the four oils were performed under the best conditions described by other authors using the commercial lipase Novozym® 435; in this case methanol was added step-wise and the reactions were carried out over 16 h at 45 °C [37]. The reaction products were then evaporated in a speed vacuum concentrator at 60 °C for 90 min to remove excess methanol and the upper layer, obtained by centrifugation during the same evaporation process, was prepared for FAMEs determination by GC and for FFA concentration determination by titration.

TLC analysis

In a first approach to verify the oil conversion, a thin layer chromatography (TLC) was performed. The transesterification products and standards were diluted 1:50 in hexane and 8 µL samples were then applied to the origin of 20 × 20 AluGram UV254 silica gel plates (Mecherey Nagel). A mixture of hexane-diethyl ether-acetic acid (80:20:1.5

v/v/v) was used as the mobile phase. Water degummed soybean oil and a sample of industrially synthesized biodiesel (kindly provided by BE- Biodiesel, Spain) were used as standards for glycerides and FAMEs mobility, respectively. 15 % percent phosphomolibdic acid in ethanol was used as colour developer through a fine spray. Spot visualization was completed by mild heating of the plate with an air dryer.

GC analysis of FAMEs

FAMEs release (%) was analysed by gas-chromatography at the Scientific and Technical Services of the University of Barcelona, using a SGE® BPX70, GC Capillary Column (0.32mm, 0.25µm, 25m), following the protocol recommended by the European Regulation (EN14103) for FAMEs determination [30,38]. Methyl heptadecanoate was used as internal standard. The solution was prepared at a concentration of 10 mg mL^{-1} in acetone. Evaporated oil samples were weighed (50 mg) in a vial and mixed by vortex with 1 mL solution of internal standard. Samples were then diluted 10-fold with acetone to reach a final concentration of 1 mg mL^{-1} of internal standard, and were transferred to a glass vial sealed with a septum cover prior to being injected into the gas chromatographer.

RESULTS AND DISCUSSION

Immobilized lipase production

Polypropylene matrix Accurel MP1000, a quite cheap support, was previously found as the best out of three supports tested for LipA, LipC and LipCmut adsorption immobilization for transesterification purposes [26], and was chosen here for immobilization improvement. To increase the rate of immobilized activity, supernatant samples of grown cultures producing each lipase extracellularly in the homologous host *Pseudomonas* PABST7.1 [32], containing equal amounts of protein were used to perform a three cycle re-loading of the support. Contrarily to the previously described protocol, where immobilization was performed under the optimal activity temperature for each enzyme [26], the process here was carried out at 4 °C for all the enzymes to prevent activity decay during the three loading repeats. Table 1 shows the amount of immobilized activity retained by Accurel MP1000 after support re-loading, by comparison with the results obtained before [26]. Activity is expressed in U per gram of support, based on hydrolysis assays performed on *p*NP-octanoate.

Table 1. Lipase activity improvement after a three cycle re-loading immobilization on Accurel MP1000

	U/g support*	U/g support** after re-loading	Activity fold-increase
LipA	36	108	3
LipC	40	150	3.75
LipCmut	63	170	2.7

* [26]; ** This work

An important increase of immobilized activity was achieved for all assayed lipases using the re-loading strategy (Table 1). Immobilized LipCmut displayed the highest specific activity, with a 2.7-fold immobilized activity increase, whereas LipA showed the lowest specific activity even after a 3-fold increased adsorption rate. The best behaviour was found for LipC, showing high specific activity and a significant 3.75-fold increased immobilization rate. Hence, the immobilization protocol developed here resulted in significantly better-retained activities, allowing testing such immobilized enzymes in transesterification reactions.

Feedstock characterization

To avoid using edible oils for FAMEs production, alternative waste cooking oil and *Mucor circinelloides* (MU) oil were tested here as feedstocks along with water-

degummed soybean oil, and commercial triolein. Except for triolein, no information about the composition of such oils was available. Therefore, the first aim was to get to know the lipid contents of these raw materials. GC-MS analyses (Supplementary Tables ST1, ST2 and ST3) were performed to determine their free fatty acid, mono, di and triglyceride percent contents (Table 2). The free fatty acid percent was also analysed by titration for comparison with the FFAs remaining after the transesterification reactions, and expressed as the acid value for each sample. Commercial triolein is reported to have 98.9% triglycerides; after titration, we established its FFA content at 1.9%. Concerning the other oils, when measured by titration, degummed soybean oil, waste cooking oil and MU oil revealed a FFA content of 1.3, 1.4 and 32.6 %, respectively.

Table 2. Lipid composition of the feedstocks: relative peak area (area %) for the MS chromatogram of TMS derivatives of fatty acids from the three oil samples analysed

Feedstock	FFAs	Monoglycerides	Diglycerides	Triglycerides	Sterols
Degummed soybean oil	1.32	0.00	0.91	95.26	2.31
Waste cooking oil	2.08	0.00	6.45	90.73	0.60
Mucor circinelloides oil	26.54	8.41	54.19	10.10	0.70

GC-MS analysis of the degummed soybean oil showed a very high amount of triglycerides. Likewise, waste cooking oil displayed high triglyceride percent, with poor degradation into diglycerides, monoglycerides and FFAs (Table 2). In fact, the calculated amount of FFAs in waste cooking oil was unexpectedly low, as it was assumed that the heat provided during the cooking process would have accelerated the hydrolysis of the triglycerides, thus increasing the FFAs content [17]. Concerning *Mucor circinelloides* oil (MU), it displayed a very low percent of triglycerides, with a very high amount of FFAs, monoglycerides and diglycerides (Table 2). Although these properties position MU oil as an inadequate raw material for biodiesel synthesis, previous assays using alkaline transesterification reported high conversion values and a high biodiesel quality [15,16,39]. Interestingly, during the identification of the mass spectroscopy ions of MU oil, an unusual unsaturated 18:X acyl glyceride, not yet identified, was detected as free, mono and diglyceride, showing a relevant peak area (Supplementary Table ST3).

FAMEs biosynthesis conditions

To set up the water and methanol concentration conditions for FAMEs synthesis using the enzymes previously immobilized, several reactions were assayed, and the resulting

FAME and FFA % contents were measured. Degummed soybean oil was the conventional substrate of choice used here to standardize the reaction conditions. In a previous work we reported that water addition during enzymatic transesterification for FAMEs synthesis could produce better results [22,38], as water is essential to maintain the specific tridimensional structure of some lipases, especially if used in soluble form [40]. Thus, the effect of water on the immobilized lipases prepared here was studied, carrying out the reactions in the presence of 0 %, 2.5 %, 5 %, and 10 % (v/v; H_2O/oil) water concentration. Moreover, each water condition was also assayed with 5 %, 7.5 %, and 15% (v/v; methanol/oil). It is important to notice that prior to setting up the transesterification reaction conditions, the hydrolytic behaviour of the immobilized *Pseudomonas* lipases in the presence of such methanol concentrations had to be assayed to make sure that no loss of activity occurred. Therefore, the immobilized lipases were incubated with different concentrations of methanol for 24 h, and their residual hydrolytic activity measured on *p*NP-octanoate, as described in Materials and Methods. No loss of activity was observed even at the maximum percent of methanol (20 %) assayed (data not shown), in agreement with previous results indicating that *Pseudomonas* species lipases display great resistance to methanol compared to other immobilized lipases tested [41]. These assays were also performed with commercial lipase Novozym® 435, described as sensitive to methanol [42].

In order to perform a fast and accurate screening of FAMEs synthesis under all the conditions of water and methanol stated above, a simple and sensitive qualitative TLC assay was used to analyse the products obtained. An example of such TLC assays is shown in Figure 1, where the products resulting from transesterification of degummed soybean oil with immobilized *Pseudomonas* LipA are presented for the various conditions studied. FAMEs appearing in the course of reactions using 5 % or 10 % of water and 5 % or 7.5 % methanol can be identified as spots migrating at the same position of an industrially synthesized biodiesel sample, used as a control. Although moderate, production of FAMEs was appreciated (Figure 1), as happened for the other enzyme preparations and oils assayed (not shown).

After FAMEs production was proven by TLC, gas chromatography analysis of the resulting products was performed. For LipA, similar results as those of the TLC analysis were obtained when FAMEs production was determined by gas chromatography. In that case, a FAMEs production increase was appreciated when 10 % water and 5 % or 7.5 % methanol were used (Figure 2). These results suggest that

Pseudomonas lipases are acting with a reaction mechanism depending on water, as happened for Callera Trans L, previously described [22], while the commercial *Candida antartica* enzyme (Novozym® 435) reached a higher production in the absence of water. Thus, the best conditions for each enzyme were determined according to the values shown in Figure 2: 5 % MetOH and 2.5% H_2O for LipA, and 5 % MetOH and 0 % H_2O for Novozym® 435. Concerning LipC and LipCmut, the best FAMEs synthesis conditions were previously established to be 5% MetOH, 10 % H_2O and 7.5 % MetOH, 10 % H_2O, respectively [30].

Free fatty acid reduction after the transesterification reaction was also evaluated by titration of the FFA concentration in the final products. As expected, when the percent of water increased in the reaction, hydrolysis of the triglycerides also increased; hence the enzymatic hydrolysis was favoured rather than the transesterification. The synthesis reaction mechanism would thus proceed through a two-step process involving an initial hydrolysis releasing FFAs, followed by an esterification reaction leading to FAMEs production, as previously reported [22,43].

Transesterification assays using alternative feedstocks

Once the best conditions for FAMEs synthesis were obtained, new reactions were set up in order to assay the usability of non-food, different origin oils as promising feedstocks for FAMEs enzymatic synthesis using low cost *Pseudomonas* immobilized lipases. Like for the degummed soybean oil tested above, the FFA content was determined by titration prior to the assays. As shown in Table 2, degummed soybean oil, triolein, and unexpectedly, the waste cooking oil, displayed a low FFA contents, whereas fungi oil showed a very high FFA contents (Table 2).

Regarding the percent of FAMEs produced, the highest conversion obtained (43.7%) was observed when the reaction was catalyzed by Novozym® 435 using *Mucor circinelloides* oil as a substrate, in comparison to the moderate FAMEs yields obtained for the other feedstocks tested (Figure 3). During the first step of the enzyme-catalyzed transesterification reaction in the presence of water, it is known that a TAG hydrolysis that releases FFAs occurs, to be then esterified by the enzyme into the corresponding FAMEs [22,44,45]. It has also been reported that the hydrolysis process is favoured by the low initial MetOH concentration when this co-substrate is added stepwise [22,30]. This concept justifies the fact that the enzymatic catalysis admits the use of high FFA contents feedstocks, which undergo esterification to produce FAMEs [22,30,45], a

phenomenon that was also observed here, where a great reduction of FFAs was found after the FAMEs synthesis reaction, reaching FFA values 39 % less than the initial rate for the fungus oil.

Contrarily, the *Pseudomonas* immobilized lipases were not capable to use the free fatty acids to synthetize FAMEs under the conditions assayed. In fact, the value of FFAs of the fungal oil did not show any difference before and after the FAMEs synthesis reactions when these lipases were used. Interestingly, an increase of the levels of FFAs was detected when the other three oils were tested with the immobilized lipases, suggesting a low esterification rate under the conditions used (Figure 3). Thus, although a good FAME synthesis rate was obtained during the standardization conditions of the reaction, it was shown here that *Pseudomonas* immobilized lipases cannot use the released FFAs for esterification to synthetize FAMEs. Moreover, the TAG hydrolysis rates were very high, especially with LipC and LipCmut, a drawback that could be overcome by combination of these enzymes with other lipases like Novozym® 435 in a multi-enzymatic reaction.

To prove that the reaction system used in this work was adequate, and to perform an additional evaluation of the four feedstocks, reactions using the reported optimal conditions for commercial lipase Novozym® 435 were assayed [37]. In a previous work, a 93% ethyl ester production was confirmed by ^{1}H NMR and HPLC using Novozym® 435 as the catalyst on oil from a different strain of *M. circinelloides* [15]. As shown in Table 3, under the conditions used here, a high FAMEs production was achieved for every feedstock tested using Novozym® 435 under optimal conditions.

Table 3. FAMEs production and FFA concentration found after transesterification reactions using Novozyme® 435 on different feedstocks under optimal reaction conditions [37]

Feedstock	**Transesterification products**	
	% FFAs	% FAMEs
Degummed soybean oil	5.0	85
Triolein	1.6	91
Waste cooking oil	1.0	85
M. circinelloides oil	3.6	76

A promising 91 % FAMEs synthesis was obtained when triolein was used as a substrate (Table 3), no matter the fact that the reaction was carried out in a very small-scale volume and without reactors. Under the same conditions, a 76 %, 85 % and 85 % FAME synthesis was obtained after the reaction using *Mucor*, degummed soybean and

waste cooking oils, respectively. At this respect, previous works reporting production of FAMEs through chemical catalysis using waste cooking oils, underlined the impossibility of producing biodiesel from oils with a FFA value higher than 2 % without acidic pre-treatment to get them reduced [17,46]. Hence, enzymatic catalysis can be adapted to the conventional chemical systems to make it possible the production of biodiesel from low cost feedstocks without the need to add steps or solvents to the process. Accordingly, the FAMEs synthesis reaction can still be further improved for the alternative substrates tested here, which may become attractive alternative, non-food feedstocks for enzymatic biodiesel production.

CONCLUSIONS

As a conclusion, new lipases and alternative feedstocks for biodiesel production have been produced, analysed and tested here. After setting up the general conditions, a first glance to the behaviour of each enzyme on every tested substrate was acquired, including their ability to use FFAs. This knowledge will allow further improvement of the specific reaction conditions for each enzyme on every substrate so that high FAMEs production rates can be achieved. In particular, this work has revealed that there are alternative, non-food and cheap feedstocks that can be used for biodiesel synthesis. Further improvement of the specific reaction conditions for each enzyme and substrate may allow increasing the promising FAMEs synthesis rates of 76, 85 and 91 % obtained here, using alternative, non-edible feedstocks.

ACKNOWLEDGEMENTS

We thank the Serveis Cientifico-Tècnics of the University of Barcelona for technical support in sequencing and GC/MS analysis.

FUNDING SOURCES

This work was financed by the Scientific and Technological Research Council (MINECO, Spain), grants CTQ2014-59632-R, CTQ2013-48995-C2-2-R and AC2015-00008-00-00, by the Pla de Recerca de Catalunya, grants 2009SGR-819 and 2014SGR-534 00327, by PCI-AECID project A203563511, and by the Generalitat de Catalunya to the "Xarxa de Referència en Biotecnologia" (XRB).

ETHICAL APROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

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FIGURE CAPTIONS

Figure 1. Example of TLC analysis showing the products of transesterification reactions performed under different water and methanol concentrations. S: degummed soybean oil; BD: industrial biodiesel (control product); C1: negative control of a transesterification reaction run without methanol; C2: negative control of a transesterification reaction run without enzyme. Reaction products of LipA: 1) 0 % H_2O, 5 % MetOH ; 2) 2. 5% H_2O, 5 % MetOH; 3) 5 % H_2O, 5 % MetOH; 4) 10 % H_2O, 5 % MetOH; 5) 0 % H_2O, 7.5 % MetOH; 6) 2.5 % H_2O, 7.5 % MetOH; 7) 5 % H_2O, 7.5 % MetOH; 8) 10 % H_2O, 7.5 % MetOH; 9) 0 % H_2O, 15 % MetOH; 10) 2.5 % H_2O, 15 % MetOH; 11) 5 % H_2O, 15 % MetOH; 12) 10 % H_2O, 15 % MetOH.

Figure 2. Gas chromatography results of FAMEs yield and FFA conversion under several conditions of water and methanol, using degummed soybean oil. % FAMES (squares) (5, 10 and 15 % MetOH) and % FFA (crosses) (5, 10 and 15 % MetOH). LipA-MP1000 (A), Novozym® 435 (B), LipC-MP1000 (C), LipCmut-MP1000 (D).

Figure 3. FAMEs yield and FFA conversion using different feedstocks. LipA-MP1000 (A), Novozym®435 (B), LipC-MP1000 (C), LipCmut-MP1000 (D).

Figure 1

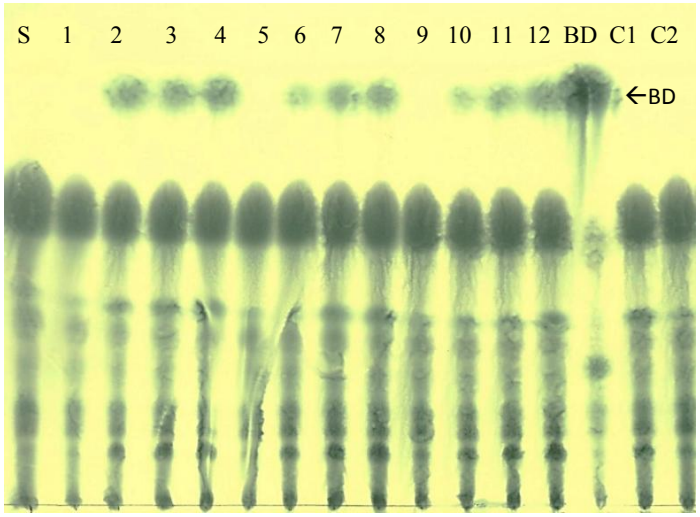
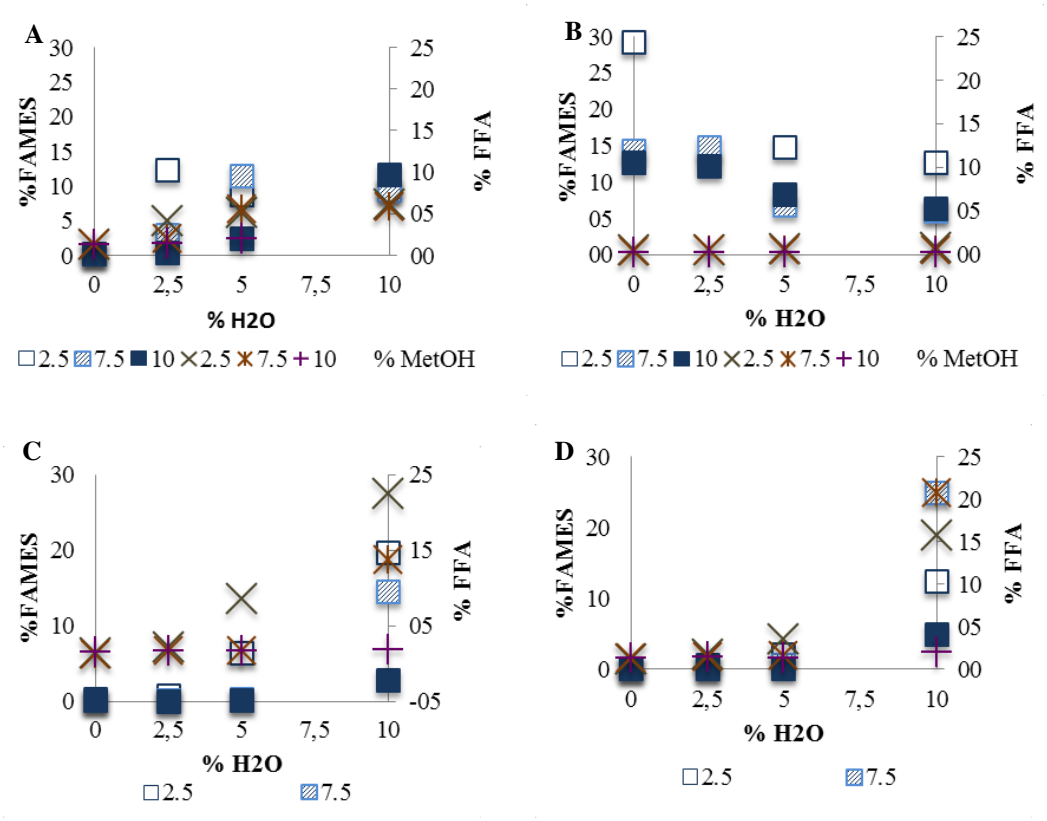


Figure 2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%FAMES
% FFA
% H2O
2.5 7.5 10 2.5 7.5 10 % MetOH
B
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C
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D
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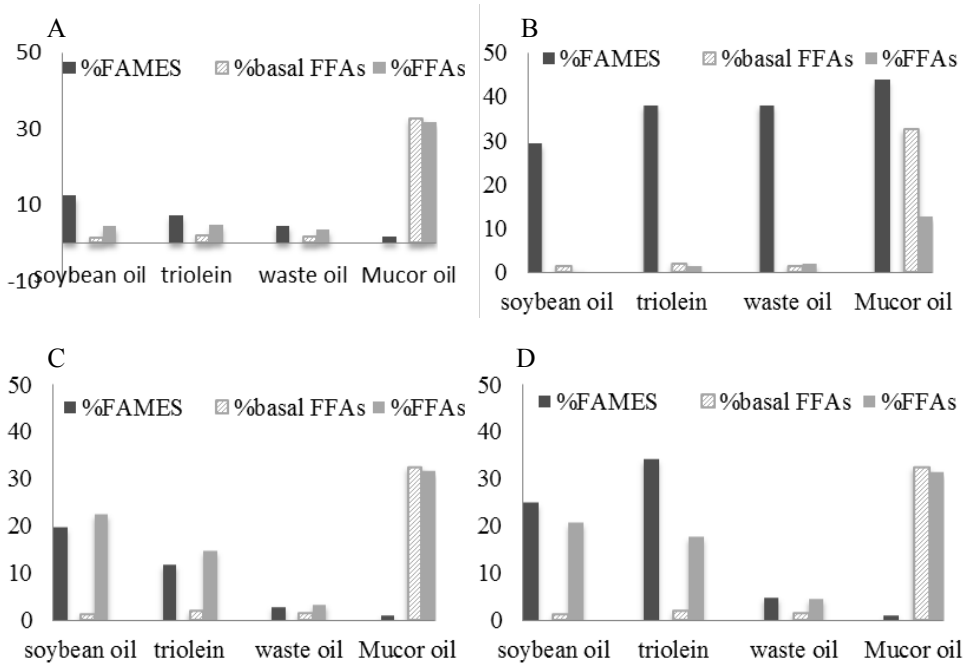
Figure 3

Figure 3



Supplementary Material

Supplementary Table ST1. Peak Identification and Relative Peak Area for the MS Chromatogram of TMS Derivatives of Fatty Acids from degummed soybean oil

peak number	compound	Ret. Time	MW	area %
1	palmitic TMS	9,2	328	0.12
2	unknown	9,39	282	0.04
3	linoleic TMS	10,21	352	0.89
4	oleic TMS	10,26	354	0.39
5	PAH	9,91	234	0.07
6	unknown	16,13	475	0.11
7	tocopherol TMS	16,98	490	0.27
8	esterol TMS	18,64	472	0.35
9	stigmasterol TMS	18,88	484	0.61
10	sitosterol TMS	19,27	486	0.97
11	1,2 OP	23,73	651	0.1
12	1,3-OP	24,02	651	0.21
13	1,2-LL+1,2 LO	24,58	688, 690	0.23
14	1,3 LL+1,3 LO	24,76	688, 690	0.07
15	1,3 OO	24,89	692	0.3
16	PPL	29,6	831	2.85
17	PLL+POL	30,79	854, 856	32.55
18	LLO+OOL+ LLS	31,43	880, 882,882	59.86

PHA: polycyclic aromatic hydrocarbon, OP: olein palmitate, LO: linolein olein, LL: dilinolein, OO: dilinolein, PPL: dipalmitin linolein, PLL: palmitin dilinolein , POL: palmitin olein linolein, LLO: dilinolein olein + OOL: diolein linolein, LLS: dilinolein stearin.

Supplementary Table ST2. Peak Identification and Relative Peak Area for the MS Chromatogram of TMS Derivatives of Fatty Acids from waste cooking oil

peak number	compound	Ret. Time	MW	area %
1	palmitic TMS	9,19	328	0.13
2	unknown	9,38	282	0.02
3	PHA	9,91	234	0.13
4	linoleic TMS	10,21	352	1.12
5	oleic TMS	10,26	354	0.83
6	sterol TMS	18,62	472	0.04
7	stigmasterol TMS	18,86	484	0.1
8	sitosterol TMS	19,26	486	0.46
9	1,2 PP	23,02	640	0.08
10	1,3 PP	23,1	640	0.11
11	1,2 OP	23,72	651	0.25
12	1,3 EP (elaidico-palmitico)	23,91	666	0.18
13	1,3 OP	24,01	651	0.86
14	1,2-LL+1,2-LO+1,2-OO	24,58	688, 690, 692	0.84
15	1,2 OS	24,64	694	0.29
16	1,3-LO+ 1,3-LL	24,77	690, 688	0.56
17	1,3-OS+1,3-OO	24,91	694, 692	3.28
18	PPO+PPL	29,64	832, 830	7.5
19	PLL+POL+OOP	30,65	854, 856, 858	23.13
20	LLL+LLO+OOL	31,43	878, 880, 882	60.1

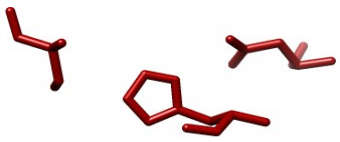
PHA: polycyclic aromatic hydrocarbon , PP: palmitin palmitate, OP: olein palmitate, OO: dilinolein, EP: elaidicpalmitic, LL: dilinolein, LO: linolein olein, OS: olei stearin, PPO: dipalmitin olein, PPL: dipalmitin linolein, PLL: palmitin dilinolein , POL: palmitin olein linolein, OOP: diolein palmitin, LLL: trilinolein, LLO: dilinolein olein + OOL: diolein linolein.

Supplementary Table ST3. Peak Identification and Relative Peak Area for the MS Chromatogram of TMS Derivatives of Fatty Acids from *Mucor circinelloides* oil

peak number	compound	Ret. Time	MW	area %
1	stearic	8,83	284	0.16
2	palmitolein TMS	9,05	326	4.02
3	palmitic TMS	9,21	328	0.21
4	linolenic	9,9	280	12.55
5	18:1	10,0	282	7.38
6	20:1	10,07	295	0.73
7	oleic TMS	10,28	354	1.49
8	unknown	13,11	--	0.03
9	monopalmitin TMS	13,56	474	0.04
10	18:1 TMS	14,66	500	4.08
11	18:1 TMS	14,78	500	0.34
12	monolein TMS	15,08	428	3.95
13	scualen	15,37		0.29
14	ergosterol TMS	18,38		0.15
15	sitosterol TMS	19,27	486	0.22
16	sterol TMS	19,67	472	0.04
17	dipalmitin	23,17	640	0.17
18	1,2-OP	23,74	651	1.1
19	1,3 OP	23,94	651	2.09
20	16:0 + 18:1	24,05	666	3.45
21	1,2- OO	24,63	692	8.84
22	1,3-OO	24,83	692	10.39
23	di 18:1	25,0	692	28.15
24	PPO+PPL	29,55	832, 830	0.25
25	OOP	30,5	858	3.85
26	OOO	31,67	884	6.00

OP: olein palmitate, OO: dilinolein, PPO: dipalmitin olein, PPL: dipalmitin linolein, OOP: diolein palmitin, OOO: triolein.

Discussion



1. Exploring a new esterase from *P. barcinonensis*

P. barcinonensis displays a set of hydrolases and an esterase, EstA, that have already been identified and characterized in our group (Prim et al., 2000; Sánchez et al., 2005). The lipolytic activity of a second enzyme was detected by zymogram as an additional band distinct from that of EstA. During a gene walking procedure, the sequence of a putative lipase/esterase gene was found next to a cellulase-coding gene. The sequence was then amplified and cloned into *E. coli* using a vector suitable for expression. The new enzyme, named Est23, was successfully over-expressed in *E. coli*, showing lipolytic activity. This new enzyme was purified and characterized during this work (Paper 1), showing preference for mid-chain substrates and having maximum activity at 37 °C and pH 7. In addition, the enzyme displayed a typical Michaelis-Menten kinetics, without any significant interfacial activation, showing the enzymatic kinetics typical of an esterase (Jaeger et al., 1999).

In a first analysis of the secondary structure, a predicted signal peptide containing a transmembrane region was identified by sequence analysis using SignalP 3.0. This was also confirmed from the structure analysis obtained from Phyre^2 webserver, where again transmembrane helices were predicted with a signal peptide (residues 1 – 25). Secreted lipolytic enzymes are mainly "true" lipase; therefore, based on those predictions, the cloned ORF could be a lipase. However, after the results obtained from the biochemical characterization, a more accurate analysis with SignalP 4.0 was carried out, indicating that Est23 does not indeed bear a true signal peptide, therefore being most probably a cell-bound esterase, as expected for an enzyme showing the properties of an esterase as Est23.

The 3D model of Est23, constructed using the closet structure (pdb c1cr6), allowed location of the catalytic triad Ser^{146}, Asp^{294} and His^{324} by similarity, and the oxyanion hole (G^{71}GPG) at the predicted positions was confirmed (**Fig. D1**). Analysis of the 2D and 3D structure of Est23, did not reveal the presence of a lid covering the active site, a fact that further supports the idea of Est23 being an esterase.

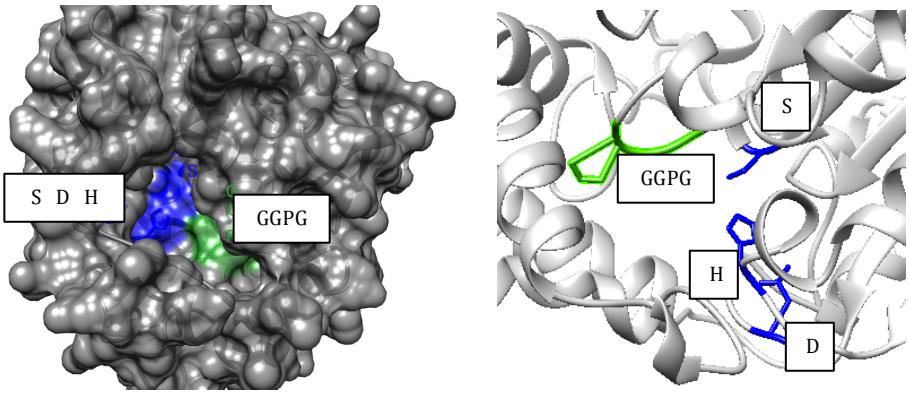


Figure D1. 3D homology model of Est23 with the catalytic residues and the oxyanion hole highlighted; A GGG(A)X-type putative oxyanion hole is shown in green ($G^{71}GPG$), and the residues constituting the catalytic triad (Ser^{146}, Asp^{294} and His^{324}) are highlighted in blue. The 3D model was generated with Phyre[2] server and obtained using pdb c1cr6. UCSF Chimera was used as molecular visualization system.

Moreover, a phylogenetic tree was constructed to assign Est23 to one of the bacterial hydrolase families described by Arpingy and Jaeger (Arpigny and Jaeger, 1999). Est23 could not be assigned to any bacterial hydrolases described till that moment, suggesting that Est23 could be part of a new group of bacterial lipases.

Est23 displays a GGG(A)X-type putative oxyanion hole, widely described as a motif involved in tertiary alcohol resolution. The flexible conformation of the GGG(A)X loop and the parallel arrangement of the carbonyl oxygen atoms to the binding pocket could enable these enzymes to accept bulky tertiary alcohol groups (Henke et al., 2002). Moreover, it was also reported that the GGG(A)X motif of some enzymes can be mutated only at the first and last position of the motif without losing activity towards the tertiary alcohol esters (Gall et al., 2010). Accordingly, the ability of Est23 for conversion and resolution of tertiary alcohols was evaluated under the conditions previously set in our laboratory (Bassegoda et al., 2010). The released products were analyzed by gas chromatography and mass spectrometry. However, no conversion was detected using the esters linalyl and terpinyl acetate alcohols as substrates. More substrates should be tested to find a better interaction with the enzyme (Fillat et al., 2014). Also new improved Est23 variants could be produced, for example changing the rigid residue P^{71} for an alanine or a glycine, both of them smaller amino acids and reported as more common in bacterial esterases (Bassegoda et al., 2010) and with a stronger effect than other amino acids over activity and enantioselectivity (Gall et al., 2010).

2. Improving LipR activity for transesterification

LipR, isolated from *Rhodococcus* sp. strain CR-53 in a previous work (Bassegoda et al., 2012a), showed an unusual fungal-like oxyanion-hole never found among bacterial lipases, close to the Y-type oxyanion hole described for *Candida antartica* lipase A (CalA), a lipase widely used in industry (Widmann et al., 2010). Moreover, LipR displayed good long-term stability and high tolerance to the presence of ions and chemical agents in the reaction mixture (Bassegoda et al., 2012a) . These features called our attention and suggested that LipR might be a good candidate for application in transesterification reactions involving TAGs (Canet et al., 2014; Duarte et al., 2015; Jaeger and Eggert, 2002). A first step forward to application of LipR was therefore to improve the expression rate. LipR was actively expressed in *E. coli* Origami™, a host chosen to favor formation of the disulfide bonds reported in the structure analysis of the lipase. A previous study showed an efficient expression of CalA, a lipase reported to have two disulfide bonds (Cys-Cys 101/273 and Cys-Cys 350/394) (Ericsson et al., 2008), using strain Origami™ as a host (Pfeffer et al., 2007). Thus, LipR was successfully expressed, purified and characterized in this strain (Bassegoda et al., 2012a). But for the enzyme application, a higher expression was required, and further expression optimization attempts were tested.

In a first and simple approach, a codon optimized *lip*R gene for expression in *E. coli* was ordered as a synthetic gene (Gustafsson et al., 2004). Several studies have confirmed that the expression level of recombinant enzymes in *E. coli* can be reduced by the presence of rare codons in the nucleotide sequence, hence improvement of the expression levels were achieved by codon optimization strategies (Fang et al., 2015). This approach was selected taking into consideration that LipR displays a very high content on uncommon G/C nucleotides. A total of 139 out of 1350 nucleotides were substituted in the optimized sequence of *lip*R. The new recombinant vector, having the optimal codon bias for *E. coli* expression was transformed and expressed in *E. coli* Origami™ and BL21 strains. However, no significant changes in expression were found (see results in **Annex 1**). Yields of genes redesigned by randomization methods are expected to be greater than yields of native genes, yet the result is uncertain, it cannot be predicted until the assay has finished. There exist many methods to construct the model, thus it becomes necessary to perform an actual experimental to evaluate the optimization method used based on statistics (Menzella, 2011; Tuan-Anh et al., 2017). When employing certain methods, the translation efficiency may be limited by other constraints rather than by the choice of the favorite codon to encode a

given amino acid in the designed gene (Menzella, 2011). In fact, in some cases, the methods used to replace rare codons by the most preferable usage codons can result in worse protein expression rates (Rosano and Ceccarelli, 2009). It has also been suggested that the approach of improving protein expression by an exchange of rare codons by more frequent codons should exclude rare codons located in highly conserved rare codon rich regions (Widmann et al., 2008).

Consequently, a change of heterologous host from *E. coli* to *Pichia pastoris* was chosen as an alternative. *P. pastoris* has long been the expression system selected for many lipases, mainly because of the existence of a strong and tightly regulated promoter, the very high cell densities achieved when growing on minimal medium, and the possibility of secreting the heterologous proteins to the media (Guillén et al., 2011; Valero, 2012). In order to ensure a successful improvement, expression of *lip*R under two promoters was assayed: the constitutive *GAP* promoter, and the *AOX1* promoter, tightly regulated and methanol induced (see results in Annex 1). Thus, *lip*R sequence was ligated to vectors bearing either one or the other promoter. Moreover, both constructions were designed by placing *lip*R sequence in the correct reading frame with that of *S. cerevisiae* α-factor pre-propeptide to facilitate secretion of the enzyme, because an extracellular production is most desirable (Cereghino and Cregg, 2000). However, just a very low signal of expression activity was found for clones under *AOX1* promoter in concentrated supernatants of recombinant *P. pastoris* grown cultures analyzed by zymogram. In fact, the highest activity signal was detected in the cell extract fractions. Finally, after 72 h cultivation of inducted cultures, no activity improvement, measured with *p*NP-decanoate, was achieved in cell extracts of clones bearing *lip*R under either the *GAP* or the *AOX1* promoters. Thus, again no improvement was achieved in LipR expression rate by changing the heterologous host to *P. pastoris*. Transformation and expression assays of LipR in *P. pastoris* were carried out at the School of Chemical Engineering of the Autonomous University of Barcelona, under the supervision of Dr. Francisco Valero.

The next objective was to seek an improvement of LipR performance instead of increasing the expression rate. Thus we directly focused on the improvement of LipR activity on long-chain substrates. For this purpose, a directed mutagenesis approach was chosen in order to elucidate and improve the residues involved in the exceptional Y-type oxyanion hole of LipR. Therefore, a rational design, structure-based strategy was developed to reveal the putative involvement of conserved residues in the oxyanion hole in fatty acid conversion

(Bassegoda et al., 2012b; Fillat et al., 2014). The structure analysis of LipR was reported in a previous work (Bassegoda et al., 2012a). Alignment of the LipR cluster of sequences constituting the family X lipases, and the 3D homology model of LipR showed different conserved amino acid sequence motifs located close to the active site of the enzyme. The putative lipase from *Burkholderia cenocepacia* J2315, included in the LipR cluster, was also isolated and cloned, confirming its lipolytic activity with a profile similar to that of LipR (Bassegoda et al., 2012a). The sequence Tyr-Asp-Ser-Leu was found in a loop close to the catalytic serine, constituting a highly conserved amino acid sequence motif. This fact and the similarity of this motif with the CalA oxyanion hole (the sequence of CalA is also included in the alignment of **Figure D2**), strongly suggests that these residues might be essential for substrate binding. Moreover, LipR shows significant sequence identity with some secretory lipases from *C. albicans*, also classified as members of the *C. albicans* lipase-like family. However, none of the lipase members of that family and bearing the Y-type oxyanion hole are bacterial lipases.

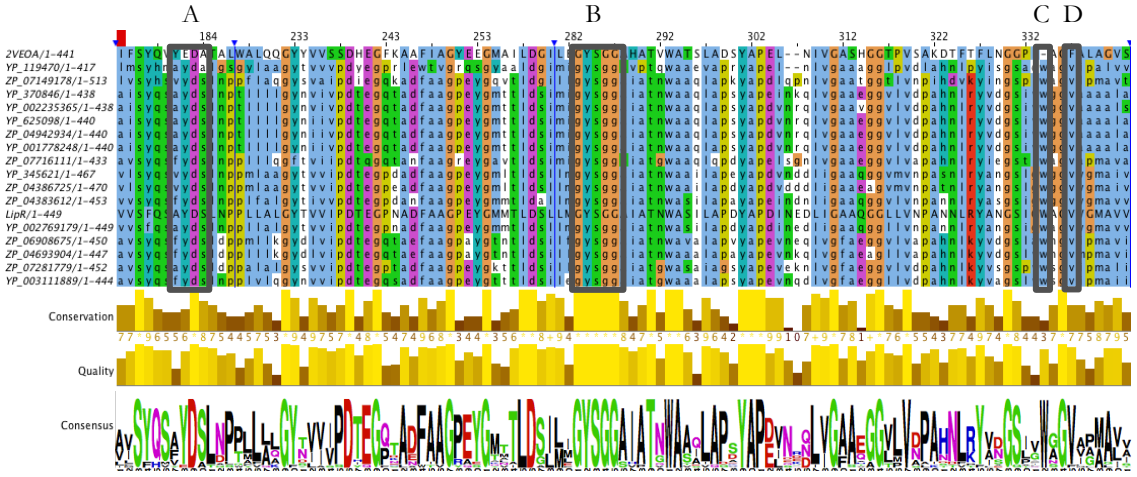


Figure D2. Blocks of sequences conserved in the sequence alignment of LipR cluster of putative bacterial lipase family X plus CalA. (A) oxyanion hole motif, (B) conserved pentapeptide GYSGG, (C) conserved tryptophan, (D) conserved valin. YP_002769179: putative lipase *R. erythropolis* PR4. ZP_04383612: putative triacylglycerol lipase *R. erythropolis* SK121. YP_345621: putative lipase *R. erythropolis* PR4. ZP_04386725: triacylglycerol lipase *R. erythropolis* SK121. ZP_07281779: putative triacylglycerol lipase *Streptomyces* sp. AA4. ZP_06908675: putative triacylglycerol lipase *S. pristinaespiralis* ATCC 25486. ZP_04693904: putative lipase *S. roseosporus* NRRL 15998. YP_003111889: putative triacylglycerol lipase *Catenulispora acidiphila* DSM 44928. ZP_07716111: putative triacyl glycerol lipase *Aeromicrobium marinum* DSM15272. YP_002235365: putative exported lipase *Burkholderia cenocepacia* J2315. ZP_04942934: putative triacylglycerol lipase *B. cenocepacia* PC184. YP_625098: putative triacylglycerol lipase *B. cenocepacia* AU 1054. YP_001778248: putative triacylglycerol lipase *B. cenocepacia* MC0-3. YP_370846: putative triacylglycerol lipase

Burkholderia sp. 383. ZP_07149178: putative triacylglycerol lipase Corynebacterium resistens DSM 45100. YP_119470: putative lipase *Nocardia farcinica* IFM 10152. 2VEO: *Candida antartica* lipase A.

On the basis of the uncommon fungal-like oxyanion hole found for LipR, a first mutagenesis approach was designed for obtaining five-oxyanion hole variants covering the five typical oxyanion holes described for bacterial lipases. Surprisingly, a zymogram assay of the LipR mutants obtained revealed that the modifications introduced produced no functional enzymes; activity was only found in the recovered wild-type variant. This indicates that the fungal-like, Y-type oxyanion hole of LipR is strictly required for activity. The residues of the GGG(X) oxyanion hole are reported as highly conserved among bacterial and fungal lipases. Previous works have reported enhancement of hydrolytic activity and thus an improvement in the enantioselectivity on tertiary alcohols by changing one of these five typical residues (Fillat et al., 2014, 2015). In that case, the reason for the low activity of the wild type enzyme was having an uncommon amino acid at the third position of the oxyanion hole (Bassegoda et al., 2010). But for LipR, this could not be the reason for the low activity found, as all possible residue combinations were tested, indicating that LipR shows a strict requirement for the Y-type oxyanion hole.

In order to better understand the role of the residues involved in the Y-type oxyanion hole of LipR, the data reported for CalA, the only crystalized lipase of this group, were analyzed for comparison. CalA is a lipase showing preference for bulky and long-chain substrates, suggesting that a wide substrate entrance tunnel must exist. It exhibits a Y-type oxyanion hole, where Tyr93 was identified as the oxyanion hole-forming amino acid (Widmann et al., 2010b). After analysis of the crystal structure obtained for CalA, a second, highly conserved residue, D^{95} was also proposed as a significant component of the oxyanion hole. This was an unexpected finding because an acidic group is not supposed to be a good candidate for stabilizing the negatively charged reaction intermediate occurring during lipase catalysis (Ericsson *et al.*, 2008). Both residues, Y^{93} and D^{95}, are located in the same loop, close to the catalytic serine, where they seem to play an important role in stabilization of the tetrahedral intermediate occurring during catalysis (Gall et al., 2010). As reported, Asp95 is critical for the catalytic activity of CalA; in fact, a complete iterative saturation mutation library led to not a single functional enzyme variant (Ericsson et al., 2008). However, the mutation Tyr93Phe, carried out to confirm that Tyr93 does not contribute to the catalytic activity, demonstrated fully retained activity, thus indicating that it is not involved in the catalytic mechanism (Sandström et al., 2009). By contrast, Glu94, located between the two relevant

oxyanion hole amino acids of CalA, was reported as a poorly conserved residue (Sandström et al., 2009).

Taking into consideration the information derived from CalA, a second mutagenesis approach was developed. Two libraries were constructed by iterative saturation mutagenesis by changing each one of the putative oxyanion hole residues, Tyr110 or Asp111, of LipR. The residue Y^{110} defines the oxyanion hole class, while D^{111} is equivalent to D^{95} of CalA (**Fig. D2**). Thus, two iterative saturation libraries were obtained and their sizes were optimized using CASTER worksheet, considering codon usage and the proper degeneracy applied (Reetz et al., 2006a). Variants for the two positions covering all groups of amino acids were obtained. As a result, complete loss of activity over different chain-length- substrates was obtained when Tyr110 was mutated. This indicates that, contrarily to CalA, Tyr110 has a critical role for the catalytic activity of LipR, probably due to the polarity of the tyrosine chain of this motif, which is very important to stabilize the transition state, while phenylalanine constitutes a non-polar amino acid. Experiments with purified enzyme would be required to further evaluate this hypothesis. The finding that the CalA mutant Tyr93Phe displayed activity could be related to the bulky size of the two amino acids, and the presence of a Glu between them, which might equally contribute to stabilization of the tetrahedral intermediate produced during binding of long-chain length substrates. This would not apply for LipR, showing mostly preference for short-chain length substrates (Bassegoda et al., 2012a).

Among the Asp111 mutants obtained, variant Asp111Gly produced a change on the chain-length- substrate preference of LipR, displaying a 5.6 fold increase of activity on *muf*-oleate. This improvement of activity on longer chain length substrates makes of this LipR variant a very attractive candidate for testing activity on biodiesel synthesis, a process requiring activity on long-chain substrates (Nielsen et al., 2008).

To define the role of D111 on the catalytic behaviour of LipR, a 3D homology model of both, wild type and mutant D111G variant were constructed. Analysis of the theoretical models achieved revealed that mutation D111G changes the distances between this residue and the catalytic serine, showing a shorter distance when Asp is part of the oxyanion hole, and probably causing also more interactions during the transition state intermediate stabilization (Paper III). On the contrary, presence of a Gly residue at position 111 originates a wider cavity, with increased distance between Gly and the catalytic Ser. A molecular docking analysis using *p*-NP-palmitate and *p*-NP-octanoate as substrates showed

the presence of a wider substrate-binding pocket in variant LipR-YGS compared to that of wild type LipR-YDS. Thus, a wider pocket is created after substitution of D111 by a glycine, increasing the size of the substrate entrance tunnel in LipR-Y**G**S, a fact that would allow stabilization and catalysis of larger substrates. The results obtained support the structural model constructed for LipR using CalA as a template and are also in agreement with a previous study of a lipase from *Candida parasilopis* (CpLIP2), where point mutations were designed using the 3D model of CpLIP2, based on homologies with the crystallographic structure of CalA. For CpLIP2, the D90N mutation allowed production of a functional protein, although not highly active on ethyl oleate (Subileau et al., 2015). Yet, compared to CpLIP2, an increase of 25 % of the activity on ethyl laurate could be observed for the D90N mutation, but no modification of the substrate specificity profile or reaction specificity (transesterification ratios) could be observed compared to wild type CpLIP2. In that case, the role of the D90 in the oxyanion hole of CpLIP2 remains unclear, but in the model obtained, D90 is not directly oriented towards the catalytic serine, contrarily to its homologous CalA_D95 and LipR_D111. In CpLIP2, the Asp residue is more buried in the core protein and may not play the same role as CalA_D95, while in the case of LipR D111, this residue is oriented towards the catalytic Ser212 (Bassegoda et al., 2012a).

Furthermore, based on the docking models, residues Trp261 and Val264 caught our attention because of their proximity to LipR oxyanion hole, and the possibility of producing additional interactions. These amino acids could even be additional residues of LipR oxyanion hole, not found in CalA but found in the other family X bacterial lipase members (Figure 2). Another residue that could also play a role in catalysis stabilization is Ala109; this amino acid appears in the LipR-YGS docking models as a protruding residue that could interfere with short- mid-chain length substrate accommodation. Accordingly, additional mutations of those and the surrounding residues would be helpful to get complete knowledge of all the amino acids involved in the stabilization of the transition state during catalysis, and thus to completely elucidate the oxyanion hole structure and the mode of action of LipR.

The structure/function analysis performed on the LipR 3D model obtained using CalA 3D structure as a template, allowed elucidating the role and improving the residues involved in the exceptional oxyanion hole of LipR, and facilitated rational design to obtain better enzyme variants by structure-based knowledge of putative involvement of conserved

blocks of amino acids in the oxyanion hole. Nevertheless, no matter the LipR improvement achieved, LipR and LipR_YGS variant still need a clear expression enhancement in order to apply them to transesterification reactions using oily feedstocks.

3. Lipase stabilization improvement by immobilization

Many lipases have been isolated and characterized in our laboratory. Among them, *Pseudomonas* lipases LipA and LipC, proposed to play an important role in biotechnological applications (Gupta et al., 2004; Jaeger and Reetz, 1998), have been characterized and improved. LipA, displays maximum activity at moderate temperatures, with a typical Michaelis-Menten kinetics, whereas LipC, is more active at low temperatures and displays partial interfacial activation (Bofill et al., 2010). These lipases were selected to be applied in transesterification of oils. A thermoestable variant of LipC (LipCmut) was also assayed (Cesarini et al., 2012).

For that purpose, the first aim was to provide higher stabilization to the enzymes through immobilization. Immobilization constitutes an improvement strategy widely used to enhance enzyme efficiency during a reaction. Accordingly, immobilization of the *Pseudomonas* lipases selected was tested over three different supports: Accurel MP1000 and EP100, using adsorption, and Celite®545 by precipitation. For immobilization, the lipases were expressed in a homologous *P. aeruginosa* host, so that they were produced in the supernatant of culture in their active form (Bofill et al., 2010). This fact constitutes an advantage to get a better preparation of enzymes through immobilization by direct adsorption to the support from the culture supernatant, thus skipping the purification step and reducing costs and time. Therefore, a fast and economic immobilization procedure by adsorption was set up.

This immobilization methodology by adsorption was further standardized. It resulted very efficient, since after 1 h incubation of the sample with the support more than 90 % of the supernatant activity of LipC and LipCmut was already immobilized into the polypropylene matrices. LipA immobilization was a little bit slower, requiring more than 5 h for a complete immobilization. A fourth enzyme was also assayed and presented in paper III, but it is not discussed in the context of this thesis. To evaluate the stability of the immobilized enzymes and to choose the best matrix for each enzyme, the preparations were assayed for transesterificacion of triolein during 24 h at the optimal temperature of each enzyme. Water (10 % w/w of oil) and methanol (15 % w/w of oil) were used as

substrates. Methanol was added in two steps (7.5 % each) to prevent possible enzyme inhibition (Azócar et al., 2010). The reaction products (FAMEs) were verified by thin layer chromatography (TLC) standardized during this work. The TLC is a qualitative but still very sensitive technique, constituting a useful tool for rapid evaluation of the reaction products (Fedosov et al., 2011). Transesterifications showing the highest product production were those involving LipC and LipCmut adsorbed on Accurel MP1000. Other works have demonstrated that smaller particle size have highest specific enzyme content (Oliveira et al., 2009; Séverac et al., 2011). Our results are in agreement with this: lipases immobilized on MP1000 show better results than those immobilized on EP100. For LipA preparations, the immobilization on Celite®545 was apparently the method showing better results than those obtained by immobilization on polypropylenes carriers. Celite® 545 supports are surely less expensive than polypropylene matrices. However, the scaling up of this immobilization process leads the problem of using large volumes of acetone. Moreover, thinking about a future application in biodiesel production, Celite® 545 has not the particle size suitable for its use in fixed bed reactors, and unfortunately the mechanical properties in stirred batch reactors are reported to be quite poor (Christensen et al., 2003). Several other studies have confirmed an increase in catalytic activity with increasing hydrophobicity of the support (Adlercreutz et al., 2013). *Burkholderia cepacia* lipase immobilized on Celite® 545 showed a yield of 98 % of FAAEs from *Jatropha* oil (Shah and Gupta, 2007). Same result was obtained using *Burkholderia fluorescens* lipase immobilized on a polyprolpylene support and using soybean oil as substrate (Salis et al., 2008).

In order to obtain more accurate results and to determine the most appropriate support, the products from transesterification reactions using the three immobilized lipases were also analyzed for FAMEs detection by GC (**Table D1**). However, the results obtained demonstrated that catalysis with these immobilized lipases as well as the reaction conditions assayed were inefficient, and surprisingly, the lowest value was obtained for LipA immobilization Celite®545. Thus, Accurel MP1000 was the matrix suggested as the best support for the three *Pseudomonas* lipases assayed in this work. Through these assays, not only the immobilized catalytic activity was determined, also the operational stability of the immobilized lipases was also evaluated with the report of the amount of product achieved during an application (Adlercreutz et al., 2013).

Table D1. FAMEs percent found in products of transesterification reactions performed on triolein for testing *Pseudomonas* immobilized lipases

Lipase	Support	% FAMEs*
LipA	MP1000	1.5
	EP100	1.7
	Celite®545	0.1
LipC	MP1000	3.2
	EP100	0.4
	Celite®545	1.1
LipCmut	MP1000	7.3
	EP100	4.1
	Celite®545	0.4
Novozym®435		17.1
**Triolein control		0.0

*Column FAMEs EN14103, [Dbwax, 30m, 0.32mm]. ** Triolein control, without enzyme

The results of FAMEs percent measured by GC demonstrated very low values of transesterificatiom. The reactions were carried out in a very low scale and under the same conditions for all enzymes; the best conditions for each immobilized lipase were not taken into consideration. However, the methodology used made it possible to determine the best support for immobilization and stabilization of *Pseudomonas* lipases to further improve the transesterification process. Subsequently, the next step was to improve the conditions of methanol, water and reaction volume, as well as the enzyme amount in order to obtain better production of FAMEs.

4. Improving the transesterification reaction

The amount of enzyme used during a catalyzed reaction is critical. Consequently, the amount of immobilized activity was the first improvement to get a higher amount of immobilized enzyme; for such purpose, the initial adsorbed preparations were re-loaded with supernatant of cultures for three additional times. Moreover, the temperature of the immobilization process was changed to 4 °C to avoid enzyme activity loose during the reloading. An increase of immobilized activity on MP1000 of 3.0, 3.75 and 2.7 fold was obtained for LipA, LipC and LipCmut, respectively. The new immobilization system suffered thus a notable enhancement and only by repeating the simple and fast process already described, greater activity rates were achieved per weight of support. In previous

studies, improvement of immobilized activity was achieved with the improvement of the expression system (Cesarini et al., 2014a).

Methanol is an important factor to be considered in enzymatic transesterifications. A better rate of FAMEs is achieved with the increase of methanol concentration. However, methanol has, in general, an inhibitory effect on lipases (Chen et al., 2008). When tested on *p*NP-derivative substrates, the three MP1000 immobilized lipases prepared during this work had not shown hydrolytic activity inhibition under 20 % methanol (data not shown), suggesting that this co-substrate might not affect the transesterification reaction progress. In a previous work, the behavior of soluble LipC and LipCmut was studied, and both lipases showed good tolerance to excess of methanol concentration (up to 30 % MetOH) (Cesarini et al., 2014a). Nevertheless, several lipases used in industrial processes, although stable in the presence of other organic solvents, are inactivated by methanol at or below the concentration optimal for FAMEs production (Azócar et al., 2010). This makes it necessary to use stepwise methanol feeding (Canet et al., 2016; Cesarini et al., 2014a) to further get optimal transesterification results (Kaieda et al., 2001). Some lipases require even six methanol additions to achieve the best yields (Clementz et al., 2016). For such reason, efforts are being devoted to the identification of novel enzymes suitable for use in one-step biodiesel production processes (Lotti et al., 2015).

The amount of water is another critical parameter for the transesterification reaction. The increase of water rises the enzyme flexibility and the expressed activity (Kaieda et al., 2001). However, after an optimum level of water is reached, hydrolytic reactions become significant and the transesterification yield is expected to be reduced (Kumari et al., 2007). The best conditions of water and methanol for transesterification reactions catalyzed by LipC-MP1000 and LipCmut-MP1000 using soybean oil as a substrate were determined as a part of a previous work in our group, and a significant increase of FAMEs % production was achieved (Cesarini et al., 2014a). Also an optimization of the reaction was achieved by scaling-up the reaction volume from 1 ml to 2 ml, and using 5 ml-glass tubes instead of 2 ml-plastic tubes, due to the unspecific binding of the enzyme to the plastic supports (Cesarini et al., 2014a). In the context of the present thesis, the best conditions for FAME synthesis using LipA-MP1000 were established. The products obtained were analyzed for FFAs and FAMEs contents after 24 h reaction. Thus the best conditions of methanol percent and percent of water were assayed and described in comparison with the commercial Novozym 435 immobilized CalB, a well-known lipase with good performance

on transesterification reactions (Azócar et al., 2010; Watanabe et al., 2001). In all cases, a clear improvement of FAMEs percent production was achieved, indicating that the conditions to carry out transesterification reactions with LipA have been improved by increasing the enzyme load on the support, the concentration and step-wise addition of methanol, and the water concentration in the reaction mixture. **Table D2** shows the transesterification results obtained using triolein as a substrate, in order to compare the new transesterification results with those presented in Table 1, where no reaction improvement had been obtained.

Table D2. FAMEs and FFAs percent obtained as products of transesterification reactions performed on triolein using the best conditions of MetOH and H_2O percent for each immobilized lipase after support reloading

Lipase	Reaction conditions		Transesterification products	
	MetOH (%)	H_2O (%)	FAMEs (%)*	FFAs (%)
LipA-MP1000	5	10	8.0	4.6
LipC-MP1000	5	10	11.8	14.8
LipCmut-MP1000	7.5	10	34.2	17.7
Novozym®435	5	0	38	1.2
Novozym®435*	step-wise***	0	91	1.6
** Triolein control			0.0	1.9

*Column FAMEs EN14103, [Dbwax, 30m, 0.32mm]. ** Triolein control, without enzyme. ***two-step: 7.5 % + 7.5 %.

5. Search for new feedstocks for FAMEs production

There exists an interest in finding alternative TAG sources for the production of large-scale biodiesel without greatly affecting the supply and cost of food, since raw materials widely used to obtain biofuels come from edible oil crops like soybean, canola and palm oils (Moser 2008; Crabbe et al. 2001; Geris et al. 2007). The enzymatic catalysis with lipase allows taking advantage of the relatively high free fatty acids (FFAs) contents of most unconventional oils and is not disabled by the sometimes high water contents of this kind of feedstocks, a fact that is not very favorable during a chemical catalysis (Kumari et al., 2007). In this context, characterization of alternative oils to be used as raw materials for FAMEs production by enzymatic catalysis was performed during this thesis.

A total of four oils were tested: commercial triolein, degummed soybean oil, waste cooking oil, and *Mucor circinelloides* (MU) oil. The contents of FFAs of these feedstocks before and after transesterification were determined by titration. The high content of FFA in some

feedstocks requires an excess of alkali during a chemical transesterification, frequently causing insoluble soap formation and thus decreasing the final yields (Shah et al., 2004). Therefore, one of the aims of this work was to evaluate the efficiency of each enzyme in converting these unconventional oils into FAMEs taking into account their contents in FFAs. Transesterification reactions were assayed using the different oils as substrates under the reaction conditions standardized before for each immobilized enzyme. Commercial Novozym® 435 was also used to evaluate the efficiency in transesterification of the different feedstocks, and the reactions were assayed under the two different conditions stated in Table D2. The reaction products for each enzyme and each feedstock were then evaluated by measuring the FAMEs % by GC and the FFAs % contents by titration.

In a global analysis, a good increase of FAMEs percent was obtained with LipA, LipC and LipCmut immobilized on Accurel MP1000. But better results were achieved when the reactions were catalyzed by Novozym® 435 commercial enzyme, especially when methanol was added step-wise during the reaction; in this case a very high value of 91 % FAMEs was obtained using triolein as a substrate, despite the very small-scale volume used for the assays. *Candida antartica* like *Rhizopus oryzae* lipase preferentially hydrolyze triglycerides at sn-1/3 position. Only a few lipases including *C. antatica* lipase also display sn-2 fatty acid preference of triglycerides (Sandstrom et al., 2012). Thus a maximum of 66 % conversion for the *Pseudomonas* lipase assayed would be expected, while the commercial Novozym® 435 enzyme used could achieve a higher percent of FAMEs production.

The MU oil resulted in a very attractive substrate to evaluate because of its high level of FFAs, mono and di- glycerides content and its very low level of triglycerides. Novozym® 435 took advantage of those initial FFAs, being capable to esterify them for FAMEs synthesis in the presence of low water concentrations. Under the first condition assayed for Novozym® 435 (water 0 % w/w of oil and methanol 5 % w/w of oil), the highest value of FAMEs production of 43.7 % was obtained using MU as a substrate, and the initial FFAs high rate of MU oil decreased till 12.7 %. However, when the commercial enzyme was assayed under step-wise ethanol adding conditions, a lower value of FAMEs of 76 % was obtained even though a greater FFAs consumption was registered: 3.6 % FFAs was detected in the final product. The contents of water of MU oil could be affecting the performance of the enzyme, since Novozym® 435 activity significantly decreases with increasing amounts of water (Haigh et al., 2014). Therefore, MU oil constitutes a very

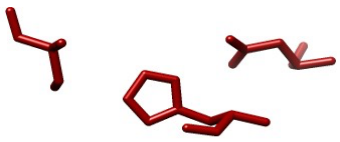
attractive feedstock for biodiesel synthesis although more reaction conditions should be optimized for this substrate.

Concerning waste frying oil, taking into consideration the quantity of FFAs, mono and di-glycerides, unexpectedly it resulted in a good quality of oil for FAMEs production. 85 % conversion of waste cooking oil was found using Novozym® 435 under optimal conditions. Nevertheless, because of the uses given to cooking oil, the waste frying oils displays a higher content of water. Thus, the global yield of the FAMEs synthesis reaction could be improved by reducing the amount of water in the initial sample.

The *Pseudomonas* immobilized lipases instead, were not capable to use these free fatty acids to synthetize FAMEs under the conditions assayed. Although they were capable to hydrolyze the TAGs of the raw materials assayed, no subsequent esterification to methanol occurred, indicating that the reaction conditions still need to be improved.

Finally, this work demonstrates how the enzymes can be improved and shows how an enzymatic process could be able to take advantage of a high level of FFAs contents contained in unconventional feedstocks, thus allowing the use of waste and unrefined oils as raw materials for transesterification. Additional optimization of the reaction conditions for each enzyme over each substrate are still required, including the use of multi-enzyme systems that could be a suitable solution for better results while reducing costs (Kourist et al., 2009).

Conclusions



1- A new gene corresponding to the second esterase of *Paenibacillus barcinonensis* has been isolated, and the encoded enzyme, designated Est23, has been expressed in *E. coli*.

2- Est23 was purified and biochemically characterized, revealing carboxylesterase activity.

3- Based on sequence analysis and alignment studies we conclude that Est23 belongs to a different sequence cluster, which probably constitutes a new family of bacterial lipolytic enzymes. Although Est23 was found to display a GGG(A)X-type oxyanion hole, described as an important motif for tertiary alcohol ester resolution, neither conversion nor enantiomeric resolution could be detected.

4- The LipR Y-type oxyanion hole sequence change from a fungal Y-type to a bacterial sequence motif has demonstrated its importance because no variants with activity were obtained.

5- A site-directed mutagenesis library of residue Tyr110 was constructed but lack of activity of all LipR variants was obtained, indicating that this residue is crucial for activity. The change of Asp111 by a Gly caused a shift in substrate specificity towards longer chain length substrates.

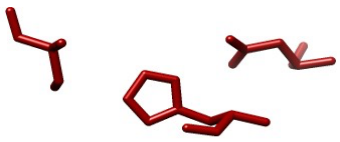
6- Three novel *Pseudomonas* sp. 42A2 lipases, including LipA, LipC and LipCmut were efficiently stabilized by a low cost procedure through three cycle reloading adsorption on propylene matrices, being Accurel MP1000 defined as the best support for transesterification reactions.

7- The immobilized lipases and commercial Novozym® 435 were applied in transesterification reactions and the best conditions were stablished.

8- Four different raw materials, including residual cooking oil and fungal oil were tested for transesterification using the previously immobilized lipases under the best conditions of enzyme load, water and methanol for ach enzyme. Between 5 and 10% water and methanol was needed in case of *Pseudomonas* lipases.

9- Characterization of FFA and glyceride contents of the raw materials used has been reported. Interestingly, *Mucor cicirnelloides* oil displays a high level of FFAs, which however can be converted in part to FAMEs. A waste frying oil demonstrated a high quality as a raw material, containing low FFAs and mostly diglycerides, thus being adequate for FAMEs synthesis.

Summary



1. Introducción

1.1 Generalidades de las Lipasas

Las lipasas (acilglicerol hidrolasas, EC 3.1.1.-) son enzimas que catalizan hidrólisis, trans e interesterificación de enlaces éster. Las esterasas y las lipasas difieren en las características de sus bolsillos de unión al sustrato, como el tamaño, la forma y la profundidad de la zona de interacción hidrófoba, así como en sus propiedades fisicoquímicas. Por lo tanto, las lipasas aceptan ésteres de ácidos grasos cortos (C4) a cadenas largas (C16), mientras que las esterasas aceptan sólo cadenas cortas (Pleiss et al., 1998). El mecanismo de reacción de las lipasas es análogo al de las serin-proteasas, teniendo la tríada catalítica constituida por los residuos de aminoácidos Asp, His y Ser, siendo este último responsable del ataque nucleofílico (Arpigny & Jaeger 1999).

Las lipasas han recibido una atención creciente debido principalmente a su capacidad para catalizar una amplia variedad de reacciones, lo que permite una amplia gama de aplicaciones en la industria (Rosenau & Jaeger, 2000). Recientemente, se reportó que las lipasas están involucradas en 671 documentos de patentes dirigidas a la resolución cinética, 456 a la producción de detergentes, 165 a la producción de biodiesel y 544 a la producción de alimentos y piensos. Respecto a las publicaciones científicas, se han encontrado 1352, 117, 439 y 315 documentos respectivamente para cada una de las aplicaciones mencionadas (Daiha et al., 2015).

El sitio activo de las lipasas contiene también otras estructuras que facilitan el proceso catalítico tal como el "bolsillo de unión-escisión de ácido graso", que es una cavidad variable, situada dentro de la cavidad de unión al sustrato, responsable del alojamiento de la cadena de acilo del éster que será hidrolizado por la enzima. Las esteresas tienen un pequeño bolsillo de unión que se adapta óptimamente al resto acilo de sus sustratos cortos. Las lipasas o "lipasas verdaderas" tienen en cambio un sitio de unión a ácido graso escindible, largo, hidrófobo, situado dentro de la cavidad de unión (en una hendidura, en la pared de un embudo de unión o en un túnel) (Pleiss et al., 1999). La longitud y la hidrofobicidad de este sitio de unión deben correlacionarse con el perfil de longitud de cadena de ácido graso de la lipasa. También son frecuentes bolsillos de unión adicionales para las otras cadenas de acilo de sustratos tales como triacilgliceroles (TAG), que

contribuyen a la unión del sustrato al sitio activo de la enzima durante la catálisis (Pleiss et al., 1998).

Durante el mecanismo de reacción, luego de la unión del sustrato éster, se forma un primer intermedio tetraédrico por ataque nucleofílico de la serina catalítica; la estabilización de este átomo de oxígeno de carbonilo aniónico que se forma como intermedio tetraédrico que se produce durante la catálisis de la lipasa, se consigue mediante la colaboración de la formación de un "*oxyanion hole*", utilizando dos enlaces de hidrógeno, que son proporcionados por dos grupos amida del esqueleto de la proteína (Henke et al , 2002). El enlace éster se escinde y el resto alcohol sale de la enzima. En una última etapa, el complejo enzima-acilo se hidroliza. Las lipasas requieren agua para hidrolizar el enlace de éster de acilo. El ataque nucleofílico por la serina catalítica está mediado por la histidina catalítica y el ácido aspártico (o glutámico) (Pleiss et al., 1998). El mecanismo para la hidrólisis o formación de ésteres es esencialmente el mismo para las lipasas y las esterasas (Bornscheuer et al., 2002a, Jaeger et al., 1999).

En cuanto a la clasificación de las lipasas, as enzimas lipolíticas bacterianas se clasificaron inicialmente en ocho familias (Arpigny y Jaeger, 1999). Esta clasificación se basa en la similitud de la secuencia de aminoácidos y la presencia de motivos conservados, como el patrón GDSL de las lipasas de la familia II. Otra clasificación describe tres clases de lipasas, que no son sólo para las lipasas bacterianas, y se construyó de acuerdo con los aminoácidos involucrados en la formación del *oxyanion-hole* (Pleiss et al., 2000).

1.2 Descubrimiento de las Lipasas

Las lipasas son ubicuas en la naturaleza y son producidas por plantas, animales y microorganismos. Las lipasas de origen microbiano, principalmente bacterianas y fúngicas, representan la clase más ampliamente utilizada como enzimas en aplicaciones biotecnológicas y química orgánica (Gupta et al., 2004). La gran versatilidad de las lipasas fúngicas deriva de géneros como *Candida*, *Geotrichum*, *Rhizopus* y *Thermomyces*, mientras que entre las lipasas bacterianas la atención suele apuntar a clases particulares de enzimas como las lipasas del género *Pseudomonas* y *Bacillus* (Arpigny y Jaeger, 1999). Las lipasas bacterianas extracelulares son de considerable importancia comercial, ya que su producción es mucho más fácil y no requieren cofactores. Aunque existen varias fuentes bacterianas productoras de lipasas, sólo unas pocas son explotadas comercialmente como cepas silvestres o recombinantes para la producción de lipasa (Gupta et al., 2004).

A pesar de que existe un mayor conocimiento de la superfamilia de las enzimas de tipo α/β-hidrolasas, hoy en día se requieren nuevas lipasas para convertir la biomasa en biocombustibles de segunda y tercera generación, nuevos biomateriales o productos químicos de alto valor añadido (Bornscheuer et al., 2012). Además, para producir procesos basados en tecnología verde rentables, se debe dar especial énfasis al desarrollo de enzimas reutilizables y estables en condiciones extremas (Gupta et al., 2015).

Los microorganismos lipolíticos se encuentran en una variedad de hábitats incluyendo suelos contaminados con aceites, desechos de aceites vegetales, desechos lácteos y alimentos deteriorados (Gupta et al., 2015). Las lipasas se han aislado y purificado de hongos, levaduras, bacterias, plantas y fuentes animales (Snellmanet et al., 2002) para su aplicación en procesos productivos más sostenibles.

La diversidad microbiana es un recurso importante para el aislamiento biotecnológico de productos de valor agregado y la implementación de procesos ecológicos. La biosfera está dominada por microorganismos, pero la mayoría de los microorganismos en la naturaleza no han sido estudiados (Gupta et al., 2004). Además, la gran mayoría que están presentes en un solo nicho medioambiental no son cultivables en el laboratorio y se estima por tanto que, en promedio, se ha identificado menos del 1% (Lorenz et al., 2002).

La creación de "librerías de metagenomas" es una estrategia prometedora con respecto a la selección de nuevos biocatalizadores adecuados. La posibilidad de buscar en todo el ADN presente en un hábitat definido (metagenoma) para las actividades deseadas, permite el acceso a un grupo de nuevas enzimas que no se pudo encontrar utilizando métodos basados en cultivos. La principal limitación para detectar realmente todo el metagenoma son los sistemas de expresión heterólogos utilizados en la construcción: podría suceder que muchos genes no sean expresados correctamente por el huésped heterólogo y, por tanto, no se detecten (Lorenz y Eck, 2005).

El enorme progreso en la tecnología de secuenciación en combinación con bibliotecas metagenómicas ha llevado a un aumento exponencial en el número de datos de secuencia (actualmente aproximadamente 20 millones de secuencias) en las bases de datos. Sin embargo, la anotación de la función de una proteína putativa se realiza automáticamente y, en consecuencia, puede conducir a interpretaciones erróneas (Davids et al., 2013). Por consiguiente, una fuente adicional de nuevas enzimas es la base de datos de estructura de proteínas Brookhaven (PDB), que contiene numerosas proteínas, donde la estructura 3D

se ha depositado, pero donde las proteínas no han sido necesariamente bioquímicamente caracterizadas (Davids et al., 2013).

Otra estrategia que ha recibido una atención considerable se basa en las búsquedas basadas en la actividad. Por ejemplo, se realizó una nueva investigación con microorganismos tolerantes a los disolventes, permitiendo el aislamiento de un microorganismo que puede sobrevivir en presencia de una gama de disolventes orgánicos y lógicamente sus enzimas son catalizadores estables y eficientes para funcionar en dichos medios disolventes (Gupta y Khare , 2009).

1.3 Lipasas ensayadas en el presente trabajo

1.3.1 Nueva esterasa Est23 de *Paenibacillus barcinonensis*

Paenibacillus barcinonensis es una bacteria del suelo que contiene un conjunto complejo de enzimas para la degradación de polisacáridos y lípidos vegetales. Este microorganismo fue aislado e identificado a partir de un suelo de arroz en el delta del río Ebro (España) (Blanco y Pastor, 1993; Sánchez et al., 2005). *P. barcinonensis* es una bacteria grampositiva formadora de endosporas, anaeróbica y facultativa.

P. barcinonensis ha demostrado ser una muy buena fuente de enzimas hidrolíticas. Hasta la fecha, cuatro celulasas (Blanco et al., 1998; Pastor et al., 2001; Sanchez et al., 2003), seis xilanasas, (Gallardo et al., 2003, 2010; Sainz-Polo et al., 2014; Valenzuela et al., 2010, 2014, 2016), y dos pectinasas (Soriano et al., 2000) ya han sido aisladas, clonadas y caracterizadas bioquímicamente. Además, algunos de ellos muestran interesantes aplicaciones biotecnológicas (Bassegoda et al., 2010, Cadena et al., 2010, Fillat et al., 2015, Valenzuela et al., 2014).

La presencia de halos de hidrólisis al crecer en placas suplementadas con tributirina indicó también la presencia de actividad lipolítica. El análisis de zimograma reveló la presencia de al menos dos enzimas que muestran actividad sobre *muf*-butirato. Se aisló, clonó y caracterizó previamente una carboxilesterasa de tipo B (Prim et al., 2000), mostrando actividad sobre sustratos de ácidos grasos de longitud de cadena corta, que se mejoró adicionalmente para la resolución de alcoholes terciarios (Bassegoda et al., 2010) . La segunda enzima que muestra actividad lipolítica fue aislada y caracterizada en el presente trabajo.

1.3.2 LipR de la cepa *Rhodococcus* sp. CR-53

La cepa CR-53 de *Rhodococcus* sp. se aisló previamente en nuestro laboratorio a partir de una muestra de suelo subtropical (Ruiz et al., 2005). Las pruebas fisiológicas más el análisis del gen 16S rRNA de la cepa revelaron un alto nivel de similitud (99%) con otras cepas descritas como *Rhodococcus erythropolis*, aunque no se obtuvo una concordancia perfecta (Falcocchio 2005). Un rasgo notable de la cepa fue su alta actividad lipolítica, siendo así una buena cepa candidata como productora de lipasa.

Se aisló la lipasa LipR de *Rhodococcus* sp. cepa CR - 53 y fue caracterizada en un trabajo previo (Bassegoda et al., 2012a). LipR se propuso como el primer miembro de la nueva familia X de la clasificación de Aspigny-Jaeger. Además, LipR presenta el mismo *oxyanion-hole* que el de CalA, clasificado como *oxyanion-hole* de tipo Y, y la secuencia tiene identidad con algunas lipasas secretoras de *C. albicans*, clasificadas como miembros de la super familia de lipasas similares a la lipasa de *C. albicans*. Curiosamente, ninguna de las lipasas utilizadas para definir las diferentes familias de lipasas bacterianas descritas presenta *oxyanion-hole* de tipo Y, por lo que este hecho proporciona pruebas de las diferencias existentes entre LipR y todos los miembros de las familias de lipasas bacterianas descritas hasta la fecha.

El análisis de la secuencia de aminoácidos de LipR reveló un alto contenido de aminoácidos no polares (59,3%) y la presencia de un péptido señal, indicando una localización extracelular. Un modelo de homología LipR 3D construido usando *Candida antarctica* lipase A (CalA) como molde permitió detectar la presencia de un enlace disulfuro entre las cisteínas Cys388 y Cys432. LipR se describió como una enzima mesofílica mostrando preferencia por grupos acilo de cadena media sin mostrar activación interfacial, y mostró buena estabilidad a largo plazo (Bassegoda et al., 2012a).

1.3.3 Lipasas LipA y LipC de *Pseudomonas* sp. 42A2

Los genes *lipA* y *lipC* aislados de *Pseudomonas* sp. 42A2 se clonaron con su foldasa específica (LipH) en el vector pBBR1MCS (Kovach et al., 1994) y se transformaron en el huésped homólogo *Pseudomonas* PABST7.1 para sobreexpresión y caracterización (Bofill et al., 2010). Ambas lipasas mostraron preferencia por sustratos de longitud de cadena de ácidos grasos medios. Sin embargo, se pudieron detectar diferencias entre LipA y LipC en términos de cinética enzimática y en cuanto al patrón del comportamiento. En

consecuencia, LipA mostró máxima actividad a temperaturas moderadas, y mostró una cinética típica de Michaelis-Menten. Por el contrario, el LipC fue más activo a bajas temperaturas y mostró una activación interfacial parcial, mostrando un cambio en la especificidad del sustrato cuando se ensayó a diferentes temperaturas. Otros ensayos de ingeniería de proteínas realizados en LipC permitió el aislamiento de LipCmut, una variante LipC termoestable que mantiene sus propiedades psicófilas (Cesarini et al., 2012).

1.4 Mejora de Lipasas por Inmovilización

Para producir una tecnología rentable basada en catálisis enzimática, se debe dar especial énfasis al desarrollo de enzimas reutilizables y estables bajo condiciones adversas. La inmovilización facilita la reutilización de enzimas y la purificación del producto, mejorando así drásticamente la economía del proceso (Gupta et al., 2015).

Por lo tanto, los biocatalizadores pueden ser mejorados por métodos físico-químicos. La inmovilización a menudo estabiliza un biocatalizador y facilita el procesamiento posterior mediante una fácil separación de la enzima. Además, la inmovilización implica comúnmente la ventaja de un aumento de la actividad catalítica en comparación con la enzima no inmovilizada (Adlercreutz et al., 2013).

Se han empleado diversas técnicas de inmovilización tales como adsorción, unión covalente, atrapamiento, encapsulación o reticulación (Fig. I4) sobre lipasas y se han utilizado para la producción de biodiesel (Tan et al., 2010).

La adsorción es la unión de la enzima en la superficie del soporte por fuerzas débiles, tales como van der Waals, interacciones hidrofóbicas o fuerzas de dispersión. Los soportes utilizados incluyen resinas acrílicas, membranas textiles, polipropileno, celite o tierra de diatomeas (Tan et al., 2010). La adsorción física es quizás el método más simple para la inmovilización enzimática. Una solución enzimática, usualmente acuosa, simplemente se pone en contacto con un material de soporte y, siempre que las interacciones enzima-soporte sean suficientemente fuertes, se producirá la adsorción. En el caso de las lipasas, las interacciones hidrofóbicas son las más comunes, pero también pueden ser útiles interacciones iónicas con materiales de intercambio iónico.

Con respecto al tamaño de los poros de los soportes, los diámetros de alrededor de 100 nm parecen ser ventajosos, aunque sólo se han llevado a cabo pocos estudios sobre la importancia de este parámetro. La mayoría de las sílices mesoporosas tienen por lo tanto

unos poros algo estrechos, mientras que algunos de los polímeros orgánicos porosos utilizados para la inmovilización de lipasas tienen una fracción considerable del volumen de poros suficientemente grandes, tales como el polipropeno Accurel EP-100 y Accurel MP- 1004 (Adlercreutz et al., 2013). Se descubrió que Accurel MP, un polímero macroporoso muy hidrofóbico de propileno, no adsorbe el glicerol, una ventaja importante durante la síntesis de biodiesel por las lipasas (Séverac et al., 2011). Por lo tanto, este método puede usarse para la purificación e inmovilización simultánea de lipasas (Adlercreutz et al., 2013).

1.5 Mejora de Lipasas por Ingeniería de Proteínas

Las lipasas son enzimas muy estudiadas, cuyo mecanismo de reacción es bien conocido, siendo a menudo enzimas estables y fáciles de manejar en el laboratorio. En consecuencia, la ingeniería de proteínas de las α/β-hidrolasas no sólo se ha utilizado para resolver problemas en la biocatálisis aplicada, sino que también se ha utilizado para estudios como pruebas de principio para establecer nuevos métodos de ingeniería de proteínas (Jochens et al., 2011).

Los primeros enfoques de ingeniería de proteínas a través de mutagénesis dirigida implicaron ciclos iterativos de cambios aleatorios de aminoácidos en una proteína, seguido por selección o selección de las bibliotecas resultantes para variantes con estabilidad enzimática mejorada, especificidad de sustrato y enantioselectividad. Los cambios en la especificidad del sustrato u otras propiedades enzimáticas pueden ser monitoreados por métodos de alto rendimiento, tales como la clasificación por células activadas por fluorescencia o la producción de color resultante por catálisis, que puede detectar decenas de millones de variantes en un corto período de tiempo. Los desarrollos más recientes se han centrado en mejorar la eficiencia de la evolución dirigida para crear bibliotecas "más inteligentes" obtenidas mediante el diseño racional (Bornscheuer et al., 2012). La solución más simple es una detección más eficiente. Otro enfoque es limitar la localización de los cambios en el sitio activo y restringir el tipo de cambios a los conocidos a partir de las comparaciones de secuencias que se producen a menudo en estos sitios en enzimas similares (Bornscheuer et al., 2012). Este concepto combina la información estructural con secuencias de proteínas y se basa en el "enfoque de consenso" que significa que la mayoría de los aminoácidos abundantes en cada posición en un grupo de enzimas homólogas contribuyen más que el promedio al rendimiento de proteínas que los aminoácidos no consensuados; Por lo tanto, la comparación de

secuencias dentro de grandes familias de enzimas puede ayudar a identificar los aminoácidos conservados como residuos de mutación caliente y, en consecuencia, se puede diseñar una mutagénesis semi-racional (Davids et al., 2013). En este contexto, el diseño de mutantes con la especificidad o propiedad de sustrato deseada se puede realizar identificando restos funcionalmente relevantes aparte de los residuos del sitio activo. Para las lipasas, esto puede lograrse usando, por ejemplo, la herramienta de navegación de la Base de Datos para Ingeniería de Lipase (*Lipase Engeneering Database* o LED) (Fischer y Pleiss, 2003).

Una estrategia que aprovecha varias regiones predefinidas consideradas cruciales para mejorar una propiedad catalítica dada es la mutación por saturación iterativa. Con la aplicación sistemática de la mutagénesis de saturación únicamente a "sitios blanco", como se sugiere por consideraciones racionales que resultan de información estructural, sólo se consideran partes definidas de una enzima. El proceso iterativo permite entonces una alta presión evolutiva en regiones confinadas del espacio de la secuencia proteica, lo que incrementa la probabilidad de éxito mientras se reducen los costos, el tiempo y el esfuerzo humano (Reetz y Carballeira, 2007).

En los últimos años, el diseño de proteínas computacional está recibiendo cada vez más atención como una estrategia novedosa para predecir los efectos de las mutaciones en la estructura, función o estabilidad de las bibliotecas de variantes enzimáticas generadas por métodos *in silico* (Bassegoda et al., 2012b; Dalby, 2007).

El principio de la evolución dirigida enfocada se extendió por ejemplo con el desarrollo de la Prueba Combinatoria de Saturación de Sitios Activos (CASSTing) (Sandström et al., 2012), entre otras herramientas. En ese caso se utiliza la información derivada de datos estructurales/funcionales para identificar aminoácidos en la bolsa de unión de sustrato con el fin de mejorar habitualmente la actividad y la selectividad de las enzimas diana. Entonces los residuos identificados para la aleatorización están mutados de una manera iterativa (Cheng et al., 2015, Reetz et al., 2006b). Para demostrar el poder de este enfoque, se usaron enzimas α/β-hidrolasas (Jochens et al., 2011).

El objetivo final de la ingeniería de proteínas es entender cómo las enzimas funcionan de forma mecánica y lo que determina propiedades tales como la estabilidad, la especificidad y la actividad. Este conocimiento conducirá a una comprensión más profunda de la

catálisis y permitirá la creación de enzimas personalizadas para la ciencia farmacéutica, la medicina y la química sintética (Jochens et al., 2011).

1.6 Aplicación de Lipasas en la transestrificación de triglicéridos

Las lipasas son activas tanto en la presencia como en la ausencia de agua, por lo que son posibles reacciones inversas de hidrólisis del éster, esterificación o transesterificación. Por lo tanto, la gama de reacciones a catalizar por las lipasas se amplía considerablemente (Kourist et al., 2010). La mayor aplicación de lipasas se basa principalmente en el interés debido su alta actividad y estabilidad en sistemas no acuosos (disolventes orgánicos, líquidos iónicos), un amplio rango de sustratos y una alta estereoselectividad (Bornscheuer, 2002a).

En la producción de éster monoalquílico o biodiésel, la reacción de transesterificación se produce entre el aceite (triacilgliceroles), que puede ser aceite vegetal, grasa animal o grasa, y un alcohol en presencia de un catalizador, ya sea químico (ácido o base) o enzimático. Es una secuencia de tres reacciones reversibles en las que los triacilgliceroles (TAGs) se convierten en diacilgliceroles y luego los diacilgliceroles se convierten en monoacilgliceroles, seguido por la conversión de monoacilgliceroles en glicerol. La transesterificación de triacilglicéridos con metanol produce ésteres metílicos de ácidos grasos (FAME) junto con glicerol (Narwal y Gupta, 2013).

La transesterificación de aceites vegetales es el método más popular para producir esta mezcla de ésteres monoalquílicos de ácidos grasos superiores o biodiesel. El componente de alta viscosidad, el glicerol, se elimina y por lo tanto el producto tiene baja viscosidad como la de los combustibles fósiles. La mezcla de estos ésteres monoalquílicos puede por lo tanto utilizarse como sustituto para tales combustibles (Ranganathan et al., 2008).

Las enzimas muestran mayores rendimientos y tienen vidas más largas que los catalizadores químicos cuando se usan en aceites ricos en AGL, como es el caso de muchas materias primas crudas (Cesarini et al., 2013). Pero los aceites crudos suelen tener también un alto contenido de fosfolípidos (gomas) que pueden inhibir las lipasas durante la producción de biodiesel (Fjerbaek et al., 2009; Shimada et al., 2002). Los fosfolípidos son los componentes principales eliminados durante el desgomado y se identificaron en extractos de lipasas inmovilizadas usadas para metanólisis de los aceites crudos, causando adsorción e inhibición enzimática (Chen et al., 2009; Lv et al., 2010). Una opción para

evitar tales problemas de inhibición es llevar a cabo el desgomado enzimático simultáneo a la transesterificación en un proceso de un solo paso (Cesarini et al., 2014b, Shimada et al., 2002).

La producción de biodiesel por proceso alcalino a escala industrial es rentable y altamente eficiente (Sotoft et al., 2010). Sin embargo, la materia prima representa más del 70% del coste total de producción. En el futuro, el aceite de *Jatropha*, el aceite microbiano, el aceite de microalgas y el aceite residual se convertirán en la principal materia prima para la producción de biodiesel (Tan et al., 2010). Una forma de disminuir el costo del biodiesel es usar productos de desecho en lugar de aceites refinados. Como se ha indicado anteriormente, éstos a menudo tienen un alto contenido de ácidos grasos libres (AGL) y gomas, lo cual es favorable para el uso de enzimas. Desafortunadamente, los aceites utilizados como alternativos a materias primas de uso alimentario son mucho más costosos y son tienen más complicaciones en ser transformados en biodiesel con catalizadores químicos (Fjerbaek et al., 2009; Sotoft et al., 2010). Por lo tanto, el uso de enzimas como las lipasas es una buena alternativa para resolver estos problemas.

2. Objetivo

El grupo de enzimas microbianas para aplicaciones industriales trabaja en la identificación y diseño de enzimas para aplicaciones biotecnológicas. El grupo de investigación trabaja por tanto en la caracterización bioquímica y manipulación genética de lipasas, esterasas, celulasas, xilanasas, expansinas y polisacáridos monoxigenasas líticas, desde las primeras etapas de muestreo, clonación y purificación hasta estudios de relación estructura-función e ingeniería enzimática, convencionales y también *in silico*.

La importancia de las lipasas está aumentando en varias industrias. Sin embargo, el uso comercial de lipasas es todavía un inconveniente en la economía de las aplicaciones industriales basadas en lipasa. Existen muchas herramientas para mejorar y adaptar las propiedades enzimáticas a los requisitos deseados de un proceso que podría conducir a la catálisis de lipasas mediante un proceso rentable. En este contexto, el objetivo principal de este trabajo fue: "Caracterizar, expresar y mejorar nuevas lipasas bacterianas para procesos industriales sostenibles".

3. Resultados y Discusión

3.1 Explorando una nueva esterasa de *P. barcinonensis*

P. barcinonensis muestra un conjunto de hidrolasas y una esterasa, EstA, que ya han sido identificada y caracterizada en nuestro grupo (Prim et al., 2000; Sánchez et al., 2005). La actividad lipolítica de una segunda enzima se detectó mediante zimograma, como una banda adicional distinta de la de EstA. Durante un procedimiento de paseo cromosómico, se encontró la secuencia de un gen putativo de lipasa/esterasa junto a un gen que codifica una celulasa. La secuencia fue amplificada y se clonó en *E. coli* utilizando un vector adecuado para la expresión. La nueva enzima, denominada Est23, se sobreexpresó exitosamente en *E. coli*, mostrando actividad lipolítica. Esta nueva enzima se purificó y caracterizó durante este trabajo (Artículo 1), mostrando preferencia por sustratos de cadena media y con actividad máxima a 37 ° C y pH 7. Además, la enzima mostró una cinética típica de Michaelis-Menten, sin ningún efecto interfacial significativo, mostrando por tanto la cinética enzimática típica de una esterasa (Jaeger et al., 1999).

En un primer análisis de la estructura secundaria, se identificó un péptido señal que contenía una región transmembrana mediante análisis secuencial usando SignalP 3.0. Esto también se confirmó a partir del análisis de estructura obtenido a partir del servidor web Phyre[2], donde de nuevo las hélices transmembrana se predijeron como un péptido señal (residuos 1 - 25). Las enzimas lipolíticas secretadas son principalmente lipasa "verdadera"; Por lo tanto, basándose en esas predicciones, el ORF clonado podría haber correspondido al de una lipasa. Sin embargo, después de los resultados obtenidos de la caracterización bioquímica, se llevó a cabo un análisis más preciso con SignalP 4.0, indicando que Est23 no tiene realmente un péptido señal real, por lo que probablemente sea una esterasa intracelular, como es de esperarse para una enzima que muestra las propiedades de una esterasa, como Est23.

El modelo 3D de Est23, construido usando la estructura más cercana (pdb c1cr6), permitió la localización de la tríada catalítica Ser146, Asp294 e His324 por similitud, y el *oxyanion-hole* (G71GPG) en las posiciones predichas fue confirmado (Fig. 1). Además, el análisis de la estructura 2D y 3D de Est23, no reveló la presencia de una tapa que cubre el sitio activo en las lipasas, otro hecho que apoya aún más la idea de Est23 es una esterasa.

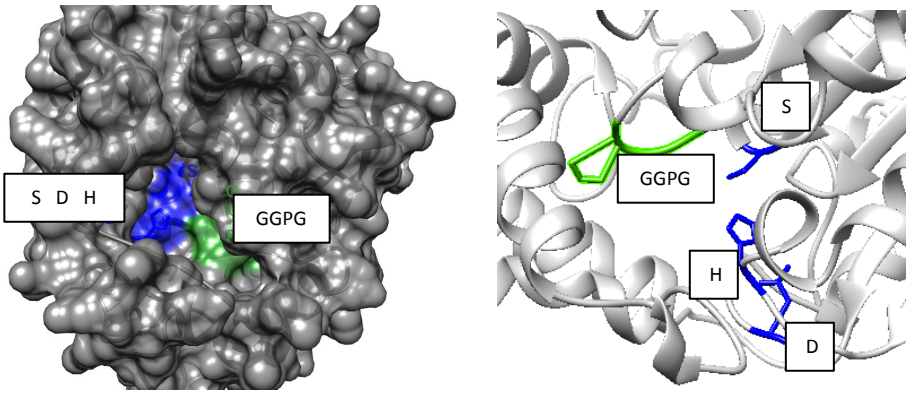


Figura 1. Modelo 3D de Est23 con los residuos catalíticos y los residuos del *oxyanion hole* resaltados; El putativo *oxyanion hole* GGG(A)X-type en verde ($G^{71}GPG$), y los residuos de la triada catalítica (Ser^{146}, Asp^{294} and His^{324}) en azul. El modelo 3D fue generado con Phyre^2 *server* y obtenido utilizando como templado el pdb c1cr6. Se visualizó la estructura con UCSF Chimera.

Se construyó un árbol filogenético para asignar Est23 a una de las familias de hidrolasas bacterianas descritas por Arpingy y Jaeger (Arpigny y Jaeger, 1999). Est23 no se pudo asignar a las hidrolasas bacterianas descritas hasta ese momento, lo que sugiere que Est23 podría ser parte de un nuevo grupo de lipasas bacterianas.

Est23 muestra un *oxyanion-hole* de tipo GGG (A), ampliamente descrito como motivo conservado en esterasas capaces de catalizar la resolución de alcoholes terciarios (Henke et al., 2002). Además, se informó también que el motivo GGG (A) X de algunas enzimas puede ser mutado sólo en la primera y última posición del motivo sin perder actividad hacia los ésteres de alcohol terciario (Gall et al., 2010). En consecuencia, se evaluó la capacidad de Est23 para la conversión y resolución de alcoholes terciarios en condiciones previamente establecidas en nuestro laboratorio (Bassegoda et al., 2010). Los productos liberados se analizaron mediante cromatografía de gases y espectrometría de masas. Sin embargo, no se detectó ninguna conversión usando los ésteres de los alcoholes de acetato de linalilo y terpenilo como sustratos. Se deben probar más sustratos para encontrar una mejor interacción con la enzima (Fillat et al., 2014). También se podrían producir nuevas variantes de Est23 mejoradas, por ejemplo cambiando el residuo rígido P71 por una alanina o una glicina, ambos aminoácidos más pequeños y reportados como frecuentes en esterasas bacterianas (Bassegoda et al., 2010) y con un efecto mayor que otros aminoácidos sobre la actividad y la enantioselectividad (Gall et al., 2010).

3.2 Mejora de LipR por evolución dirigida

Para la aplicación de LipR se requería una mejora en cuanto al rendimiento de la enzima. Por lo tanto, se planteó una mejora centrada directamente en el aumento de la actividad LipR en sustratos de cadena larga específicamente. Para este propósito, se eligió un enfoque de mutagénesis dirigida con el fin de elucidar y mejorar los residuos implicados en el excepcional *oxyanion-hole* de tipo Y de LipR. Por lo tanto, se desarrolló una estrategia basada en el diseño racional para revelar el rol de residuos conservados en el *oxyanion-hole* en la conversión de ácidos grasos (Bassegoda et al., 2012b; Fillat et al., 2014). El análisis de la estructura de LipR se informó en un trabajo anterior (Bassegoda et al., 2012a). La alineación de secuencias que constituyen las lipasas de la familia X, del grupo LipR, y el modelo de homología 3D de LipR mostraron diferentes motivos de secuencia de aminoácidos conservados situados cerca del sitio activo de la enzima. La secuencia Tyr-Asp-Ser-Leu se encontró en un bucle cercano a la serina catalítica, constituyendo un motivo de secuencia de aminoácidos altamente conservado (Fig. 2). Este hecho y la similitud de este motivo con el *oxyanion-hole* de CalA, sugiere fuertemente que estos residuos podrían ser esenciales para la unión del sustrato. Además, LipR mostró una identidad de secuencia significativa con algunas lipasas secretoras de *C. albicans*, también clasificadas como miembros de la familia *C. albicans* de tipo lipasa. Sin embargo, ninguno de los miembros de lipasa de esa familia y que portan el *oxyanion-hole* de tipo Y son lipasas bacterianas.

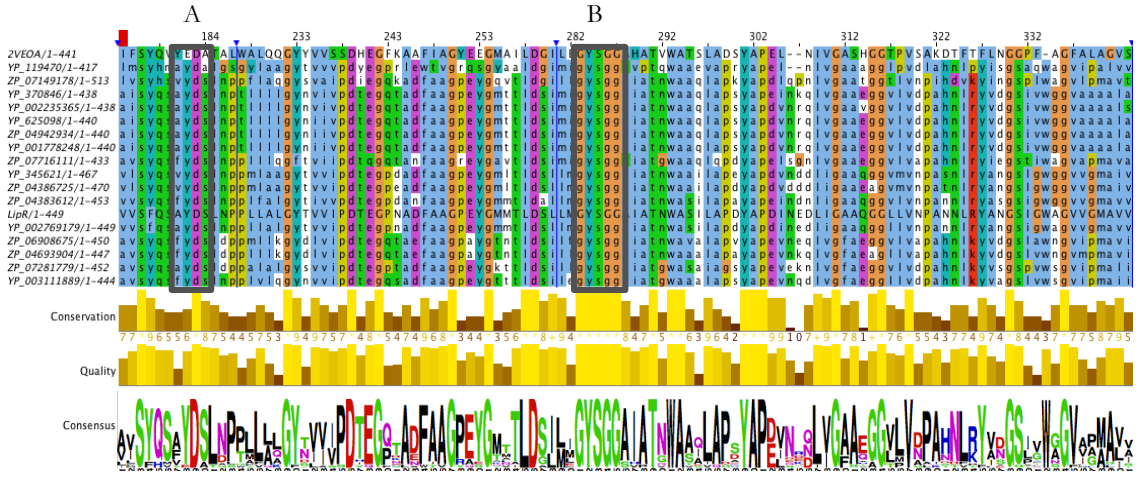


Figura 2. Bloques de secuencias conservadas en los alineamientos de secuencias entre LipR, las lipasas putativas de la familia X y CalA. (A) motivo *oxyanion hole*, (B) pentapeptido GYSGG. YP_002769179: putativa lipasa *R. erythropolis* PR4. ZP_04383612: putativa triacilglicerol lipasa *R. erythropolis*

SK121. YP_345621: putativa lipasa *R. erythropolis* PR4. ZP_04386725: triacilglicerol lipasa *R. erythropolis* SK121. ZP_07281779: putativa triacilglicerol lipasa *Streptomyces* sp. AA4. ZP_06908675: putativa triacilglicerol lipasa *S. pristinaespiralis* ATCC 25486. ZP_04693904: putativa lipasa *S. roseosporus* NRRL 15998. YP_003111889: putativa triacilglicerol lipasa *Catenulispora acidiphila* DSM 44928. ZP_07716111: putativa triacilglicerol lipasa *Aeromicrobium marinum* DSM15272. YP_002235365: putativa lipase *Burkholderia cenocepacia* J2315. ZP_04942934: putativa triacilglicerol lipasa *B. cenocepacia* PC184. YP_625098: putativa triacilglicerol lipasa *B. cenocepacia* AU 1054. YP_001778248: putativa triacilglicerol lipasa *B. cenocepacia* MC0-3. YP_370846: putativa triacilglicerol lipasa *Burkholderia* sp. 383. ZP_07149178: putativa triacilglicerol lipasa Corynebacterium resistens DSM 45100. YP_119470: putativa lipasa *Nocardia farcinica* IFM 10152. 2VEO: *Candida antartica* lipase A.

Basándose en el *oxyanion-hole* de tipo fúngico encontrado en LipR, se diseñó un cebador para realizar la mutagénesis, obteniéndose cinco variantes con cinco secuencias de *oxyanion-hole* que cubrían las cinco más típicas descritas en lipasas bacterianas. Sorprendentemente, un ensayo de zimograma de los mutantes LipR obtenidos reveló que las modificaciones introducidas no produjeron enzimas funcionales. La actividad se logró determinar sólo en la variante recuperada de tipo salvaje. Esto indica que el *oxyanion-hole* de tipo fúngico, de tipo Y es estrictamente necesario para la actividad de LipR. Los residuos del *oxyanion-hole* GGG (X) se reportan como altamente conservados entre las lipasas bacterianas y fúngicas. Trabajos previos han informado la mejora de la actividad hidrolítica y, por tanto, una mejora en la enantioselectividad de alcoholes terciarios mediante el cambio de uno de estos cinco residuos típicos (Fillat et al., 2014, 2015). En ese caso, la razón de la baja actividad de la enzima de tipo salvaje se debía a que tenía un aminoácido poco común en la tercera posición del *oxyanion-hole* (Bassegoda et al., 2010). Sin embargo, para LipR, esto no podría ser la razón de la baja actividad encontrada, lo que indica que LipR muestra un requisito estricto para el *oxyanion-hole* de tipo Y.

Se desarrolló un segundo enfoque de mutagénesis. Se construyeron dos bibliotecas mediante mutagénesis de saturación iterativa cambiando cada uno de los residuos putativos del *oxyanion-hole*, Tyr110 o Asp111, de LipR. El residuo Y110 define la clase de *oxyanion-hole*, mientras que D111 es equivalente a D95 de CalA (Fig. D2). Así, se obtuvieron dos bibliotecas iterativas de saturación y se optimizaron sus tamaños utilizando la hoja de trabajo CASTER, considerando el uso de codones y la degeneración apropiada aplicada (Reetz et al., 2006a). Se obtuvieron variantes para las dos posiciones cubriendo todos los grupos de aminoácidos. Como resultado, se obtuvo una pérdida

completa de actividad sobre substratos con diferentes longitud de cadena, al ensayar los mutantes de Tyr110.

Entre los mutantes Asp111 obtenidos, la variante Asp111Gly produjo un cambio en la preferencia de longitud de cadena de sustrato de LipR, mostrando un aumento de 5,6 veces de actividad en *muf*-oleato. Esta mejora de la actividad en sustratos con mayor longitud de cadena hace de esta variante de LipR una lipasa muy atractiva para la síntesis de biodiesel, un proceso que requiere lipasas con actividad en sustratos de cadena larga (Nielsen et al., 2008).

3.3 Mejora de la estabilidad de Lipasas por inmovilización

Para la aplicación de las enzimas seleccionadas se planteó primeramente proporcionar una mayor estabilización a las enzimas mediante inmovilización. La inmovilización constituye una estrategia de mejora ampliamente utilizada para aumentar la eficacia de una determinada enzima durante una reacción. Por consiguiente, se ensayó la inmovilización de las lipasas de *Pseudomonas* seleccionadas sobre tres soportes diferentes: Accurel MP1000 y EP100, por adsorción, y Celite®545 por precipitación. Para la inmovilización, las lipasas se expresaron en un huésped homologo de *P. aeruginosa*, de modo que se produjeron en el sobrenadante del cultivo en su forma activa (Bofill et al., 2010). Este hecho constituye una ventaja para obtener una mejor preparación de enzimas mediante inmovilización por adsorción directa al soporte a partir del sobrenadante del cultivo, omitiendo así la etapa de purificación y reduciendo los costes y el tiempo. Por lo tanto, se estableció un procedimiento de inmovilización de forma rápida y económica por adsorción.

Esta metodología de inmovilización por adsorción fue estandarizada, resultando muy eficaz, ya que después de 1 h de incubación de la muestra con el soporte más del 90% de la actividad del sobrenadante de LipC y LipCmut ya estaban inmovilizadas en las matrices de polipropileno. La inmovilización de LipA fue un poco más lenta, requiriendo más de 5 h para una inmovilización completa. Para evaluar la estabilidad de las enzimas inmovilizadas y elegir la mejor matriz para cada enzima, se ensayaron las preparaciones para transesterificacion de trioleina durante 24 h a la temperatura óptima de cada enzima. Se utilizaron como sustratos agua (10% p/p de aceite) y metanol (15% p/p de aceite). Se añadió metanol en dos etapas (7,5% cada una) para evitar la posible inhibición enzimática (Azócar et al., 2010). Los productos de reacción (FAME) se verificaron mediante cromatografía en capa fina (TLC) estandarizada también durante este trabajo. La TLC es

una técnica cualitativa pero muy sensible, que constituye una herramienta útil para la evaluación rápida de los productos de reacción (Fedosov et al., 2011). Los productos de transesterificación más altos fueron de aquellas reacciones catalizadas por LipC y LipCmut adsorbidas en Accurel MP1000. Otros trabajos han demostrado que el tamaño de partícula más pequeño tiene el contenido enzimático específico más alto (Oliveira et al., 2009; Séverac et al., 2011). Nuestros resultados están de acuerdo con esto: lipasas inmovilizadas en MP1000 muestran mejores resultados que los inmovilizados en EP100. Para las preparaciones de LipA, la inmovilización en Celite®545 resultó el método de inmovilización que mostraba aparentes mejores resultados que los obtenidos por inmovilización sobre portadores de polipropilenos.

3.4 Aplicación de Lipasas a la tranesterificación de aceites y Caracterización de materias prima

Se establecieron las mejores condiciones para la síntesis de FAME utilizando LipA-MP1000. Los productos obtenidos se analizaron para los contenidos de AGL y FAMEs después de 24 h de reacción. Por lo tanto, se ensayaron las mejores condiciones de metanol y agua, y se describieron en comparación con la enzima comercial de Novozym® 435, inmovilizado de CalB, una lipasa bien conocida con buen rendimiento en las reacciones de transesterificación (Azócar et al., 2010; Watanabe et al., 2001). En todos los casos se logró una clara mejora del porcentaje de producción de FAME.

Existe un interés en encontrar fuentes alternativas de TAG para la producción de biodiesel a gran escala sin afectar el suministro y el costo de los alimentos, ya que las materias primas ampliamente utilizadas en la actualidad para obtener biocombustibles provienen de cultivos de aceite comestible como la soja, la canola y los aceites de palma (Geris et al., 2007). La catálisis enzimática con lipasa permite aprovechar los contenidos de ácidos grasos libres (AGL) relativamente altos de la mayoría de los aceites no convencionales y no se desactiva debido los altos contenidos de agua que pueden presentar estos tipos de materias primas, hecho que en cambio no es muy favorable durante una catálisis química (Kumari et al., 2007). En este contexto, durante esta tesis se realizó la caracterización de aceites alternativos a ser utilizados como materias primas para la producción de FAMEs por catálisis enzimática.

Se probaron un total de cuatro aceites: trioleína comercial, aceite de soja desgomado, aceite de cocina de fritos y aceite del hongo *Mucor circinelloides* (MU). El contenido de AGL

de estas materias primas antes y después de la transesterificación se determinó mediante titulación. El alto contenido de AGL en algunas materias primas requiere un exceso de álcali durante una transesterificación química, causando con frecuencia formación de jabón insoluble y disminuyendo así los rendimientos finales (Shah et al., 2004). Por lo tanto, uno de los objetivos de este trabajo fue evaluar la eficiencia de cada enzima en la conversión de estos aceites no convencionales en FAMEs teniendo en cuenta su contenido en AGL. Las reacciones de transesterificación se ensayaron utilizando los diferentes aceites como sustratos en las condiciones de reacción estandarizadas para cada enzima inmovilizada. También se usó Novozym® 435 para evaluar la eficiencia en la transesterificación de las diferentes materias primas. Los productos de reacción para cada enzima y cada materia prima fueron entonces evaluados midiendo el contenido de FAME en % por cromatofrafía de gases y el de contenido de AGL por titulación.

En un análisis global, se obtuvo FAMEs con las enzimas LipA, LipC y LipCmut inmovilizadas en Accurel MP1000. Sin embargo, se obtuvieron mejores resultados cuando las reacciones fueron catalizadas por la enzima comercial Novozym®435, especialmente cuando se añadió metanol en dos etapas durante la reacción, obteniéndose un valor muy alto de 91% de FAMEs utilizando trioleína como sustrato, valor obtenido a pesar que los ensayos se hicieron a muy baja escala, con muy poco volumen de reacción.

El aceite de MU resultó en un sustrato muy atractivo de evaluar debido al alto nivel en el contenido de ácidos grasos libres, mono y diglicéridos que presentó, mientras que el nivel de triglicéridos fue muy bajo. Novozym® 435 utilizó de forma favorable los AGL iniciales, siendo capaz de esterificarlos para la síntesis de FAMEs en presencia de concentraciones bajas de agua. Bajo la primera condición ensayada para Novozym® 435 (agua 0% p/p de aceite y metanol 5% p/p de aceite), el valor más alto de la producción FAMEs del 43,7% se obtuvo utilizando MU como sustrato y los AGL iniciales disminuyeron hasta 12,7%. Sin embargo, cuando se ensayó la enzima comercial bajo condiciones de adición de metanol en dos pasos, se obtuvo un valor menor de FAMEs comparando con el obtenido con los otros aceites, 76% de FAMEs, aunque sin embargo se registró un mayor consumo de AGL, detectándose un 3,6% de AGL en el producto final. El contenido de agua del aceite de MU podría estar afectando el rendimiento de la enzima, ya que la actividad de Novozym® 435 disminuye significativamente con cantidades crecientes de agua (Haigh et al., 2014).

Respecto al aceite residual de fritos, teniendo en cuenta la cantidad de AGL, mono y diglicéridos, inesperadamente resultó en un aceite de buena calidad para la producción de FAME. Se encontró una conversión del 85% del aceite utilizando Novozym® 435 en condiciones óptimas. Sin embargo, debido a los usos dados al aceite de cocina, los aceites de desecho de frituras presentan un mayor contenido de agua. Por lo tanto, el rendimiento global de la reacción de síntesis de FAME podría mejorarse reduciendo la cantidad de agua en la muestra inicial.

Las lipasas de *Pseudomonas* inmovilizadas no fueron capaces de usar estos ácidos grasos libres para sintetizar FAMEs en las condiciones ensayadas. Aunque fueron capaces de hidrolizar los TAGs de las materias primas ensayadas, no se produjo esterificación subsiguiente, lo que indica que las condiciones de reacción todavía necesitan mejorarse.

Por último, este trabajo demostró cómo se pueden mejorar el desempeño las enzimas en las reacciones y cómo un proceso enzimático podría aprovechar un alto nivel de contenido de AGL en materias primas no convencionales, permitiendo así el uso de aceites desechos y sin refinar como materias primas para la transesterificación. Se requiere una optimización adicional de las condiciones de reacción para cada enzima sobre cada sustrato, incluyendo el uso de sistemas multienzimáticos que podrían ser una solución adecuada para obtener mejores resultados (Kourist et al., 2009), a la vez que se reducirían los costes disminuyendo la cantidad de enzimas comerciales al sr utilizadas con enzimas no comerciales.

4. Conclusiones

1- Se ha aislado un nuevo gen correspondiente a la segunda esterasa de *Paenibacillus barcinonensis*. La enzima codificada denominada Est23 se ha expresado en *E. coli*.

2- Est23 se purificó y se caracterizó bioquímicamente, revelando actividad carboxilesterasa.

3- En base a análisis de secuencias y estudios de alineación se concluye que Est23 pertenece a un grupo de lipasas de secuencia diferente por lo que probablemente constituya una nueva familia de enzimas lipolíticas bacterianas. A pesar de que se encontró que Est23 mostraba un *oxyanion-hole* de tipo GGG(A)X, descrito como un motivo importante para la resolución de éster de alcohol terciario, no se pudo detectar conversión ni resolución enantiomérica.

4- El cambio en la secuencia del *oxyanion-hole* de tipo Y, de tipo fúngico encontrado en LipR a motivos de secuencia típicos en lipasas bacteriana, ha demostrado su importancia, debido a que no se obtuvieron variantes con actividad.

5- No se detectó actividad en todas las variantes de LipR obtenidas a partir de una librería de mutagénesis dirigida al sitio del residuo Tyr110, indicando que este residuo es crucial para la actividad. El cambio de Asp111 por Gly causó un cambio en la especificidad de sustrato hacia substratos de mayor longitud de cadena.

6- Tres lipasas de *Pseudomonas* sp. 42A2, incluyendo LipA, LipC y LipCmut se estabilizaron eficientemente mediante un procedimiento de bajo coste por adsorción y recarga en tres ciclos en matrices de propileno, siendo Accurel MP1000 definido como el mejor soporte para reacciones de transesterificación con estas enzimas.

7- Se establecieron las mejores condiciones en reacciones de transesterificación de las lipasas de *Pseudomonas* inmovilizadas.

8- Con las reacciones estandarizadas se logró ensayar cuatro materias primas diferentes, incluyendo aceite de residual de fritos y un aceite de origen fúngico, en reacciones de transesterificación catalizadas por las lipasas previamente inmovilizadas, utilizando las mejores condiciones de carga enzimática, agua y metanol determinada para cada enzima.

Fueron necesarios entre 5 y 10% de agua y metanol en la reacción catalizadas por las lipasas de *Pseudomonas*.

9- Se ha descrito la caracterización en cuanto a contenidos de AGL y glicéridos de las materias primas utilizadas. Curiosamente, el aceite de *Mucor cicirnelloides* mostró un alto nivel de AGL, que sin embargo se pudieron convertir en FAMEs. El aceite de desecho de fritos demostró una alta calidad como materia prima, conteniendo AGL y diglicéridos bajos, siendo por tanto un aceite adecuado para la síntesis de FAMEs.

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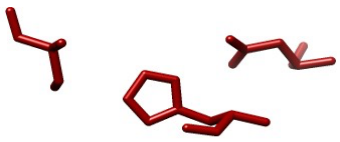

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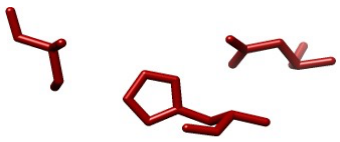
Annexes



1. Lipase Expression: Attempts for Improving LipR Expression

1.1 Summary

Recombinant enzymes have been widely expressed in bacteria, filamentous fungi and yeasts. There is no universal expression system for heterologous proteins. All expression systems have some advantages as well as some disadvantages (Rai and Padh, 2001). Therefore, in order to choose the appropriate expression system for the production of a recombinant enzyme, activity and yield are the main factors to consider.

E. coli is by far the most widely employed host when post-translational modifications of proteins is not desired, due to the vast knowledge about its genetics, physiology and complete genomic sequence, which greatly facilitates gene cloning and cultivation. *E. coli* can grow rapidly to high densities in simple and inexpensive media. The efficiency of expression could be reduced depending on differences in codon utilization by bacteria (Rai and Padh, 2001). Some codons are particularly rare in *E. coli*, and they have been shown to cause translational errors as well as lower protein expression. Codon optimization could improve the translational fidelity and thus enhance the expression of enzymes (Gustafsson et al., 2004).

The methylotrophic yeast *P. pastoris* is considered an excellent host for the production of enzymes from different sources. *P. pastoris* expression systems display perfect protein processing mechanisms, including signal peptide cleavage, protein folding, post-translational modifications inside the cell, and the ability of secreting its products into the medium with normal function (Cereghino and Cregg, 2000; Cregg et al., 2000). Besides, the strong promoter for alcohol oxidase, AOX1, is tightly regulated and induced by methanol when used for expression of the gene of interest. Many recombinant enzymes have been successfully expressed in *P. pastoris* (Bollok et al., 2009).

The low activity shown by cloned LipR prompted us to try to improve its expression in *E. coli* and *Pichia pastoris* systems, testing the results by measuring the hydrolytic activity. This objective was defined in an attempt to improve LipR production and activity for application of this enzyme in biocatalytic processes.

1.2 Materials and Methods

1.2.1 Strains, plasmids and chemicals

E. coli DH5α, *E. coli* BL21(DE3) and *E. coli* Origami™ (Table A1) were purchased from Novagen, and were routinely cultured overnight at 37°C in Luria-Bertani broth or on Luria-Bertani agar plates. For culturing different recombinant strains of *P. pastoris* X-33 (Invitrogen, Carlsbad, CA), buffered minimal methanol (BMM) medium (100mM buffer phosphate pH 6.0, 1.34% yeast nitrogen base, 0.00004% biotin, 0.5% methanol), buffered glycerol-complex (BMG) medium (100mM buffer phosphate pH 6.0, 1.34% yeast nitrogen base, 0.00004% biotin, glycerol) and buffered glucose-complex (BMD) medium (100mM buffer phosphate pH 6.0, 1.34% yeast nitrogen base, 0.00004% biotin, 2% glucose) were prepared. All media were supplemented with zeocin (100 mg/mL, Invitrogen, San Diego, CA). Plasmids pGaston (Hence 2003) and pET22 (Invitrogen) were used as expression vectors in *E. coli,* and plasmids pPICZαA and pGAPZαA (Invitrogen) were used as expression vectors in *P. pastoris*. The recombinant plasmid pGaston-LipR was constructed previously (Bassegoda et al., 2012). Syntheses of PCR primers were performed by Sigma and the synthetic *lip*R gene was synthetized by Genscript (USA) (Table A2). Restriction enzymes, Taq DNA Polymerase and T4 DNA ligase were purchased from Thermo scientific. All chemicals were of analytical grade and obtained from commercial suppliers. Plasmids pPICZαA A and pGAPZαA, zeocin and strain X-33 were provided by Dr. Francisco Valero from the Department of Chemical Engineering, UAB.

Table A1: Strains used in this work

Strain	Genotype	Reference
E.coli DH5α	φ80dlacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17(rk- mk+) relA1 supE44 Δ(lacZYA-argF)U169	(Hanahan, 1983)
E.coli BL21 (DE3)	*hsd*S *gal* (λ*c*I*ts*857 *ind*1 *Sam*7 *nin*5 *lac*UV5- T7 gene)	(Studier and Moffatt, 1986)
E. coli Origami™	Δ(*ara–leu)7697* Δ*lacX74* Δ*phoA PvuII pho*R *araD139 ahpC gale galK rps*LF'[*lac*+ *lacI*)*pro*] *gor522*::Tn*10 trx*B (KanR, StrR, TetR)	(Bessette et al., 1999)
Pichia Pastoris X-33	wild type (mut$^+$:operable AOX1 and AOX2 genes)	invitrogen

Table A2: List of primers used for amplification and sequencing. The sequence of synthetic gene *lipR* is also shown.

Primer	Sequence (5´- 3´)
Fw LipRsinATG E	attaGAATTCGCATCAAAGATTCTCTTTC
Rv LipRstop K	tttGGTACCTCAGCCCGCCGCGTG
AOX fw	GACTGGTTCCAATTGACAAGC
AOX rv	GCAAATGGCATTCTGACATCC
α-factor fw	TACTATTGCCAGCATTGCTGC
Optimized *lipr* sequence	ATGGCGAGCAAGATTCTGTTCCAGGGTGCGGTGGCGCTGACCGCGATTGCGCTGACCGGCATTACCGCGCCGACCGTGGC GGCGCAGCCGAGCGGCGACAGCTTCTACCAGTATGATGGTAGCGCGCCGCTGAGCAGCTACCAACCGGGTGCGGTGCTGA AGACCCGTACCCTGCCGTATCACGTGGTTAACATCCCGACCCCGCTGCAGGCGGTGCAAGTTCTGTACCGTAGCACCGATGC GCTGGGTCGTCCGAGCGCGAACGTTACCAGCATCCTGCGTCCGCCGAACGCGCAGCCGGATAAAGTGGTTAGCTTCCAAAG CGCGTATGACAGCCTGAACCCGGAGGATGGTCCGAGCCGTGCGATTGCGGGTAACACCCCGATTGGCAGCCTGACCGCGAG CGCGTATGACAGCCTGAACCCGGAGGATGGTCCGAGCCGTGCGATTGCGGGTAACACCCCGATTGGCAGCCTGACCGCGA GCGGTCGTAACGTGGCGTTTGGTGGCACCCTGGCGAGCGGTGAAGCGGTTATGTTTGGTCCGCTGCTGGCGCTGGGTTAC ACCGTGGTTATCCCGGACACCGAAGGTCCGAACGCGGATTTTGCGGCGGGTCCGGAATATGGCATGATGACCCTGGACAG CCTGCGTGCGGCGCGTAACACCCCGGAAACCGGTATCAGCGATAGCGCGGAAATTGGTCTGATGGGCTATAGCGGTGGCG CGATTGCGACCAACTGGGCGAGCATTCTGGCGCCGGATTATGCGCCGGACATCAACGAGGATCTGATTGGTGCGGCGCAG GGTGGCCTGCTGGTGAACCCGGCGAACAACCTGCGTTACGCGAACGGTAGCATTGGTTGGGCGGGTGTGGTTGGTATGG CGGTGGTTGGTCTGGCGCGTGCGTACGACATCGATTTCGACCCGTATCTGGGTGACCGTGGTCGTGAGCTGCTGCCGCGTA TTGAAGATGCGAGCATCGGCAACGTGATTCTGCAGTATCCGGGTGTTACCTGGCAAGACCTGGCGAAGCCGGAGTACGCGA ACCCGAACAGCGTGCCGGAATATGTGGATGTTGCGAACAAAATCAACATGGGTAACGCGCCGATCCCGACCATTCCGATGTT TATTGCGCAAGCGGCGAACGGTGCGCTGGAAGGCACCCAGCCGGGTGGCCCGGGTGTGGGTCCGGGTGATGGTGTGATGG TTGCGGGCGATGTTCGTAGCCTGGCGACCCGTTACTGCGATGCGGGTCTGAGCATCCAGTACGATCAATATGACACCATTAGC CACGTTCCGGGTGGCGCGCTGTGGCTGCCGGGTGCGATCACCTGGCTGAACGACCGTTTTAACGGTAGCCAACTGCCGAATA ACTGCAGCAACATCGCGCCGGGTAATAGCCTGGCGCCGGAGGAACATGCGGCGGGTTGA

1.2.2 Codon optimization of *lipR* and expression in *E. coli*

The gene sequence of LipR was optimized based on the codon usage bias of *E. coli*. The codon-optimized gene was synthesized by Genscript (USA). The expression vector of the *lipRopt* cloned in pET22 vector was transformed to *E. coli* Origami™ and *E. coli* BL21, and the recombinant clones were cultured in 100 ml LB medium supplemented with ampicillin (100 μg/mL) and incubated at 37°C. Exponential growth cultures (OD_{600nm} 0.8) were induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 18 °C for 16 h. Cells were disrupted by using a French Press, to obtain cell extracts. Recombinant clone *E. coli* Origami™ pGaston-LipR was cultured following the same conditions but the induction was done with 2% rhamnose and incubated at 37°C.

1.2.3 Cloning and transformation of recombinant pPICZαA and pGAPZαA into *P. pastoris*

For cloning LipR in the appropriate plasmids, the corresponding DNA fragment was first amplified with high fidelity polymerase (Thermo scientific), the amplicon ligated to pJET easy vector (Thermo scientific), and transformed into *E. coli* DH5α, as described by the supplier. This construction expressed *lipR* with its own signal peptide. For expression of LipR lipase in *Pichia pastoris*, the digested fragment was directionally cloned into the MCS of pPICZαA and pGAPαA vectors, and introduced in *E. coli* DH5α to verify by sequencing (UB Genomics Service) and then, the plasmidic DNA was isolated.

To integrate the recombinant pPICZαA-LipR and pGAPZαA-LipR plasmids into the *P. pastoris* X-33 genome, 5 μg recombinant plasmids were linearized by *Sac*I and B*sph*I respectively, followed by electroporation into *P. pastoris* into a pre-chilled 0.2 cm electroporation cuvette, and incubated on ice for 5 min prior to electroporation on a gene pulser (Biorad, USA). Electroporation was performed at 2 kV, 25 μF and 200 Ω. Approximately 1 ml of fresh medium was immediately added to the cuvette after electroporation. Subsequently, the electroporated cells were plated on YPD agar plates containing zeocin (100 μg/ml), and incubated for 3-7 days at 28 °C. Individual transformant colonies were then picked from the plates and plated as above described for three more times. After that, colony PCR was done using external primers in order to confirm the integration of the plasmid into de genome of *P. pastoris*.

1.2.4 Expression of LpR in *Pichia pastoris* and growth conditions

Cultures in shake flasks were performed for each clone as follows: 250-ml volume baffled Erlenmeyer flasks containing 40 mL of BMG medium were inoculated from 24 h falcon cultures to an initial OD_{600} of 0.1, and incubated at 28°C and 130 rpm agitation. After 18–20 h, cells were harvested and used to inoculate 250-ml volume baffled erlenmeyer flasks with 40mL BMM medium to an OD_{600} of 1 for pPICZαA-LipR clones and with 40 mL BMD medium to an OD_{600} of 0.1 for pGAPZαA-LipR clones. Cultures were incubated for 48 h at 28°C, 80% humidity and 130 rpm agitation. For pPICZαA cultures, one pulse of methanol to a final concentration of 0.5% v/v was added to the cultures every 8 h during the first 16 h and then 16 h and 8 h after. All media were supplemented with zeocin (100 mg/mL). For pGAPZαA cultures, one pulse of glucose to a final

concentration of 20 g/L was added each 24 h. The concentrations of methanol and glucose were monitored by HPLC and by measures in YSI-2700 SELECT respectably, using pre-filtered culture samples. Cell cultures were then harvested and the supernatants were ten times concentrated using an ultrafiltration unit with 11 kDa cut off membrane. Cells were then disrupted by Hi-Press and the cell lysates were obtained (Garcia-Ortega et al., 2015). The untransformed X-33 strain of *P. pastoris* was used as a negative control.

1.2.5 Qualitative and quantitative lipase activity determination

Lipase activity was qualitatively detected using fluorimetric paper assays or zymogram analysis, as described (Diaz et al., 1999). This fast method consists in transferring a small aliquot (5 µL) of each sample to be analysed onto filter paper, followed by addition of 5 µL 25 mM methyl-umbelliferone (*muf*)-butyrate stock solution. UV illumination of the filter paper allows identification of lipase activity due to substrate hydrolysis and *muf* release. SDS-PAGE coupled to zymogram was used to detect lipase activity after protein separation. In order to detect hydrolysis activity, *muf*-butyrate was used as a substrate since better results were obtained than using other *muf*-derivatives.

Colorimetric assay was performed to quantitatively determine lipase activity. Thus, activity of crude cell extract and supernatant fractions was analysed by measuring the release of *para*-nitrophenol (*p*-NP) from *p*-NP-decanoate substrates, as previously reported (Ruiz et al., 2004). Alternatively, activity was analysed by measuring the release of *muf* from *muf*-derivative fatty acid substrates (Sigma), using a spectrofluorometer (Variant, Spain), as previously described (Prim et al., 2003). One unit of activity was defined as the amount of enzyme that released 1 mol of *p*-NP or *muf* per minute under the assay conditions used.

1.3 RESULTS

1.3.1 Codon optimization of LipR gene for expression in *E. coli*

The evaluation of LipR as immobilized biocatalyst for triglyceride transesterification using the enzyme expressed under the rhamnose promoter of pGaston previously obtained (Bassegoda et al., 2012) provided very low activity values, resulting in insufficient enzyme quantity for FAMEs production. This led us to try gene overexpression for higher enzyme production. Several studies have confirmed that the expression level of

recombinant proteins in *E. coli* can be decreased by rare codons. Consequently, an increase of expression level can be achieved by codon optimization and substitution of rare codons by preferred codons (Gustafsson et al., 2004). Therefore, the gene sequence of LipR was optimized based on the codon bias of *E. coli*. A total of 139/1350 nucleotides were substituted in the optimized sequence of *lipR*. According to Gene Script web server, CAI (Codon Adaptation Index) was measured to be 0.67. A CAI value lower than 0.8 would not have a chance to produce valuable expression levels in the desired host. Codon optimization of *lipR* resulted in an increased codon usage bias for *E. coli* by upgrading the CAI to 0.96. Cell extracts of *E. coli* Origami™ pET22-LipRopt and *E. coli* BL21 pET22-LipRopt were obtained. A preliminary analysis of the newly obtained recombinant clones was performed preparing crude cell extracts and analyzing their lipolytic activity using the qualitative fluorometric paper assay (Fig. A1). Surprisingly, not fluorescence emission was observed for the LipRopt recombinant clones.

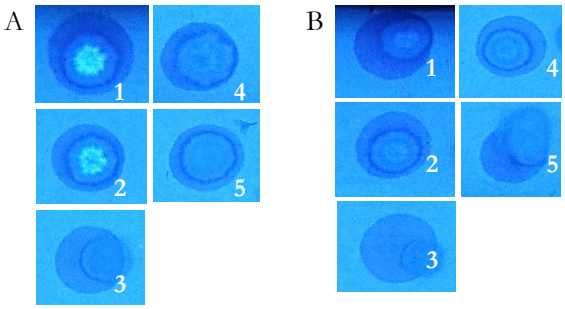


Figure A1. Fluorometric paper assay using *muf*-butyrate as a substrate. Lipolytic activity of crude cell extracts soluble fraction (A) and insoluble fraction (B) from: Origami™ pGaston-LipR (1), BL21-LipR (2), buffer control (3), Origami™ pET22-LipRopt (4), BL21 pET22-LipRopt (5).

The fluorometric paper assay revealed lipolytic activity only for clones where no codon bias modification had been applied, Origami™ pGaston-LipR, but no activity was observed in the case of the clone lacking the signal peptide

The same results were obtained from SDS-PAGE and zymogram analysis of the crude cell extracts and the insoluble fractions lipase activity production in each clone was analyzed (not shown).

1.3.2 Expression of LipR in *P. pastoris* X-33

lipR gene was amplified from pGaston-LipR plasmid and cloned into pJET. The gene of interest was then sub-cloned into two vectors: pPicZαA, containing the *AOX1* promoter

tightly regulated, methanol-induced, and the α-factor secretion signal for secretion of the recombinant protein; and pGapZαA, containing glyceraldehyde 3-phosphate dehydrogenase (*GAP*) gene promoter, which provides strong constitutive expression on glucose at a level comparable to that seen with the *AOX1* promoter (Cos et al., 2006). Although the *AOX1* promoter has been successfully used to express numerous foreign genes, there are circumstances in which this promoter may not be suitable (Cereghino and Cregg, 2000). Thus, a construction using pGAPZαA was also assayed. But since the *GAP* promoter is constitutively expressed, it is not a good choice for production of proteins that may be toxic to the yeast as could be the case for a lipase. The two constructs were sequenced with external primers (Table A2) and compared with the published *lip*R sequence for confirmation. In both constructs, *lip*R was fused to the gene of the alpha factor from *Saccharomyces cerevisiae*, which should allow secretion of the expressed lipase into the medium.

The linearized recombinant plasmids pPICZαA-LipR and pGAPZαA/LipR were then transformed to *P. pastoris* X-33 (Mut^+, the methanol utilization *plus* phenotype). Five positive recombinant *P. pastoris* X-33 pPICZαA-LipR clones (results presented just for the best two clones) and three positive recombinant pGAPZαA-LipR clones of *P. pastoris* X-33 containing multi-copy *lip*R gene cassettes, as demonstrated by good resistance in 100 μg/ml zeocin after being sub-cultivated for three times, were selected for higher scale production of LipR in shaking flasks. A two-stage fermentation was strategy adopted for expressing LipR: at the cell growth stage, biomass was accumulated in BMGY medium until the cell density reached an OD_{600} between 4.0 and 6.0. This step was done to avoid the potential toxicity caused by the expression inducer methanol. At the inducible expression stage, LipR expression was induced in BMMY medium by feeding with methanol, which acts as both, protein expression inducer and carbon source for further biomass production.

During shaking flask fermentation of *P. pastoris* X-33 pPICZαA-LipR clones for 72 h and *P. pastoris* X-33 pGAPZαA-LipR clones for 48 h, we monitored the OD_{600} of these strains every 24 h (Table A3). As expected, *P. pastoris* X-33 pPICZαA-LipR clones grew significantly slower than *P. pastoris* X-33 pGAPZαA-LipR clones. After 48 h incubation, cultures achieved a desirable density for measuring lipase ativity.

Table A3. Experimental optical density (OD) of the recombinant *P. pastoris* cultures

Strain	OD_{600nm} 24 h	OD_{600nm} 48 h
X-33-pPICZαALipR 9	6.2	11.0
X-33-pPICZαALipR 24	5.0	20.8
X-33	4.4	11.0
X-33-pGAPZαALipR 9	9.4	35.0
X-33-pGAPZαALipR 11	11.3	40.0
X-33-pGAPZαALipR 12	11.5	38.0

Supernatants were then separated by centrifugation, and cells were disrupted for obtaining crude cell extracts. The resulting concentrated supernatants and cell extracts were analyzed by SDS-PAGE and zymogram (Fig. A2). Apparently, most LipR produced by X-33 pPICZαA-LipR (Mut^+) was not secreted into the extracellular medium. Most activity signal was observed in cell extracts. In the case of those clones, a band with activity on *muf*-butyrate, with a size of 46kDa could be observed in the crude cell extracts. Nevertheless, when *P. pastoris* pGAPZαA-LipR clones were assayed, neither crude cell extracts nor the supernatant fractions showed a band bearing lipolytic activity (Fig. A3).

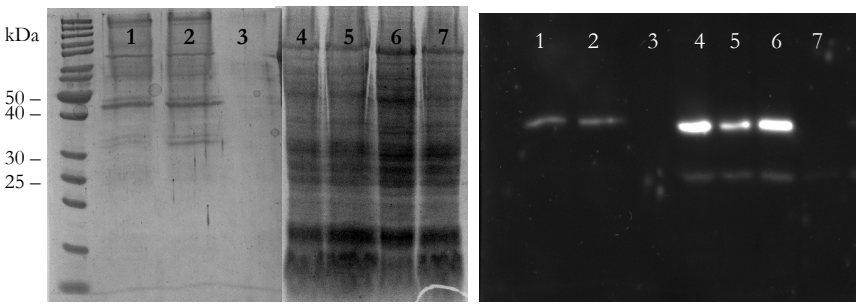


Figure A2: SDS-PAGE and zymogram analysis of X-33 pPicZαA-LipR transformants containing the *AOX1* promoter. (A) Coomassie-stained SDS-PAGE: culture supernatants from *P. pastoris* X-33 pPICZαA-LipR clone 9 (1), *P. pastoris* X-33 pPICZαA-LipR clone 24 (2), *P. pastoris* X-33 (3), cell extracts from *P. pastoris* X-33 pPICZαA-LipR clone 9 (4), *P. pastoris* X-33 pPICZαA-LipR clone 24 (5), culture of *P. pastoris* X-33 pPICZαA-LipR clone 9

(6), cell extract from *P. pastoris* X-33 (7). (B) Zymogram analysis on *muf*-heptanoate of the same gel shown in (A).

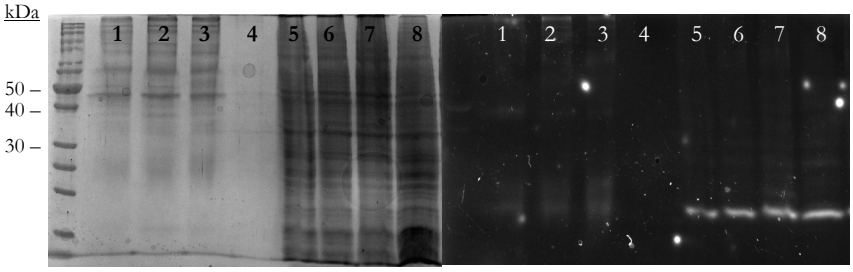


Figure A3: SDS-PAGE and zymogram analysis of *P. pastoris* X-33 pGAPZαA-LipR transformants containing the *AOX1* promoter. (A) Coomassie-stained SDS-PAGE: culture supernatants of *P. pastoris* X-33 pGAPZαA-LipR clone 9 (1), *P. pastoris* X-33 pGAPZαA-LipR clone 11 (2), *P. pastoris* X-33 pGAPZαA-LipR clone 12 (3), *P. pastoris* X-33 (4), cell extracts from *P. pastoris* X-33 pGAPZαA-LipR clone 9 (5), *P. pastoris* X-33 pGAPZαA-LipR clone 11 (6), *P. pastoris* X-33 pGAPZαA-LipR clone 12 (7), and *P. pastoris* X-33 (8). (B) Zymogram analysis on *muf*-heptanoate of the same gel shown in (A).

The activity of crude cell extracts from *P. pastoris* X-33 stain and *P. pastoris* X-33 pPICZαA-LipR clones were also tested using the quantitative assay with *p*NP-decanoate as a substrate. Cell extract of recombinant *P. pastoris* X-33 pPicZαA-LipR clone 24 showed the highest activity of 60 mU/ml (Figure A4). The activity of *E. coli* pGaston-LipR cell extract usually obtained is around 200 mU/ml. These results indicate that no activity enhancement occurred when *P. pastoris* expression systems was used. Therefore, changing to *P. pastoris* as the heterologous host strain for expression of LipR did not result in an improvement of the activity obtained.

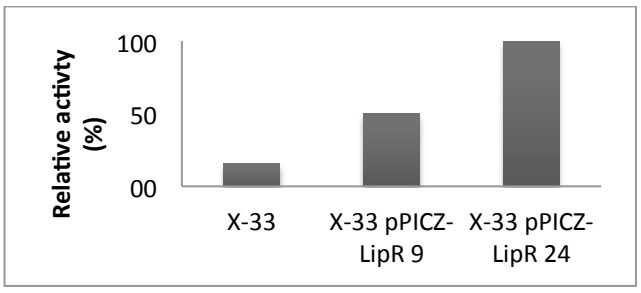


Figure A4. Relative activity of LipR obtained from cell extracts of *P. pastoris* recombinant clones

1.4 CONCLUSIONS

Two different fermentation approaches were tested with regard to the ability of expressing LipR from *Rhodococcus* in the methylotrophic yeast *Pichia pastoris* in higher yields than in *E.coli*, as an effort to increase the number of enzymes suitable for application in FAMEs synthesis. The results show that the tested yeast transformants do not lead to sufficiently high enzyme yields. A codon optimization in *E. coli* was also assayed, but no active LipR was obtained. Therefore, LipR could not be tested for transesterification assay.

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2. Journals Impact Factor

Papers presented in this thesis are published or submitted to International journals indexed at Journal of Citation Report (JCR) as following:

"Fast and economic immobilization methods described for non-commercial *Pseudomonas* lipases"
Silvia Cesarini, Bélen Infanzón, F.I.Javier Pastor, Pilar Diaz
BMC Biotechnology (2014) 14: 27.
IF: 2.034 for year 2014. Belonging to Q3 in the category Biotechnology and applied microbiology (84/163).

""Unusual carboxylesterase bearing a GGG(A)X-type oxyanion hole discovered in *Paenibacillus barcinonensis* BP-23"
Belén Infanzón, Susana V. Valenzuela, Amanda Fillat, F. I. Javier Pastor, Pilar Diaz
Biochimie (2014) 104:108-16.
IF: 2.953 for year 2014. Belonging to Q2 in the category Biochemestry and Molecular Biology (128/290).

""Exploring the unusual Y-type oxyanion hole of *Rhodococcus* sp CR53 lipase LipR through rational evolution"
Belén Infanzón, Pablo Sotelo, Josefina Martínez, Pilar Diaz
Submitted to **Enzyme and Microbial Technology.**
IF: 2.034 for year 2015. Belonging to Q3 in the category Biotechnology and Applied Microbiology (84/163).

"Alternative oils tested as feedstocks for enzymatic FAMEs synthesis using low cost immobilized lipases"
Belén Infanzón, Silvia Cesarini, Josefina Martinez, F.I.Javier Pastor, Pilar Diaz
Submitted to **Biotechnology Progress.**
IF: 2.164 for year 2015. Belonging to Q2 in the category Food Science & Technology (34/125) and to Q2 in the category Biotechnology & Applied Microbiology (77/161).

Thesis director
Dr. P. Diaz

3. Coauthority of Publications

The PhD student Belén Infanzón Ramos participated at papers presented in this work as stated below:

Silvia Cesarini, Bélen Infanzón, F. I. Javier Pastor, Pilar Diaz

"Fast and economic immobilization methods described for non-commercial *Pseudomonas* lipases"

BMC Biotechnology. DOI: 10.1186/1472-6750-14-27

The PhD student conducted 25% of the experimental work and actively participated in the experimental design and drafting of the manuscript.

Belén Infanzón, Susana V. Valenzuela, Amanda Fillat, F. I. Javier Pastor, Pilar Diaz

"Unusual carboxylesterase bearing a GGG(A)X-type oxyanion hole discovered in *Paenibacillus barcinonensis* BP-23"

Biochimie. DOI:10.1016/j.biochi.2014.06.003.

The PhD student conducted the 80% of the experimental work and actively participated in the experimental design and drafting of the manuscript.

Belén Infanzón, Pablo Sotelo, Josefina Martínez, Pilar Diaz

"Exploring the unusual Y-type oxyanion hole of *Rhodococcus* sp CR53 lipase LipR through rational evolution"

Submitted to E*nzyme and Microbial Technology*

The PhD student conducted all the experimental work, actively participated in the experimental design and prepared the draft of the manuscript.

Belén Infanzón, Silvia Cesarini, Josefina Martinez, F. I. Javier Pastor, Pilar Diaz

"Alternative oils tested as feedstocks for enzymatic FAMEs synthesis using low cost immobilized lipases"

Submitted to *Biotechnology Progress*

The PhD student conducted all the experimental work, actively participated in the experimental design and prepared the draft of the manuscript.

Thesis director

Dr. P. Diaz