NEW LIPASES

FOR INDUSTRIAL APPLICATIONS

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Alla perseveranza

e a quel giorno mai arrivato.

Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.

In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.

Beyond this place of wrath and tears
Looms but the Horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate:
I am the captain of my soul.

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Riassunto

Gli enzimi, in quanto biocatalizzatori, suscitano da sempre un forte interesse da parte delle industrie. Un catalizzatore per definizione è una molecola in grado di ridurre il tempo di una reazione, abbassando l'energia di attivazione del processo in analisi. Già considerando solo questo aspetto, tali molecole sono di forte attrattiva per un mondo, come quello delle industrie, dove un minimizzare i tempi vuol dire un aumento dei profitto in un'ottica di aumento della produttività. In oltre, essendo una proteina, il suo uso al posto di reagenti chimici e/o trattamenti con alte temperature/pressioni porta numerosi vantaggi sia nell'ottica di processi ecocompatibili a basso impatto ambientale, sia per quanto riguarda un discorso di abbattimento dei costi. Per quanto riguarda il primo aspetto, i processi chimici industriali sono caratterizzati da una produzione massiva di scorie le quali devono essere smaltite in modo adeguato. Tale processo di smaltimento prevede alti costi che possono andare ad incidere anche significativamente sull'intero costo del processo. Costi che vengono abbattuti e notevolmente ammortizzati nel caso in cui si usi un processo enzimatico, in quanto si va ad utilizzare una molecola biologica al posto di sostanze chimiche, o comunque si riduce al minimo l'uso di quest'ultime come cofattori o substrati di reazioni dell'enzima (quasi mai tossici).
Contemplando i processi industriali in siffatto modo entriamo nell'ottica di processi *ecofriendly*. Il trend dei processi industriali con l'uso di enzimi è in continua ascesa. Vi è una domanda di enzimi da parte delle aziende maggiore dell'offerta rappresentata dalle nuove proteine messe a disposizione dalla ricerca. Quindi è forte l'interesse a isolare, identificare e caratterizzare nuovi enzimi con caratteristiche peculiari. Svariate classi di enzimi trovano utilizzo nelle applicazioni industriali. Tra di esse le lipasi occupano un'importante fetta di mercato nell'ambito degli enzimi industriali. Questo perché le lipasi grazie alla loro "promiscuità" possono catalizzare un ampio spettro di reazioni. Il loro ruolo biologico è quello di idrolizzare trigliceridi portando al rilascio di una molecola di glicerolo e di molecole di acidi grassi liberi, nell'ambito del metabolismo dei lipidi. In ambito industriale invece si è visto che le potenzialità di questi enzimi vanno ben oltre, riuscendo ad espletare sia reazioni di idrolisi che di sintesi, variando substrati e condizioni di reazioni. Tra le principali reazioni catalizzate dalle lipasi vi sono:

- ***Idrolisi***
- ***Esterificazione***
- ***Acidolisi***
- ***Alcolisis***
- ***Amminolisi***
- ***Interestificazione***

Grazie alle loro peculiarità le lipasi già da tempo sono usate in svariati processi biotecnologici quale l'uso come additivi nei detergenti commerciali, l'utilizzo nella produzione di biopolimeri, l'utilizzo nella produzione di molecole ad alto valore aggiunto come possono essere gli acidi grassi polinsaturi (PUFAs), l'utilizzo

nell'industria di prodotti lattiero-caseari dove le lipasi vengono usate per modificare e caratterizzare il sapore degli alimenti, l'uso nella produzione di biodiesel, e tanti altri. Nel presente lavoro di tesi in primo luogo è stato razionalizzato quali potevano essere le "fonti" potenziali più promettenti da cui isolare e identificare nuovi microorganismi capaci di esprimere lipasi extracellulari dal forte potenziale biotecnologico. Nel far ciò sono stati utilizzati due differenti approcci rientranti in studi di Bioprospecting: Il culture e il Genome Mining.
Nell'ambito del culture mining vi è la ricerca per fini di studio e di commercio di nuovi organismi o nuove molecole ad alto valore aggiunto in una determinata area in modo da esplorare tutto il potenziale caratterizzato dalla biodiversità presente nell'habitat definito. In quest'ottica due differenti "habitat" sono stati presi in considerazione per l'identificazione e l'isolamento di microorganismi capaci di produrre enzimi lipolitici extracellulari. In primo luogo è stato preso in considerazione l'ambiente marino. Esso è riportato essere un enorme reservoir di microorganismi non ancora identificati e caratterizzati, presentando un'enorme potenzialità, quasi del tutto inespressa, per quanto riguarda molecole e meccanismi di reazioni enzimatiche d'interesse. Si calcola che solo il 2% delle specie marine sia conosciuto, inoltre molti microorganismi non possono essere coltivati in laboratorio. In quest'ottica un campione di acqua marina è stato prelevato dal porto di Pozzuoli, Italia. In particolare in una zona ben definita e circoscritta denominata "Darsena Vecchia" in cui non vi è un forte ricambio di acqua dovuto alle correnti. Tale zona è fortemente inquinata da idrocarburi e da oli provenienti da sversamenti delle imbarcazioni. Vi è ragion di credere che, nel processo di adattamento, alcune specie, per poter utilizzare come unica fonte di carbonio oli complessi, abbiano sviluppato la capacità di esternalizzare enzimi lipolitici in modo da produrre molecole più semplicemente fruibili per il metabolismo e la sopravvivenza del microorganismo. Il campione di acqua marina proveniente dalla "Darsena Vecchia" è stato soggetto di analisi e, dopo diluizioni seriali e screening per la produzione di attività lipasica, sono stati identificati alcuni microorganismi capaci di produrre lipasi extracellulari. Il DNA genomico di tali microorganismi è stato estratto, e mediante PCR sono state amplificate le regioni ITS. L'analisi di quest'ultime ha portato all'identificazione di un nuovo strain di *Galactomyces geotrichum*, chiamato *Galactomyces geotrichum* GTP. Crescite in terreno liquido hanno confermato una produzione di attività lipasica extracellulare. La produzione di lipasi è riportata come una produzione inducibile, quindi varie formulazioni di terreni di crescita, anche variando la presenza di oli, sono stati testati. L'ottimizzazione delle condizioni di crescita, che ha visto il passaggio dalla crescita in beuta alla crescita in fermentatore, hanno portato ad avere un massimo di produzione pari a 9000 U/L, migliorando di gran lunga la produzione basale del ceppo. Oltre alla produzione extracellulare è stata identificata anche un'attività lipasica correlata alla membrana cellulare, confermando altri studi presenti in letteratura su lipasi di membrana in altri ceppi di *G. geotrichum.* Nella condizione ottimizzata per la produzione di lipasi extracellulari, l'attività correlata al micelio è pari a 19.6 unità per grammi di biomassa liofilizzata. Queste due attività, extracellulare e correlata al micelio, sono state sottoposte a studi di caratterizzazione saggiandone l'attività a differenti valori di pH, a differenti temperature e contro differenti substrati, i quali variavano per la lunghezza della catena carboniosa, in quanto una delle principali caratteristiche di interesse ricercate nelle lipasi è la capacità di idrolizzare substrati aventi acidi grassi a catena lunga. Esperimenti preliminari inoltre sono stati effettuati nell'ottica di utilizzare tali enzimi nell'industria dei detergenti come additivi. L'attività enzimatica è stata saggiata in presenza di vari detergenti commerciali.

Sempre nell'ottica del bioprospecting sono stati presi in analisi campioni di compost provenienti da un centro di compostaggio dove sono riformulati scarti provenienti da frantoi e in particolare viene trattata sia la sansa che le acque di vegetazione. I campioni analizzati provenivano da 4 differenti fasi del processo di compostaggio il quale avviene ad alte temperature:

1) **Stoccaggio sansa vergine (67°C)**

2) **Bioreattore - Active Composting Composite (51°C)**

3) **Curing (51°C)**

4) **Vaglio rotante (51°C)**

Tale "habitat" risulta interessante nell'ambito della ricerca di microrganismi produttori di lipasi per due aspetti fondamentali. In primo luogo siamo in presenza di reflui provenienti da frantoi oleari, potenzialmente un'ottima sollecitazione ambientale per un microorganismo a produrre lipasi extracellulari. E in secondo luogo tali campioni di compost vengono lavorati ad elevate temperature, quindi è possibile pensare che i microrganismi presenti, in grado di vivere a 50-60°C, siano in grado di produrre enzimi termostabili/termofili. Caratteristiche queste molto richieste in ambito industriale dove alcuni processi devono essere necessariamente svolti ad alte temperature. In alternativa basti pensare ad uno dei più comuni utilizzi delle lipasi in ambito commerciale, ovvero l'uso come additivi nei detergenti, laddove i comuni cicli di lavaggio in lavatrice vengono effettuati per lo più con temperature comprese tra i 50 e i 60°C.
I quattro campioni, che si presentavano sotto forma di terriccio disomogeneo sono stati risospesi in tampone salino in beuta e incubati in agitazione a 60°C in modo da stimolare la crescita delle specie presenti. Una volta raggiunta una significativa densità ottica sono state fatte varie diluizioni in modo da riuscir a visualizzare su piastra colonie singole rappresentative di tutte le specie presenti. Le crescite sono state effettuate su due diversi tipi di terreni: LB e PDA. Questo per stimolare da una parte unicamente la crescita di batteri e dall'altra la crescita di funghi e lieviti. Dopodiché, tramite replica plating e stato effettuato uno screening di attività lipasica in modo da individuare i microorganismi produttori di lipasi. Un segnale positivo è stato visualizzato da colonie provenienti dallo stoccaggio della sansa vergine e dal vaglio rotante. In seguito ad estrazione del DNA genomico l'amplificazione della potenziale regione 16s rRNA o della potenziale regione ITS dei microorganismi in analisi è stata effettuata tramite PCR in modo da identificare i microrganismi isolati. Le analisi hanno riportato ad un unico microorganismo: *Alicyclobacillus acidocaldarius.*
Quest'ultimo è un batterio gram positivo non patogeno della famiglia "*Alicyclobacillaceae*" con un optimum di temperatura compresa tra i 60 e i 65°C. In letteratura viene spesso associato al fenomeno del deterioramento di cibo e frutta. Il genoma di un ceppo affine è sequenziato e depositato in banca dati e presenta 9 sequenze codificanti per putativi enzimi lipolitici, 5 lipasi e 4 fosfolipasi. In letteratura non vi è alcuno studio riguardo la caratterizzazione di lipasi extracellulari di questo microorganismo, ciò fa sì che sia ancor più interessante andare ad isolare e caratterizzare le lipasi espresse da tale ceppo. Il batterio è stato cresciuto in differenti terreni monitorando la capacità di produrre attività lipasica extracellulare nel tempo.

Sfruttando il fatto di avere a disposizione il genoma sequenziato e annotato è stata effettuata un'analisi bioinformatica per scegliere quale sequenza utilizzare per una produzione ricombinante in un ospite eterologo. Le 5 sequenze codificanti per putative lipasi presenti nel database sono state utilizzate per allineamenti multipli con le sequenze geniche delle lipasi commerciali più utilizzate nelle applicazioni industriali. La percentuale di copertura di sequenza e la percentuale di identità di sequenza sono state utilizzate come parametri per scegliere la sequenza da utilizzare per l'espressione ricombinante in *Pichia pastoris.* Scelta la sequenza con le percentuali maggiori, è stato sintetizzato il gene Il gene è stato sintetizzato, ottimizzando il *codon usage* per l'ospite eterologo scelto, e clonato in un opportuno vettore di espressione. Tale costrutto è stato utilizzato per trasformare, tramite il metodo dell'elettroporazione, *P. pastoris.* Tramite piastre contenente tributirrina è stato identificato il miglior produttore. La crescita in beuta di tale ceppo, rinominato *AliLip,* ha confermato la presenza di produzione di attività lipasica extracellulare.
Un altro metodo per isolare e identificare attività enzimatiche di interesse è il "genome mining". Con l'avanzamento delle tecniche di sequenziamento, negli ultimi anni è sempre crescente la quantità di sequenze presenti nei database, aumentando il divario tra numero di sequenze annotate codificanti putative attività enzimatiche e le sequenze effettivamente codificanti per proteine caratterizzate sperimentalmente. Vi è quindi questo enorme reservoir di informazioni dove andare a "pescare" nuovi enzimi di interesse. In quest'ottica rientra il fungo *Pleurotus ostreatus*, microorganismo edibile, del quale è presente in banca dati il genoma sequenziato e annotato. In esso sono presenti 44 sequenze codificanti putative lipasi. Data l'abbondanza di sequenze codificanti putative lipasi e dato il fatto che, essendo un organismo GRAS, potrebbe essere facilitato un futuro utilizzo di tali proteine in applicazioni industriali rientrati nell'ambito alimentare, *P. ostreatus* risulta essere una potenziale fonte di nuove lipasi. In primo luogo è stata verificata l'effettiva presenza di lipasi extracellulari nel terreno di crescita di *P. ostreatus.* Varie formulazioni di terreni sono state usate per indurre la produzione di lipasi. In presenza di olio d'oliva, di OMV (olive mill wastewater) e di glucosio è stata riscontrata produzione di attività lipasica, e in particolare il massimo di produzione è stato riscontrato usando come induttore l'olio d'oliva e l'OMW. I brodi di coltura delle crescite con questi due induttori sono stati analizzati tramite zimografia per visualizzare le proteine con attività lipasica. L'analisi per spettrometria di massa delle bande positive nella zimografia ha portato all'identificazione delle lipasi effettivamente espresse da *P. ostreatus* nelle condizioni sperimentali usate. Due di queste sequenze erano presenti in entrambe le condizioni e per questo motivo sono state scelte per la successiva produzione ricombinante in un ospite eterologo. I geni codificanti le due lipasi sono stati sintetizzati ottimizzando il *codon usage* per l'espressione in *P. pastoris.* Le due sequenze sono state clonate in un opportuno vettore di espressione. Tali costrutti sono stati utilizzati per la trasformazione dell'ospite eterologo scelto. Il successivo screening per l'identificazione del miglior produttore è stato effettuato mediante piastre con tributirrina. Le crescite in beuta dei due ceppi, rispettivamente chiamati *PleoLip214* e *PleoLip369* hanno portato ad un massimo di produzione lipasica di 4000 U/L e 700 U/L rispettivamente. Le due proteine sono state purificate e caratterizzate. La loro attività è stata saggiata a differenti valori di pH, a differenti temperature, in presenza di differenti detergenti, solventi e ioni. Inoltre è stata determinata la specificità di substrato dei due enzimi saggiandone l'attività contro substrati a diversa lunghezza.

Una potenziale applicazione industriale è stata ipotizzata per PleoLip241, sulla base dei dati comparativi della caratterizzazione dei due enzimi. Esperimenti sono stati volti a dimostrare il possibile utilizzo di PleoLip241 nel pretrattamento della lana per migliorare il processo di colorazione, senza andare ad alterare le caratteristiche del tessuto quali elasticità, flessibilità, morbidezza. L'enzima va a idrolizzare lo strato superficiale del tessuto composto da lipidi. Eliminata La barriera idrofobica rappresentata da quest'ultimi grazie all'azione della lipasi, si avrà un aumento della tingibilità della lana. L'ottimizzazione di questo processo può portare alla diminuzione della quantità necessaria nei processi di colorazione della lana, in quanto a parità di concentrazione di colorante nella soluzione di reazione, la lana pretrattata con le lipasi presenterà una maggior quantità di colorante adsorbita sulle fibre della stessa.

Summary

Lipases are enzymes widely used in the Industry. There is a strong interest in searching for new enzymes endowed with peculiar characteristics. In this framework, two approaches were carried out: Culture and Genome Mining.
At first, a new *Geotrichum candidum* lipase producing strain was isolated from marine polluted water. The effect of calcium and carbon and nitrogen sources on lipase secretion was analysed. The maximum lipase activity was reached after 58 h of cultivation in a bioreactor, in an optimized medium, at 28°C, and was found to be 9,000 U/L. The presence of lipase activity was assessed both in the culture broth and associated with the mycelium pellet. The two enzymatic pools (secreted and cell-associated), were both characterized testing the effect of pH and temperature on their activity and stability. Furthermore, substrate specificity was also assessed.
Four different samples from the composting centre CESCO were used to screen for new lipase producing microorganisms. The samples were suspended in buffer at pH 8 and incubated at 60°C. When the growth reached a significantly optical density, serial dilutions were carried out in order to select individual colonies. Lipase producing microorganisms were then identified and subjected to a genomic DNA extraction. The amplification of the putative 16s rRNA/ITS region led then to the identification of *Alicyclobacillus acidocaldarius* strain. Several growth media were tested in order to obtain an extracellular lipase activity of about 700 U/L. Nine putative lipolytic enzyme sequence were annotated in the online genome available for this strain. Following a bioinformatics analysis, one sequence was chosen for the recombinant expression in the heterologous host *Pichia pastoris*, that reached a maximum of extracellular lipase activity of 300 U/L.
In the second approach, an edible fungus was taken in analysis. This is the first report on the characterization of lipases from *Pleurotus ostreatus*. The database of the annotated genome reports 44 lipase putative coding-genes. For this reason *P.ostreatus* represent a potential source of lipases. Different growth media were tested to induce the extracellular lipase activity. The media with the best activity values were used to carried out a zymogram analysis to visualize positive bands. Following the hydrolysis and mass spectrometric analysis of the positive bands, five lipases were found in a culture media of *P. ostreatus*. Two sequences were chosen for recombinant expression in the heterologous host *P. pastoris*. The two lipase coding sequences were synthesised and cloned in a *P. pastoris* expression vector. The selected clone PleoLip241 reached a maximum of activity of 4,000 U/L after 9 days at 28°C, whereas, in the same conditions, the other selected clone, PleoLip369, reached a maximum of activity of 700 U/L. The enzymes were purified, characterised and the K_M and K_{cat} values were determined using different substrates. These enzymes showed a high affinity towards medium and long chains esters. Pleolip241 was tested in the treatment of the wool.

Introduction

1 Lipases

Lipases (EC 3.1.1.3) are ubiquitous enzymes found in all types of living organisms, from fungi and bacteria to plants and animals. Lipases exert their natural function towards the hydrolysis/synthesis of carboxylic ester bonds of long-chain triacylglycerols (Kumar et al., 2016). In 1856, Claude Bernard first discovered a lipase in pancreatic juice as an enzyme that hydrolysed insoluble oil droplets and converted them to soluble products. The presence of lipases was observed as early as in 1901 for *Bacillus prodigus, Bacillus pyocyaneus* and *Bacillus fluorescens* by the microbiologist Sir Eijkman (Momsia and Momsia, 2013).

Lipases belong the α/β-hydrolase superfamiliy, and despite their high diversity in sequence and function, share a common architecture, the α/β-hydrolase fold (Ollis et al., 1992) and conserved active site signatures, the GxSxG and GxDxG motifs (Fischer and Pleiss, 2003)(Barth et al., 2004). Two conserved features found in all α/β-hydrolases are the active site, consisting of the catalytic triad of S-D(E)-H, and the oxyanion hole. Depending on the amino acids involved in forming the oxyanion hole, the enzymes can be classified into three classes, the GGGX-, GX-, and the Y-class (Fischer and Pleiss, 2003). In most lipases, a mobile lid covers the substrate binding site. In this closed structure, the lipase is assumed to be inactive. Upon activation of the lipase by contact with a hydrophobic solvent or at a hydrophobic interface, the lid opens (Rehm et al., 2010).

So far, a huge number of lipolytic enzymes have been discovered in several microorganisms. The most productive species belong to the genera *Aspergillus*, *Rhizopus*, *Mucor*, *Penicillium*, and *Geotrichum.*

Nowadays, due to the lipases huge potential in industrial applications, there is an ever increasing demand of new lipase producing microorganisms in order to isolate new lipases with features of interest (Borkar et al., 2009).

1.2 Reaction mechanism of lipases

Lipases are glycerol ester hydrolases (E.C. 3.1.1.3). They are interfacial enzymes which hydrolyse ester linkages of glycerides at water-oil interface. During hydrolysis, lipases pick acyl group from glycerides forming lipase-acyl complex, which then transfers its acyl group to OH group of water (Fig 1) (Martinelle et al., 1995). As the equilibrium between the forward and reverse reactions is controlled by the water activity (aw) of the reaction mixture, under limiting water conditions the reverse reaction, the esterification, can occur. Furthermore, lipases can acylate alcohols, sugars, thiols and amines synthesizing a variety of stereo-specific esters, sugar esters, thioesters and amides catalyzing esterification, transesterification and interesterification reactions (Fig 2) (Dellamora-Ortiz et al., 1997)(Singh et al., 2003). Under low aw, different transesterification reactions can also be achieved. The term transesterification refers to the exchange of groups between an ester and an alcohol (alcoholysis), between an ester and an acid (acidolysis), between an ester and an amine (aminolysis), or between two esters (interesterification) (Borrelli and Trono, 2015).

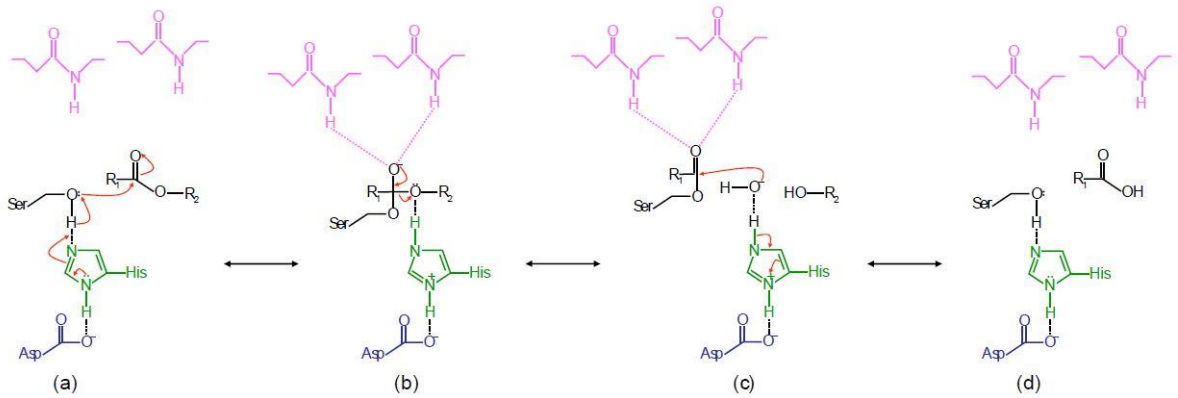

Fig. 1 Catalytic triad mechanism in lipase reaction.

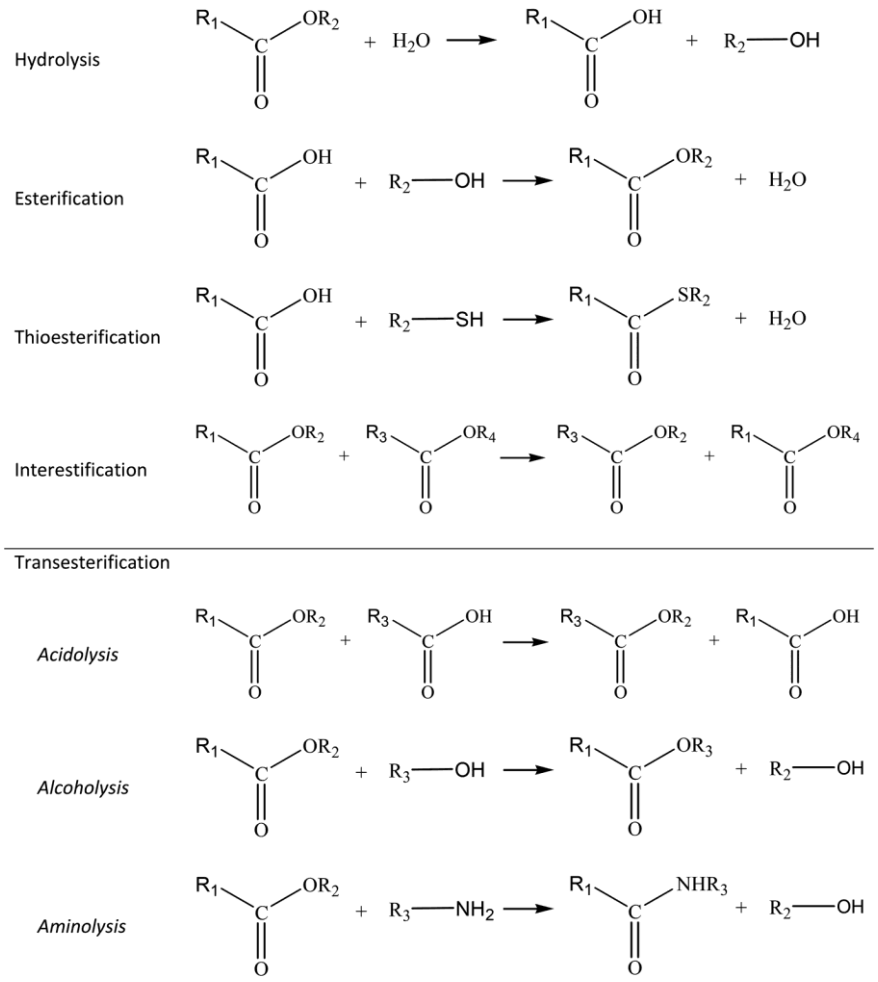

Fig. 2 Example of lipase's "promiscuity".

2 Enzymes in Industry

Enzymes are an excellent alternative to classical organic techniques in the selective transformation of complex molecules. They possess many features that favour their use as excellent biocatalysts. They impart specificity to a reaction in which a chemical process is typically non-specific. In addition, the use of enzymes can decrease side reactions and simplify post-reaction separation problems. Enzymes-catalysed processes offer cost-effectiveness too, in comparison with traditional downstream processing in which energy consumption and toxic by-products might often present problem (Pandey et al., 1999). Despite these advantages, enzyme industrial applications has been hampered mainly owing to issues linked to their stability, efficiency, and production costs (Choi et al., 2015).

2.1 Lipases in Industry

Lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential. There are many examples of lipases already used in industrial processes (Table 1). It is easy to note how these enzymes are used in a wide range of industrial fields as production of detergents, biopolymers, and biodiesel. Lipases are also used in the food industry, in the production of PUFAs (polyunsatured fatty acids), in the cosmetics industry, and in the waste treatment and oil degumming.

Table 1 List of some commercial lipases.

Source	Application	Commercial name	Producing company
Penicillium camembertii	Refining of oils and fats	Lipase G	Amano Enzyme
Thermomyces lanuginosus	Baking industry	Lipopan®	Novo Nordisk
Mucor miehei	Oils and fats industry	Lipozyme®	Novo Nordisk
Rhizomucor miehei	Dairy industry	Palatase	Novo Nordisk
Thermomyces lanuginosus	Pet food industry	Novozyme® 871	Novo Nordisk
Pseudomonas mendocina	Detergents	Lumafast®	Genencor International
Pseudomonas alcaligenes	Detergents	Lipomax®	Genencor International
Humicola lanuginose	Detergents	Lipolase TM	Novo Nordisk
Candida rugosa	Organic synthesis	Lipase AY “amano”30	Amano
Candida rugosa	Organic synthesis	Resinase	Novozymes
R. niveus	Oleochemistry	Newlase F	Amano
Penicillium roqueforti	Food processing	Lipomod™338P-L338P	Biocatalysts

The recent success of sequencing programs and the high velocity reached, has given the opportunity to identify an increasing number of gene sequences that encode new variants of microbial enzymes. Thus, most of these genes have been cloned and successfully expressed in heterologous microbial hosts. The enzyme industry has taken advantage of heterologous expression systems to increase the reproducibility and the yields of the production of enzymes, which are the major obstacles to their use at an industrial scale (Borrelli and Trono, 2015). The most frequently heterologous hosts used in the recombinant lipase production are bacteria, yeasts and fungi (Fig 3). Prokaryotic hosts, such as *E. coli,* are the most widely used systems for recombinant expression for a lot of advantages, i.e. the short growth

times, the availability of a large number of cloning vectors and their well characterised genetics. However, they have many drawbacks, in particular the lack of post-translational modifications, such as phosphorylation, glycosilation and correct formation of disulfide bridges. These aspects can lead to an incorrect protein folding in the case of proteins from eukaryotic organisms. Indeed, *E. coli* Is mostly used for the production of lipases from the bacteria strains belong to the genera *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Serratia* and *Burholderia.*
To overcome the issues of the heterologous expression in prokaryotic hosts, the yeasts and filamentous fungi are common used to produce lipases.

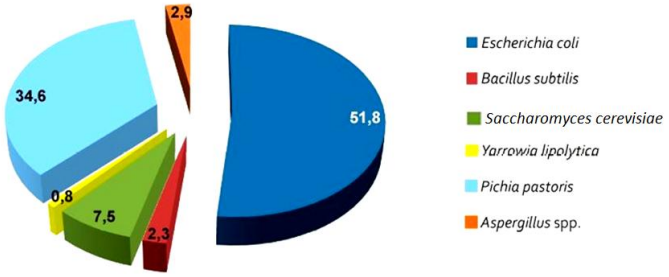

Fig 3 Distribution of lipase genes expressed in different heterologous host.

2.1.1 Industry of detergents

The most commercially important field of application for hydrolytic fungal lipases is as additives in detergents in industrial laundry and household detergents. Lipase can reduce the environmental load of detergent products as it saves energy by enabling the use of a lower wash temperature (Hasan et al., 2010). In this field, the market is looking for enzymes that are stable over a broad range of pH and compatible with surfactants and oxidizing agent at lower concentrations and with broad substrate specificity (Jellouli et al., 2011). In particular, when used in detergents, it is interesting to study the lipase interfacial activation (In fig 4) in order to understand the interaction between the enzyme and the all compounds of detergents mixture. Several lipases showed an increase of lipolytic activity in the presence of different detergents. A possible reason could be a new rearrangement of the protein folding that allows a better interaction between the active site and the substrate, thanks to an open conformation of the lid. Indeed, several lipases present this mobile structure, the lid, that exist in two different conformation, open and close.

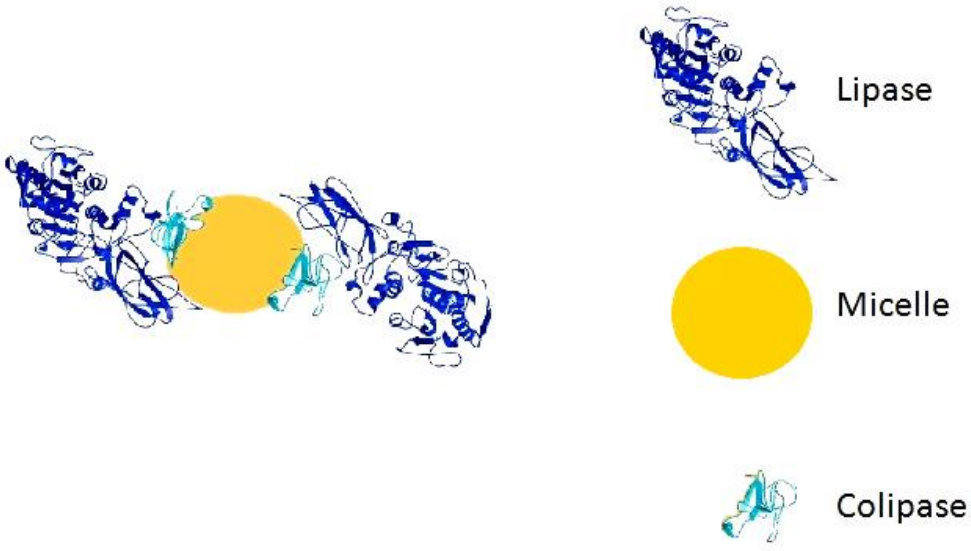

Fig 4 Schematic depiction of pancreatic lipase activation in solution by colipase and a duodenal mixed micelle (Hermoso et al, 1997).

2.1.2 Biodiesel production

In the last years, the global warming started to represent a huge problem for the public opinion. The depletion of fossil fuels is a critical aspect of the economical politics of all the countries. In this framework, intensive investigations are carried out in order to make possible a higher use of sustainable biofuels instead of fossil fuels. Biomass, from which various biofuels are produced, has an important role among other alternative energy sources including wind energy, solar energy, geothermal energy, etc. Biodiesel is one of the most important biofuels since it is a clean energy source and represents a valid alternative to petroleum-based diesel fuels. Biodiesel has different advantages and disadvantages. Transportability, high combustion efficiency, low sulphur and aromatic content, high cetane number and biodegradability are advantages of the biodiesel (Ayhan. Demirbas, 2008). Disadvantages of biodiesel are high viscosity, lower energy content, high cloud and pour point, high nitrogen oxide emission, lower engine speed and power, injector cooking, high price and engine erosion (Ayhan Demirbas, 2008). Biodiesels are a mixture of FAAEs that are usually produced by esterification of FFAs or transesterification of TAGs. Currently, the world supply of biodiesels comes almost exclusively from base-catalysed transesterification of TAGs with short-chain alcohols, such as methanol and ethanol. A downstream process is also needed for the treatment of the large quantities of wastewater produced from the process. The latter is very expensive and non-ecologically friendly. If compared with the chemical process, the enzymatic lipase-mediated transesterification became very attractive. In fact, lipases catalyse TAG transesterification reactions and FFA esterification with an alcohol, thus making possible the production of biodiesel from used oils or fats with high FFA levels. Moreover, lipases produce FAAEs without forming soaps, thus allowing easier glycerol recovery and fuel purification. In addition, these enzymes require mild reaction conditions and they are highly selective. In Table 2, some commercially lipases used in biodiesel production are reported.

Table 2 Example of a recombinant lipase used in biodiesel production.

Microorganisms source	Lipase name	Temperature (°C)
Pseudomanas aeruginosa	Lipd25A	33
Bacillus amyloliquefaciens	LipBA	35
Candida sp	Lip2	30
Candida antarctica	CALB	30
Rhizomucor miehei	GH2	30
Thermomyces lanuginosus	Tll	30
Geobacillus thermoctenulatus	BTL2	40-50
Fusarium heterosporum	FHL	25

2.1.3 Biopolymers production

About goods and services production, the new trend in the last years is to use the point of view of the “Bio-economy”. All the processes and products have to be eco-friendly and sustainable. In this framework, the common plastics are not accepted anymore. Thus, the market needs a new system of packaging. One solution is to produce biodegradable and compostable polymers. Among biopolymers, the poly(lactic) acid (PLA) (Fig 5) represent a valid alternative.

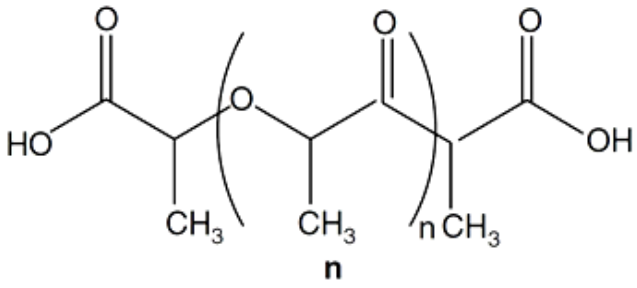

Fig 5 Poly(lactic) acid structure.

In addition, polymers based on lactic acid have gained a great interest in the field of medical application because they decompose in the body to non-harmful and non-toxic compounds. The synthetic routes to obtain PLA are the direct condensation of free acid or the ring opening polymerization of esters of the acid. The first method presents as the main disadvantage the low molecular weight of the resultant polymer, due to the equilibrium among the free acid, the oligomers and the water produced during the reaction. The second method requires the use of heavy metals-based catalysts, such as oxides of Zn and Sn; however, in this way, the contamination of final polymers is possible. These processes also needed different conditions of temperature and vacuum. In order to overcame these problems, the use of an enzyme as a biocatalyst, such as a lipase, represents a valid alternative. The main advantage of the production of PLA using lipases is the creation of a high specific and environmental friendly method that can be implemented under mild conditions (Kobayashi, 2010). In this way, it is possible to produce a biodegradable packaging materials (Fig 6).

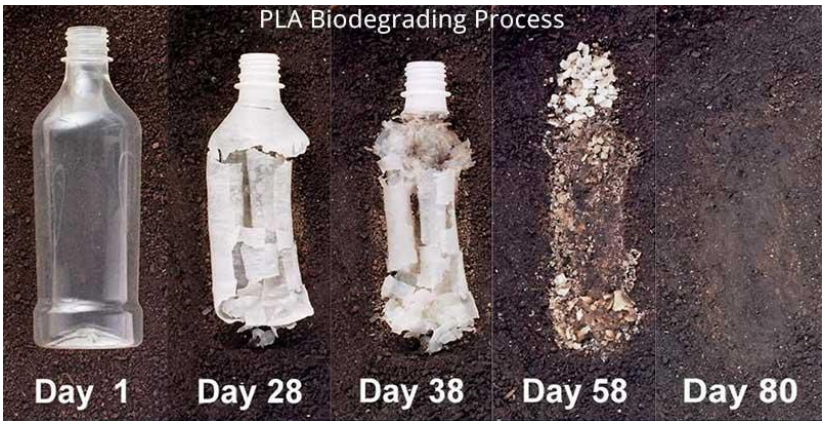

Fig. 6 Time laps of PLA natural degradation.

2.1.4 Food Industry

Lipases find more utilizations in the food industry (Table 3). They are used for the synthesis of esters, including short-chain esters, as flavour compounds, and sugar esters as emulsifiers. They are used in the dairy industry to accelerate cheese ripening and to improve cheese yield, respectively, and in the baking industry to improve volume, softness and shelf-life of bread and other baked products. The traditional commercial lipases in the dairy industry are from ovine and porcine pancreas or from pre-gastric tissues of young ruminants (P300, P400 and P600, Get Culture), although many microbial lipases have also been used efficiently, such as lipases from *A. niger* (Lipase A, Amano), *C. rugosa* (Lipase AY, Amano), *P.*

roqueforti (Lipase R, Amano; Lipomod™, Biocatalysts), *P. camemberti* (Lipase G, Amano), *R. javanicus* (Lipase M, Amano), *R. miehei* (Piccnate, Gist-Brocades), and *R. oryzae* (Lipase DF, Amano) (Borrelli and Trono, 2015). Lipases are also used to synthesize nutritionally important structured lipids from low-cost oils. These include i) low-calorie TAGs with short-chain or medium-chain FAs at the *sn*-1 and *sn*-3 positions, long chain FAs at the *sn*-2 position (SLS-TAGs, MLM-TAGs), as well as TAGs with only medium-chain FAs (MMM-TAGs); ii) TAGs that are rich in ω-3 PUFAs; iii) anti-obesity DAG oil; iv) human milk fat substitutes.
Lipases have also been used in selective removal of unhealthy *trans*-FAs from partially hydrogenated vegetable oils and in the synthesis of vitamins and phenolic esters that present higher antioxidant activities and stabilities compared to the corresponding free molecules (Ferreira-Dias et al., 2013).

Table 3 Common lipases used in the food industry.

Microorganisms source	Lipase name	Application
Candida antarctica	CALB	Ethyl lactate synthesis
Rhizopus oryzae	ROL	Ehyl butyrate synthesis
Penicillum cyclopium	MDL	Synthesis of MAGs and DAGs
Geotrichum sp.	LIP1-LIP2	Increase in loaf volume and in crumb structure uniformity
Geobacillus zalihae	Lipase T1	Dag production from palm stearin
Streptomyces racemochromogenes	PLD	PS and PG production from lecithin
Geotrichum sp.	GSL	EPA and DHA enrichment in fish oil
Metagenomic library	LipIAF5-2	Short chain ester synthesis
Metagenomic library	Lipase Est_p6	Milk flavour production
Fusarium venenatum	PLA_1	Mozzarella cheese yield improvement

3 Source of new lipases

Nowadays, the market demand for lipolytic enzymes is higher than the number of new enzymes offered by research. Taking into account the high biotechnological potential of the lipases, the *aim* of this PhD project has been the isolation and characterization of microorganisms able to produce new lipases for industrial applications. To reach this goal, in the context of bioprospecting approach two different methods were used: culture and genome mining.

3.1 Culture mining

The bioprospecting includes the entire research and developmental processes from sample extraction by publicly funded scientific and academic research institutions, to the full scale commercialization and marketing by commercial interests, such as biotechnology companies (Leary et al., 2009). It is expected that 20% of the global chemicals (fine chemicals, specialties, polymers, etc.) and 60 % of fine chemicals will be produced using biotechnology by 2020, which represents 800 billion US$ (Dionisi et al., 2012). In this framework, the culture mining is integrated as the basic research activity devoted to the search for novel biocatalysts. This specific methodology can be employed in different habitats. Many scientific projects have focused on sea water. Marine ecosystems present an enormous pool of biodiversity, thus it is a natural reservoir for acquiring an inventory of useful biocatalysts. The sea sources of enzymes are represented by microorganisms, fungi, plants or animals. Marine microorganisms can be found as intracellular or extracellular symbionts, and their

hosts are mostly marine animals (vertebrates or invertebrates). These symbiotic microorganisms must possess an arsenal of enzymes and pathways to fulfil the requirements of the host organisms (Debashish et al., 2005). In addition, only a few percentages of marine microorganisms has been isolated and characterised. For all of these reasons, it is strongly interesting to consider the sea water as a source of new enzymatic activities of interest. In this study, a sample from Mediterraneo sea water was collected and used for a bioprospecting analysis in order to isolate new lipase producing microorganisms.
With the aim to find thermostable/thermophilic enzymes, which can be widely used in different industrial processes, the olive oil mills wastes, generally treated at high temperatures, can be considered as a potential source of microorganisms capable of both growing at high temperatures and producing lipolytic enzyme. Thus, different samples from a composting centre were analysed as potential sources of lipase producing microorganisms.

3.2 Genome mining

Nowadays, thousands of microorganism genome sequences are publically available; they contain an even larger number and diversity of secondary metabolite gene clusters that await linkage to their encoded natural products. With the development of high-throughput sequencing methods and the wealth of DNA data available, a variety of genome mining methods (Fig 7) and tools have been developed to guide the discovery and the characterisation of these compounds (Ziemert et al., 2016). From a point of view of the classical variant of genome mining, the annotated genomes present on database can be taken as base for further studies. Among those, the genome of *P. ostreatus* resulted strongly interesting. *P. ostreatus* is a white rot basidiomycete for which the genome has been sequenced and annotated [http://genome.jgi-psf.org/PleosPC15_1/PleosPC15_1. home.htmL] (Riley et al., 2014; Wang et al., 2008). It represents a potential source of new lipases considering that it presents in its genome 44 putative lipase coding-genes. In addition, *P. ostreatus* is a GRAS organism and this aspect may facilitate the future use of these proteins in the food industry. For all these reasons, this biological system was chosen to investigate new lipases.

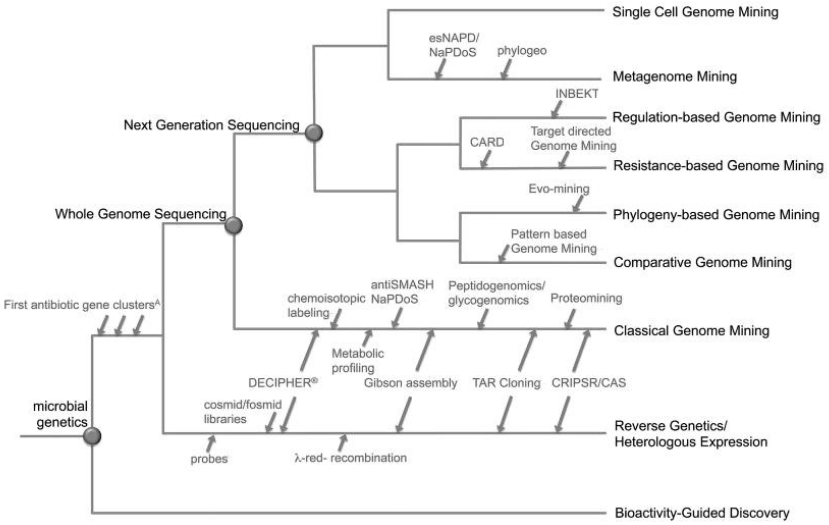

Fig 7 Evolution of genome mining (Ziemer et al, 2016)

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Chapter 1

Culture mining approach as method to isolate new lipase producing microorganisms

Section 1 (submitted to Applied Biochemistry and Microbiology)

A bioprospecting approach from Pozzuoli harbor: isolation of a lipase producing *Geotrichum candidum* GTP

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Abstract

Considering industrial potential of lipolytic enzymes, there is a strong interest in searching new enzymes endowed with peculiar characteristics. A new *Geotrichum candidum* lipase producing strain was isolated from marine polluted water. The effect of carbon and nitrogen source and of calcium on lipase secretion was analyzed. Maximum lipase activity production of 9,000 U/L at 28°C was reached after 58 h of cultivation in bioreactor in an optimized medium. The presence of lipase activity both in the culture broth and associated with the mycelium pellet was assessed. The two enzymatic pools (secreted and cell-associated), were both characterized testing the effect of pH and temperature on their activity and stability. Furthermore, substrate specificity was assessed.

Keywords: bioprospecting; *Geotrichum candidum*; lipase; polluted seawater

INTRODUCTION

Lipases are triacylglycerol acylhydrolases (EC 3.1.1.3) that catalyze the hydrolysis and the synthesis of triacylglycerol to glycerol and fatty acids. These enzymes belong to the α/β hydrolase family with a structure composed of a central β-sheet, containing a catalytic serine-aspartate/glutamate-histidine triad[1]. Lipases occur widely in nature, with microbial lipases (from bacteria, yeast and fungi) representing commercially relevant enzymes with a wider applicability than plant and animal lipases[2]. Lipases are considered as the third largest enzyme group, after proteases and carbohydrases, based on their market value. There is a large potential for these enzymes in industrial applications such as additives in foods, pharmaceuticals, cosmetics, leather, dairy industry (cheese ripening, flavour development), fine chemicals, detergents, paper manufacture and waste-water treatment[2, 3]. In this framework, there is a strong interest in bioprospecting towards novel lipolytic organisms. Microorganisms able to produce lipolytic activity can be found in different habitats, including oil-contaminated environments[4], seeds, wastes of vegetables oils[5], deteriorated foods, dairy products[6], soils[7] and seawater[8]. Marine ecosystems represent a large reservoir of organisms with unique biochemical traits. Survival under marine conditions must have necessitated the development of quite novel microbial cellular biochemistry and metabolism, thereby ensuring that biome is likely to possess unique enzymatic systems which could possibly match many industrial requirements[9]. Moreover, the coastal marine water is subject to contamination by oil from a variety of sources. The presence of this class of contaminants makes this environment a potential source to find microorganisms capable of degrading lipids by producing enzymes with lipase activity.

In this study a bioprospecting approach was applied to isolate interesting lipase producing microorganisms from polluted coastal marine water of “Darsena vecchia” in Pozzuoli, Naples (Mediterranean Sea), a small backwater zone with no tidal current, also called Fisherman’s mole. This body of water is enclosed by land with a very small mouth, affording access to Pozzuoli commercial harbor where a high volume of small/medium size boat traffic is present. The depth of this area ranges between 20 cm to 2.3 m.

The applied screening strategy allowed to isolate a *Geotrichum candidum* strain able to secrete lipases. This is the first report in which a *G. candidum* strain was isolated from marine habitat. In fact, *G. candidum* strains have already been reported as lipase producing organisms, however they were isolated from different environments, such as dairy products [10], soil[7] and oil-mill waste[5]. In most of the studies, the production of both secreted and

mycelium-bound lipases with different substrate specificities has been described[7, 11, 12]. These results are due to both the genetic variability of the microorganism as well as to the different panels of lipase enzymes produced as a function of the used culture conditions [10, 12]. Moreover, a further level of complexity is linked to the various assay methods used to assess lipase substrate specificity.

The herein described results report comparison between mycelium-bound and secreted lipases from a novel *G. candidum* GTP strain isolated form Pozzuoli harbor.

MATERIALS AND METHODS

Screening and isolation of lipase producing organisms

A 1.2 l marine water sample was collected in the "Darsena Vecchia" of Pozzuoli, Naples, Italy 40°49'N latitude 14°07'E longitude. The sample was filtered using a 0.22 µm pore size membrane. The retained fraction was resuspended in sterile water (16 ml) and subjected to an enrichment culture technique. A 2 ml aliquot of this sample was inoculated in two different rich basal media , LB (10 g NaCl l^{-1} (Merck), 5 g yeast extract l^{-1} (BD Difco), 10 g tryptone l^{-1} (BD Difco)) and YEPD (10 g yeast extract l^{-1}(BD Difco); 1 g peptone l^{-1} (BD Difco); 2 g glucose l^{-1} (Sigma-Aldrich)) and incubated at two different temperatures (15°C and 28°C) to promote the growth of all species present. These microbial cultures were used as inocula for the first step of the enrichment culture technique (Fig. 1). In each step the media concentration decreased up to zero until using only water and 1% olive oil in the culture medium. Samples from both culture media were spread on chromogenic agar plate (containing 1% olive oil, phenol red and $CaCl_2$, pH 7). Plates were incubated at 15°C and 28°C. A yellowish halo indicates the presence of lipolytic activity. The organisms forming the largest halo after 24h incubation were further isolated by repeated streaking.

Identification of the selected lipase producing strain

The strain was identified based on the analysis of the ITS1 region. The genomic DNA was extracted according to the "Smash and Grab" protocol. PCR was carried out in 50 µl reaction volumes containing 100 ng of genomic DNA, 2.0 mM $MgCl_2$, 0.5 µM of each primer (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2 5'-GC TGCGTTCTTCATCGATGC-3') 50 mM Tris/HCl (pH 8.3), 500 µg/ml of bovine serum albumin, 250 µM dNTPs and 0.5 U of Taq DNA polymerase (New England Biolabs). An initial denaturation and enzyme activation step of 2 min at 96°C was followed by amplification for 30 cycles at the following conditions: 60 seconds at 96°C, 60 seconds at 58°C, 60 seconds at 72°C. A final 10 minute extension at 72°C completed the protocol. PCR products were visualized by agarose gel electrophoresis. The DNA concentration was estimated by nanodrop . The PCR amplification fragment was purified by using a QIAquick gel extraction kit (Qiagen S.p.A.) and sequenced (Primm srl). The identification was carried out by using BLAST software[13]. Phylogenetic tree created by neighbour joining clustering method in Mega 7.0[14].

Lipase production

Lipase production was obtained through sequential adjustment of YEPD. The final medium was: 30 g yeast extract l^{-1}(BD Difco); 1 g peptone l^{-1} (BD Difco); 2 g glucose l^{-1} (Sigma-Aldrich); 10 mM $CaCl_2$ (Merck); 2% palm oil. The growth was carried out at 28°C in 1 l flask in agitation (150 rpm) with a work volume of 250 ml. The same medium was used for the growth in bioreactor (New Brunswick™ BioFlo® 7l, Eppendorf) testing two different conditions of aereation: 1 LPM (Litre per minute) gas flow and 200 rpm; 3 LPM (Litre per minute) gas flow and 500 rpm.

Microscopy

Optical microscopy was performed by means of an Olympus BX51 research system microscope (Olympus Corporation, Japan). A single colony was dissolved in sterile water and deposited on glass slides. The organism was visualized at 100-fold magnification.

Lipase assay

Lipase activity was determined spectrophotometrically (UVIKON 922 UV/Vis Spectrophotometer; BioTek Instruments) using *p*-nitrophenyl decanoate as substrate (Sigma-

Aldrich). The *p*-nitrophenyl decanoate was dissolved in 20% isopropanol. The assay mixture consisted of 880 µl 50 mM Tris/HCl (pH 8), 100 µl of the sample (or 1 mg of pellet) and 20 µl of 10 mM the *p*-nitrophenyl decanoate (ε_{405}= 3,390 $M^{-1}cm^{-1}$). The activity was assayed following the release of *p*-nitrophenol in 1 minute at RT. One unit of lipase activity was defined in International Units. All measurements were carried out in triplicate.

Protein concentration

Protein concentration was determined by Bradford Assay (Biorad) using BSA as standard, using the manufacturer instruction.

Effect of pH on lipase activity and stability

The optimal pH of lipase activity was determined using *p*-nitrophenyl decanoate in the range of 2 to 12 by using different pH buffer solutions (McIlvaine buffer, pH 2-8; 50 mM Tris/HCl, pH 8-10; sodium carbonate, pH (10-12) at room temperature (25°C). Stability at pH was assessed as described above after incubation at the different pHs.

Effect of temperature on lipase activity and stability

The optimal temperature of the enzyme activity was evaluated at temperature ranging from 30° to 60°C in 50 mM Tris/HCl buffer, pH 8. Lipase thermostability was determined by incubating the enzyme in the same temperature range.

Substrate specificity

p-nitrophenyl esters (Sigma-Aldrich) containing different acyl chain length (C2, C4, C5, C8, C10, C12, C14, C16, C18) were used to determine the substrate specificity.

Activity in commercial detergents

The samples were incubated at 30°C in 1% commercial detergents diluted in tap water to simulate washing condition. The detergents already containing enzymes in their formulation were incubated 30 minutes at 80°C to inactivate the endogenous enzymes. The commercial detergents used were: General (Henkel) (enzymes-free); ACE(+) (P&G) (enzymes-added); Biopresto (Henkel) (enzymes-added); Dixan (Henkel) (enzymes-added).

Lipase recovery

The culture supernatant containing extracellular lipases was concentrated and dialyzed against 20 mM Tris/HCl pH 8 by means of a 10 kDa T-Series cassettes tangential flow filtration device (Pall® Life Sciences).

Mycelium was recovered through filtration on cloth gauze, washed twice with water and lyophilized. Dry cells were resuspended in 20 mM Tris/HCl pH 8 at a final concentration of 5 mg/mL.

The sample was loaded onto a Resource Q column (GE Healthcare) equilibrated in 20 mM Tris/HCl pH 8 using an ÄKTA explorer chromatographic device (GE Healthcare). A four steps NaCl concentration gradient was applied (0- 0.4 -0.6- 1M). All fractions containing lipase activity were pooled and concentrated using a 10,000 MWCO PES Macrosep® Advance Centrifugal Filter (Pall® Life Sciences).

RESULTS

Isolation of lipase producing organisms

With the aim to isolate extracellular lipase producing microorganisms from seawater samples from “Darsena vecchia”, a proper enrichment culture method was set up. The principle of the enrichment culture is to provide growth conditions favorable for the organisms of interest and as unfavorable as possible for competing organisms.

The collected microbial community was first inoculated in two different rich basal media (LB and YEPD) and incubated at two temperatures (15°C and 28°C) (Fig. 1). Once microbial communities reached a high enough density, an aliquot of these cultures was inoculated in the same media containing a lower concentration of nutrients and in the presence of olive oil as a selective nutrient source. With this procedure it was possible to promote growth of microorganisms able to use olive oil as carbon sources due to their ability to secrete extracellular lipases. The concentration of rich basal media was progressively reduced up to use olive oil (1% in water) as the only carbon and energy source. Subsequent screening on chromogenic plates supplemented with olive oil was performed to isolate microorganisms able to secrete lipases (rejecting esterase producing microorganisms). The four enriched microbial communities (corresponding to the two starting different media incubated at the two different temperatures) were processed as described. Among all species grown in the four conditions, only colonies initially grown on YEPD at 15°C were able to produce lipase activity halos on chromogenic phenol red/olive oil plates. With this procedure, 12 discrete "colonies" were selected. One strain (labeled D6) showing the highest lipolytic activity, based on halos diameters, was selected and picked out for further study (Fig. 2) . The selected strain was grown for 7 days in liquid medium, yielding a production in the culture broth of about 80 U l^{-1} of lipase activity after 4 days.

Organisms’ identification and characterization

A morphological analysis of the D6 isolate was performed. The fast growing colony is white, flat, with cottony aerial mycelium and somewhat fimbriate margin when grown on a rich agar plate YEPD medium (Fig. 3a). The organism was also visualized by microscopy at 100-fold magnification. The micrograph shows that the strain has true (septate) hyphae with side branches bearing blastoconidia often with di- or trichotomous branching. The lateral branches soon disarticulate into short-cylindrical cells. According to this information, the selected strain appears to belong to fungi kingdom (Fig. 3b). In addition, D6 isolate was analysed through sequencing of its ITS1 region and Data bank interrogation. The isolate displays an

identity of 97% with *G. candidum* strains. The clustering of the new isolate with other strains belonging to *Galactomyces* and *Geotrichum* spp is reported in Fig. 3c, where it is possible to observe that the D6 strain is strictly related to *G. candidum* strain DBMy703.

The D6 strain was identified as a new strain of *G. candidum* and is hereinafter indicated as *G. candidum* GTP. Even if other studies on lipolytic activity from *G. candidum* are already present in the scientific literature[5], this fungus shows a large genetic variability[10], thus confirming the interest in characterizing lipase activity production from this selected strain.

Optimization of lipase production.

Lipases production is known to be influenced by the nitrogen source[15], and is generally related to the presence, in the culture broth, of a lipidic carbon source[16]. In addition it is reported that calcium can have a stabilizing effect on lipases[5]. On the basis of these considerations several parameters were taken into account to select the best conditions for optimizing lipase production. The effect of calcium concentration, lipidic source, temperature and concentration of nitrogen source was evaluated (Table 1). As for calcium concentration (runs 1-4) its presence is related to an increase of lipase activity, even if the lower is the calcium concentration, the higher is the lipase production. To improve lipase production olive oil was replaced by palm oil (Table 1, run 5) due to its higher content of long chain saturated fatty acids respect to olive oil[17]. It is noteworthy that the palm oil is not completely dissolved at 15°C, thus the microorganism was also grown at 28°C (Table 1, runs 6-7). While the change of temperature from 15 to 28°C had no effect on the lipase production with olive oil, a fourfold increase of lipase activity production with palm oil at 28°C was observed.

As reported in literature lipase production is also influenced by the presence and concentration of an organic nitrogen source, like yeast extract[5, 18]. The growth carried out in the presence of an increased concentration of yeast extract showed a huge increase of lipase production with a peak of activity of 6,000U l^{-1} after 6 days (Table 1, run 8). When lipase production was scaled up in a 7 l bioreactor, a peak of lipase activity of 9,000 U l^{-1} after only 2 days was observed (Table 1, run 9) (Fig. 4). During the setup of the experiments in order to find the best condition of aeration, some parameters were tested as gas flow and agitation. The best lipase secretion (Supplementary Figure 1) was obtained at a relatively low aeration.

The lipase production level displayed by *G. candidum* GTP is in agreement with those reported for *G. geotrichum* Y05 strain, producing 9,600 U l^{-1} [19] and for *G. candidum NRRLY-552* strain[20], ranging from 8,000 to 30,000 U l^{-1}.

As already reported for the majority of *G. candidum* strains[7, 21] also the newly isolated strain *G. candidum* GTP produced mycelium-bound lipases (MBL). From a comparison between extracellular lipases (EL) and MBL, it is evident that in the tested conditions the most of the lipase activity is localized in the extracellular medium. In fact, a lipase activity of 19.6 units per gram of lyophilized biomass (16.5 g l^{-1}) was measured after two days of growth (about 300 U l^{-1}) respect to a lipase secretion of about 9,000 U l^{-1}.

Both enzymatic pools have been characterized testing the effect of pH, temperature and presence of detergents (Supplementary materials) for their activity and stability. Furthermore, substrate specificity of the two enzymatic pools was assessed.

Characterization of extracellular lipases (EL) and mycelium bound lipase (MBL)

pH and temperature dependence

The two enzymatic pools display a different pH activity profile. In fact, EL showed 100% of activity in the 8-9 pH range (Fig. 5a), while MBL maximum activity was narrower and shifted to pH 7 (Fig. 5b). As for temperature dependence, EL and MBL did not display any significant difference (Fig. 6).

Enzyme stability

The two samples were also assayed for their stability at different pHs (Table 2). The values indicated a higher stability of MBL than EL, in particular at pHs where the former display its maximum activity (Table 2).

As for the stability at different temperatures (Table 2), in both cases there was a decrease in the stability with temperature increase, with MBL displaying a slightly higher stability than EL.

When the stability of the enzymes was assessed in the presence of different liquid laundry detergents, MBL confirmed its higher stability respect to EL (Supplementary Figure 2). The MBL enhanced stability could be due to the natural immobilization of the enzymes on the mycelium[22–24].

Substrate specificity

Substrate specificity of both enzymatic pools was tested using the nitrophenyl derivatives of fatty acids with different length chain, in a range from 2 to 18 carbon atoms. It was evident that EL display a higher specificity against long chain derivatives ($C \geq 10$) respect to MBL which preferentially hydrolyzed short chain derivatives ($C < 10$), being C2 the preferred substrate (Fig. 7).

The substrate specifity of *G. candidum* GTP lipases are different respect to what already reported in literature for other *G. candidum* strains. In fact, Brabcovà et al. (2010) reported for *G. candidum* 4013, isolated from soil, the production of extracellular lipases displaying high specificity against medium chain length substrates, and cell-bound lipases showing high specificity against long chain length substrates. Another *G. candidum*, isolated from soil, produces mycelium bound lipases endowed with a preference for long chain length substrates, and in particular for unsaturated fatty acids containing a cis-9 double bond[7]. These findings may be related to the biodiversity of *G. candidum* strains from different environments[10], and to the culture conditions inducing the production of different lipase isoforms[21].

Secreted lipases from *G. candidum* GTP were fractionated by means of anionic exchange chromatography to verify the presence of multiple isoforms. The presence of at least two different secreted lipases, displaying almost similar substrate specificity, was observed in this conditions (Supplementary Figure 3). The secretion of two lipases has been already reported for other *G. candidum* strains[11, 12]. On the other hand, Sugihara and coworkers[25] reported the production of four lipase isoforms from *G. candidum* ATCC 34614.

CONCLUSIONS

Applying a bioprospecting approach searching for lipase secreting microorganisms into sea water collected in the "Darsena Vecchia" of Pozzuoli harbour, a new *G. candidum* strain, named *G. candidum* GTP, was isolated. *G. candidum* strains were already reported as lipase producing organisms, however they were isolated from different environments, such as dairy products[10], soil[7] and oil-mill wastes[5].

Lipase production from *G. candidum* GTP was assessed studying the effect of carbon and nitrogen source, and of calcium. The scale up in bioreactor with optimized medium allowed reaching a production peak of extracellular activity of 9000 U l^{-1}.

Properties of secreted and natural immobilized lipases on mycelium were compared underlining the increased stability of mycelium-bound lipases, and the existence of a preferred localization of true lipases in the extracellular medium.

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List of Supporting Informations

Supplementary Figure 1: Activity profile and DO profile during the growth in bioreactor with different aeration (**a**) 1LPM, 200 rpm; (**b**) 3LPM, 500 rpm.

Supplementary Figure 2: Stability of *G. candidum* GTP lipases in commercial detergents. Confidence intervals are given for 95% reliability.

Supplementary Figure 3 Absorbance at 280 nm and lipase activity profile of the extracellular lipases on anion exchange chromatography. Green arrows indicate lipase isoforms. Substrate specificity of lipase isoform 1 **(b)** and of lipase isoform 2 **(c)**. Confidence intervals are given for 95% reliability.

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Table 1. Optimization scheme of the growth conditions. The maximum of activity of each condition was reported with related time.

Run	Medium Composition	°C	U l^{-1}	Day
1	YEPD + 1% OLIVE OIL	15	*150*	4
2	YEPD + 1% OLIVE OIL + 10 mM $CaCl_2$	15	*240*	4
3	YEPD + 1% OLIVE OIL + 50 mM $CaCl_2$	15	*180*	4
4	YEPD + 1% OLIVE OIL + 90 mM $CaCl_2$	15	*80*	4
5	YEPD + 1% PALM OIL + 10 mM $CaCl_2$ (oil not completely dissolved at this temperature)	15	*100*	4
6	YEPD + 1% OLIVE OIL + 10 mM $CaCl_2$	28	*240*	4
7	YEPD + 1% PALM OIL + 10 mM $CaCl_2$	28	*430*	4
8	YEPD +2% YEAST EX.+ 1% PALM OIL + 10 mM $CaCl_2$ (GAL BROTH)	28	*6,000*	6
9	GAL BROTH in BIOREACTOR	28	*9,000*	2

Table 2. Effect of temperature and pH on EL and MBL by G. geotrichum GTP. For each temperature and pH values is indicated the t ½ (incubation time after which the enzyme keeps 50% of activity).

	pH			Temperature			
t ½	7	8	9	30°C	40°C	50°C	60°C
EL	*2 Days*	*6 Days*	*6 Days*	*5 Days*	*2 Days*	*35 min*	*25 min*
MBL	*8 Days*	*8 Days*	*8 Days*	*8 Days*	*3 Days*	*45 min*	*15 min*

FIGURE LEGENDS

Fig. 1. Schematic representation of culture enrichment method adopted to isolate lipolytic microorganisms.

Fig. 2. Colony growth on nutrient **(a)** and chromogenic **(b)** agar plate.

Fig. 3. (a) D6 colony morphology on YEPD agar plate; **(b)** D6 micrography (100-fold amplification), Scale bar 100 □m; **(c)** Phylogenetic tree created by neighbour joining clustering method in Mega 7.0.

Fig. 4. Lipase activity profile of *G. geotrichum GTP* during the growth in bioreactor and in the shaken-flask. Confidence intervals are given for 95% reliability.

Fig. 5. Relative activity of EL **(a)** and MBL **(b)** at different pH values. The highest value of activity is considered as 100 % of activity. Confidence intervals are given for 95% reliability.

Fig. 6. Relative activity of EL **(a)** and MBL **(b)** at different temperature values. The highest value of activity is considered as 100 % of activity. Confidence intervals are given for 95% reliability.

Fig. 7. Substrate specificity of EL **(a)** and of MBL **(b)** produced by *G. candidum GTP.* Confidence intervals are given for 95% reliability.

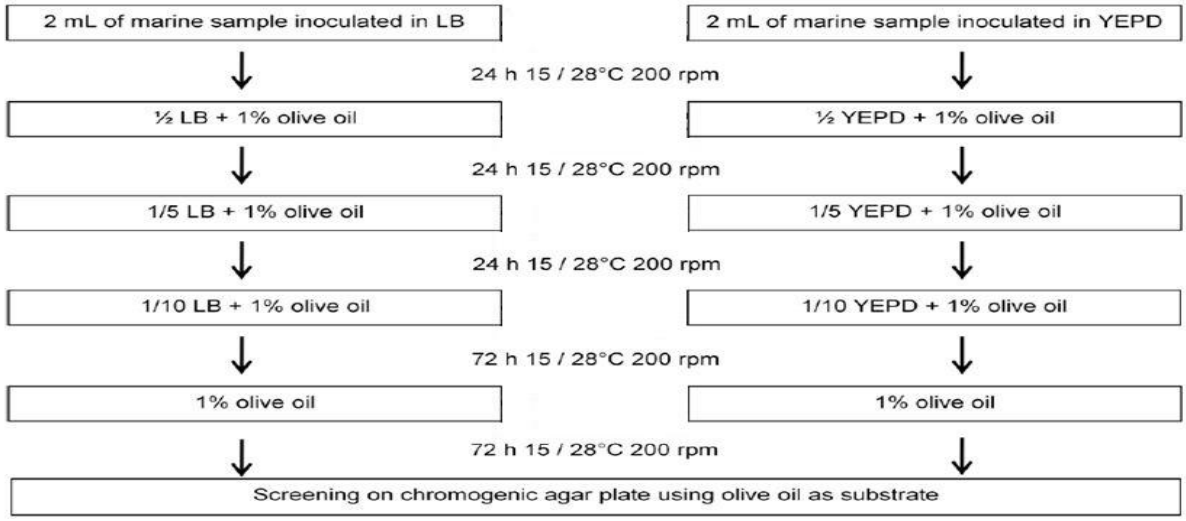
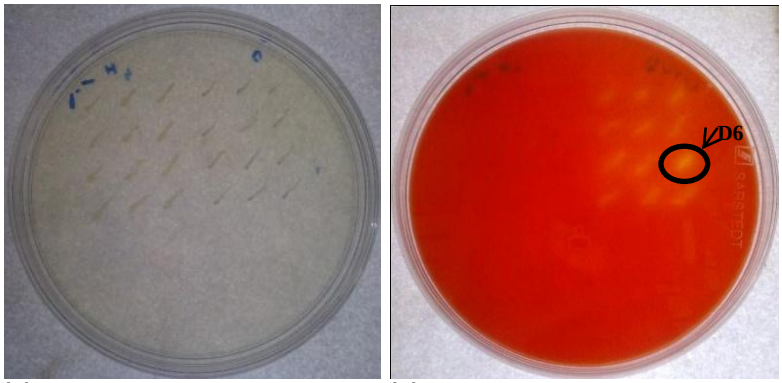
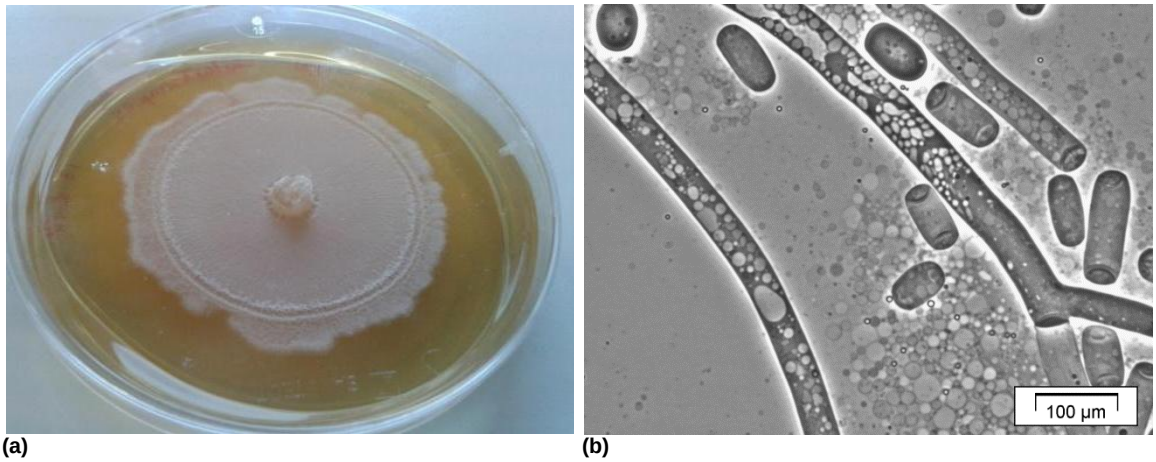

2 mL of marine sample inoculated in LB
2 mL of marine sample inoculated in YEPD
24 h 15 / 28°C 200 rpm
½ LB + 1% olive oil
½ YEPD + 1% olive oil
24 h 15 / 28°C 200 rpm
1/5 LB + 1% olive oil
1/5 YEPD + 1% olive oil
24 h 15 / 28°C 200 rpm
1/10 LB + 1% olive oil
1/10 YEPD + 1% olive oil
72 h 15 / 28°C 200 rpm
1% olive oil
1% olive oil
72 h 15 / 28°C 200 rpm
Screening on chromogenic agar plate using olive oil as substrate

Fig. 1

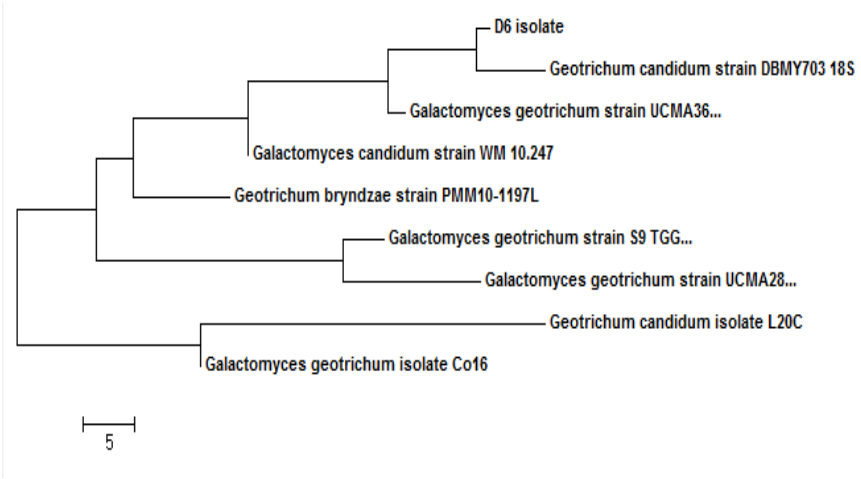

D6

(A) **(B)**

Fig. 2.



(a) (b)



(c)

Fig. 3.

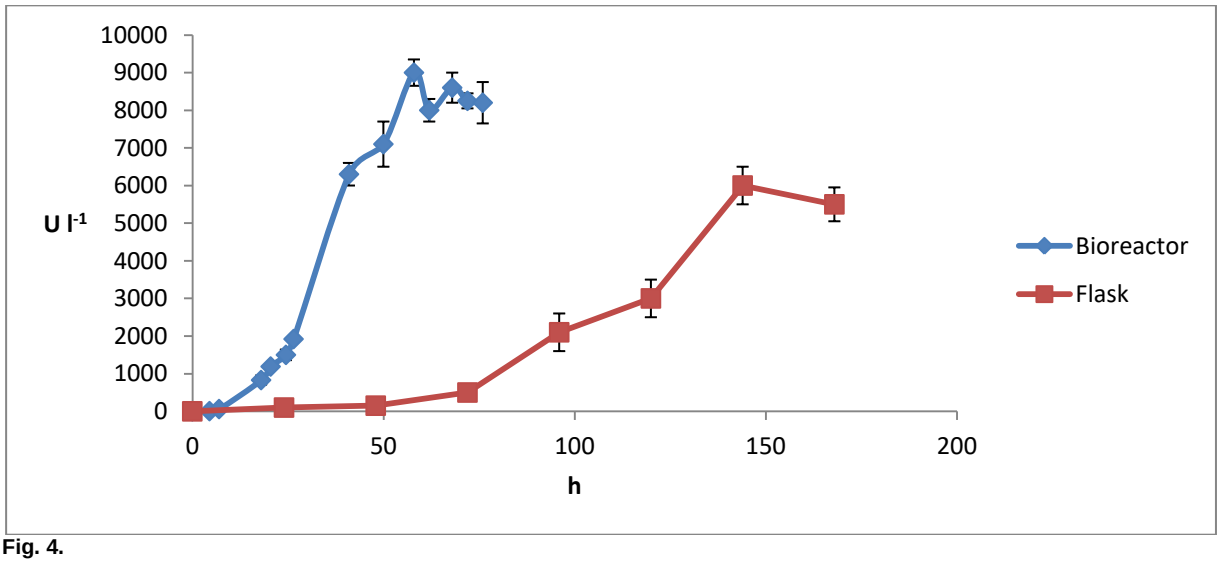


Fig. 4.

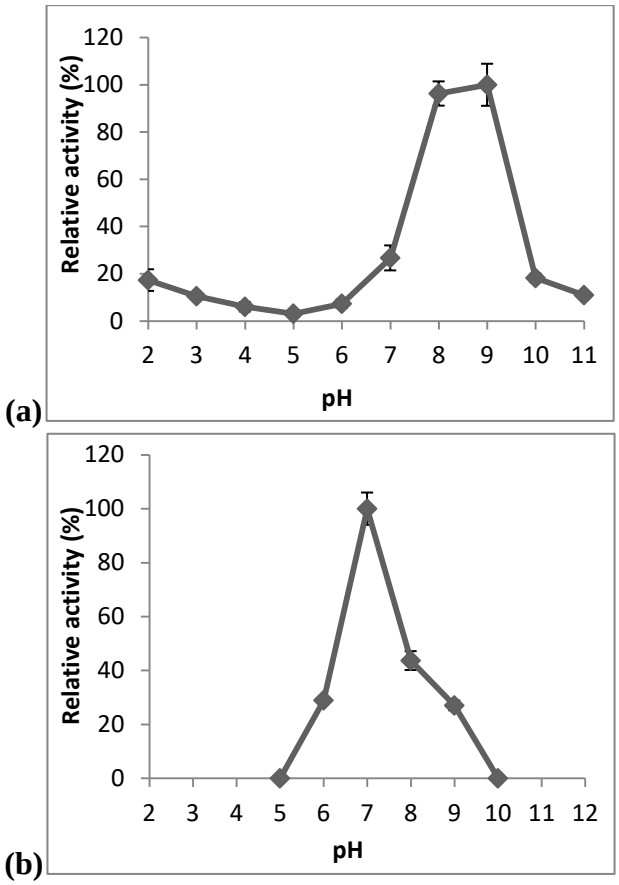

(a)
Relative activity (%)
120
100
80
60
40
20
0
2 3 4 5 6 7 8 9 10 11
pH
(b)
Relative activity (%)
120
100
80
60
40
20
0
2 3 4 5 6 7 8 9 10 11 12
pH

Fig. 5.

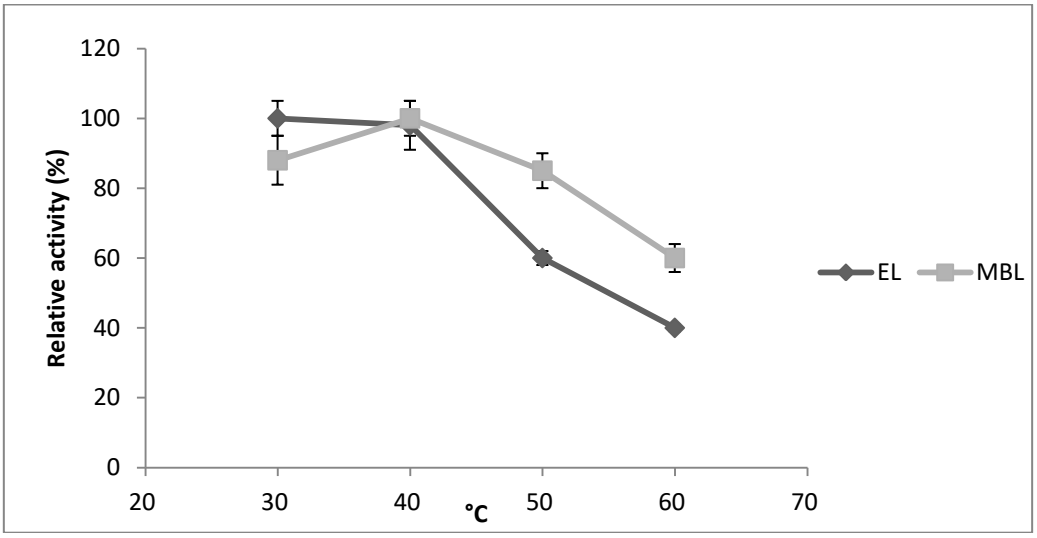

Relative activity (%)
120
100
80
60
40
20
0
20 30 40 50 60 70
°C
EL
MBL

Fig. 6.

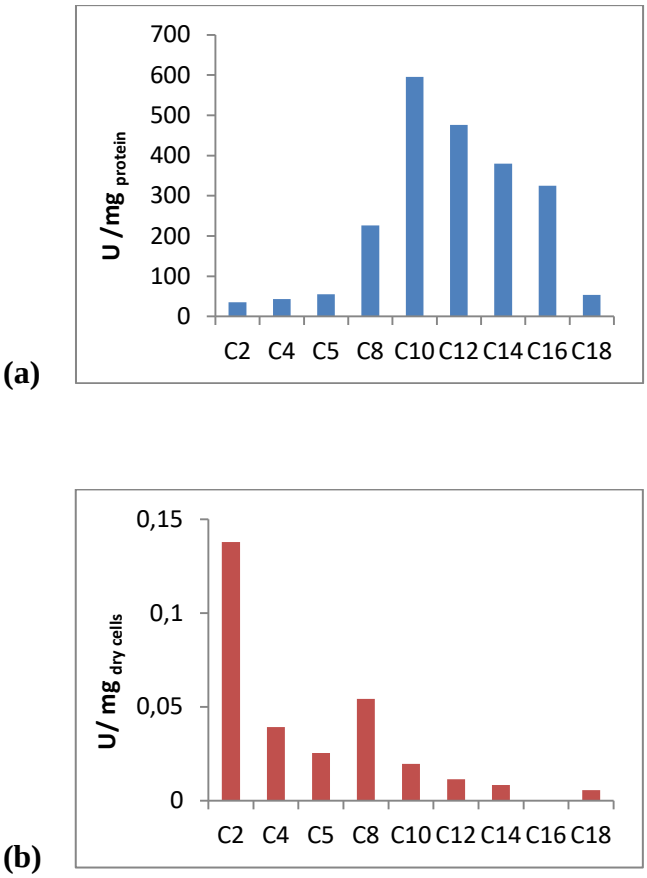


Fig. 7.

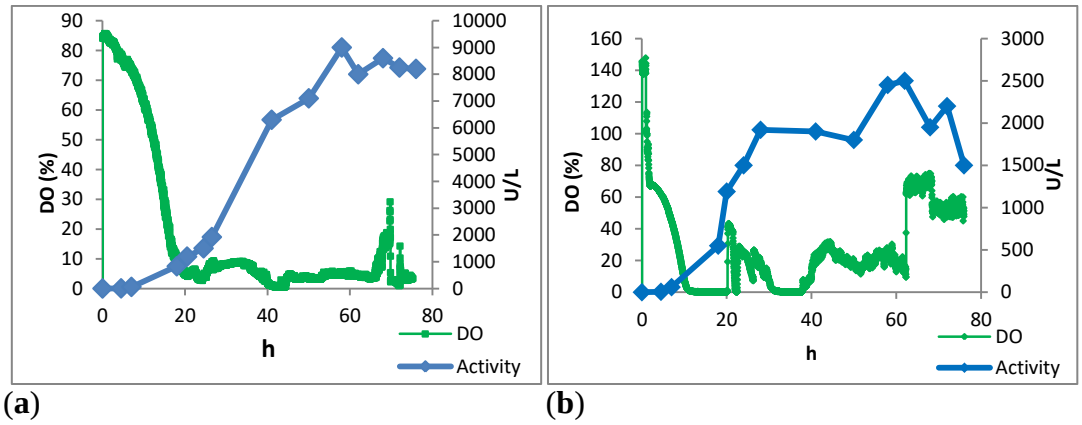
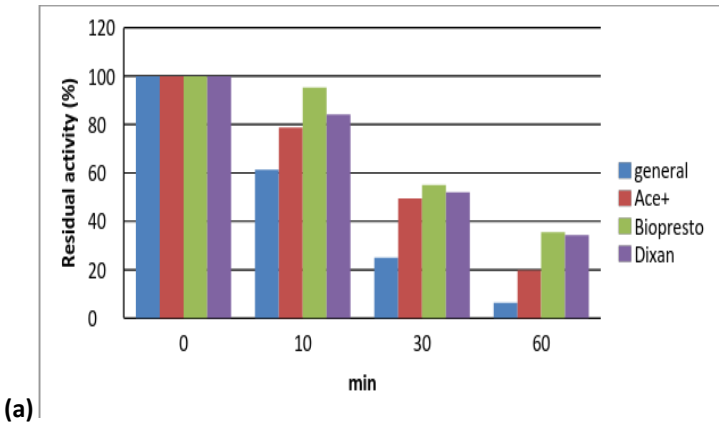
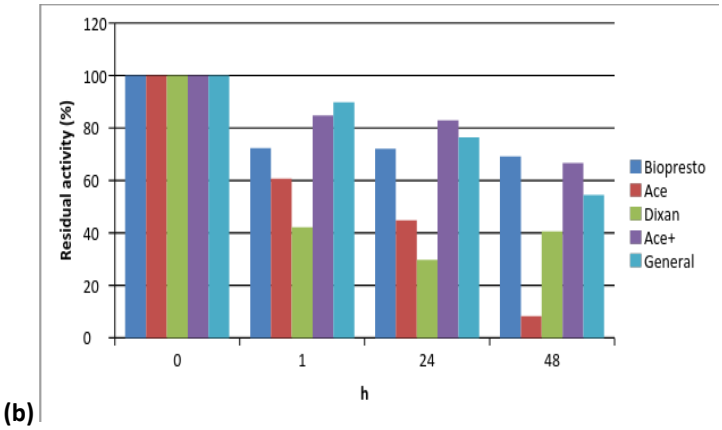


Figure S1 Activity profile and DO profile during the growth in bioreactor with different aeration (**a**) 1LPM, 200 rpm; (**b**) 3LPM, 500 rpm.



(a)



(b)

Figure S2: Stability in commercial detergents of EL (a) and MBL (b)

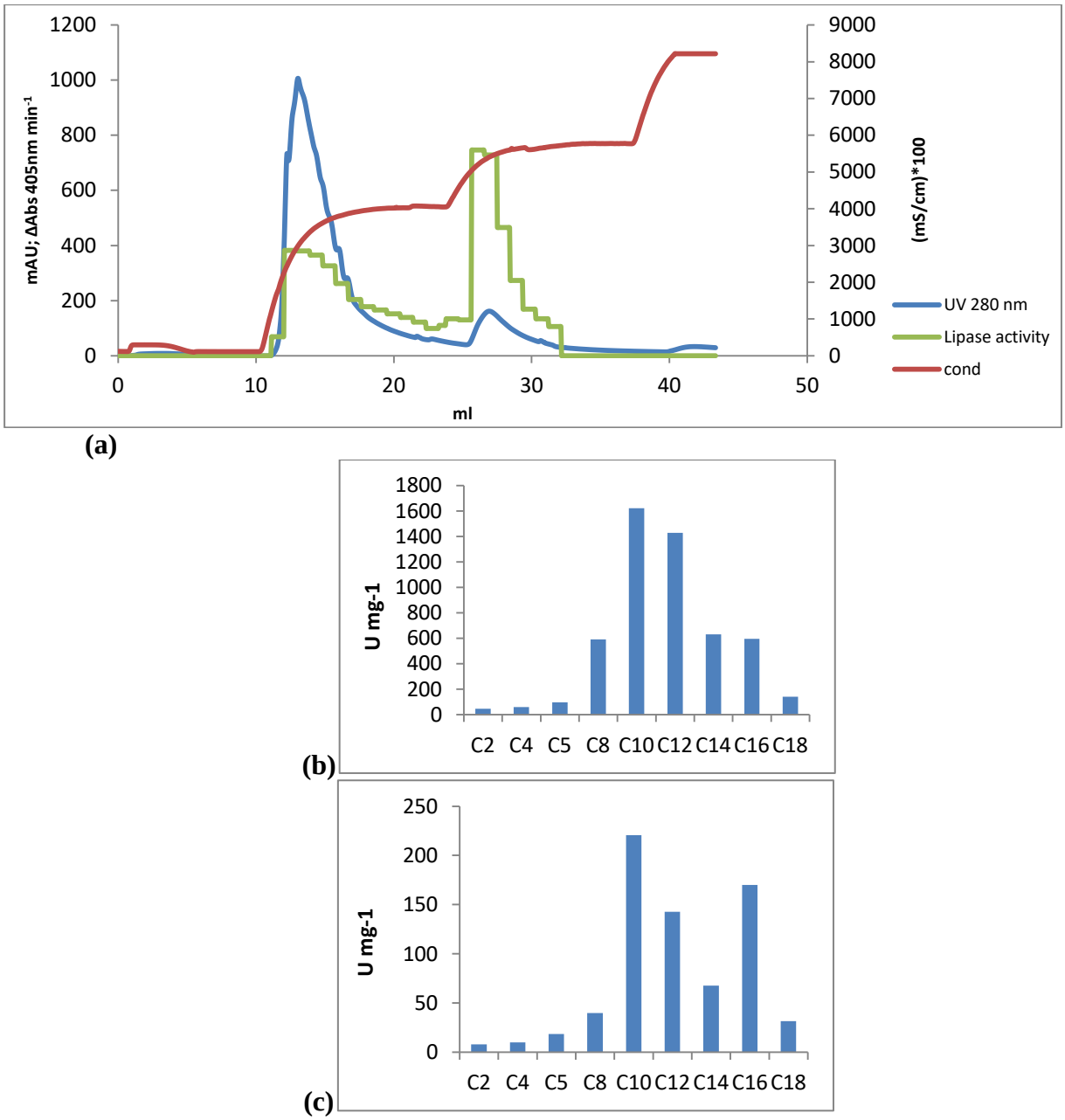


Figure S3 (**a).** Absorbance at 280 nm and lipase activity profile of the extracellular lipases on anion exchange chromatography. Green arrows indicate lipase isoforms. Substrate specificity of lipase isoform 1 **(b)** and of lipase isoform 2 **(c)**.

Section 2

Revaluation of industrial waste to produce an high value compounds: Compost as new lipase producing microorganisms.

Abstract

One of the best goal in the biotechnology industry is to convert the wastes, derivatives by industrial process, in a high-value compounds. Among these, is possible to cite the enzymes, that are already used in a wide range of industrial applications. In this study the revaluation of waste from olive oil industry was carried out in order to isolate a new lipase producing microorganisms, than new lipases. Four samples from CESCO composting center were analyzed and a themophilic lipase producing microorganism was isolated: *Alicyclobacillus acidocaldarius*. Several medium compositions were tested to improve extracellular lipase production reaching a maximum of 700 U/L. A recombinant production approach was also tested. *Pichia pastoris* was used as heterologous host.

1. Introduction

The huge amounts of wastes proceeded by human activities is increasing an it is becoming a global problem for the environment protection because of their composition and storage manner. It is necessary to stimulate the demand for materials with productive reuse, repurposing, and remanufacturing potential. Several companies offer services as the waste recovery operation to divert these materials away from landfills, trash heaps, and incinerators and into productive economic uses that support sustainable, long-term local economic growth. Sometimes the wastes from several processes can be reused as raw materials in the industrial production, as a replacement for natural raw materials (Radulescu et al., 2011).

In Mediterranean basin the industrial wastes, due to the oil production, represent a strong problem. The environmental contamination associated with the production of olive mill wastewaters in different European productive contexts, regardless of the extraction system adopted by the olive oil mills (continuous cycle in three-stage, continuous cycle in two-stages, traditional with press), still represents an unresolved problem (Caputo et al., 2003; Roig et al., 2006; Salomone and Ioppolo, 2012). In this framework, the revaluation of the waste, as the compost obtained from the treatment of oil mill waste, is strongly interesting the market, as a possible source to produce high value compounds (Fernández-Bolaños et al., 2006), as new enzymes with a biotechnological potential. CESCO is a composting centre where oil mill wastes are treated. Fig 1 reports the composting processes flowchart. The compost was worked at high temperature, with the range from 51 to 67°C. This represent an interesting aspect in order to use this compost as source of new enzymes. In fact, the thermostable enzymes are widely used in industry. In this study the compost from the composting centre CESCO, where was treated oil mill waste, was analysed in order to isolate new lipase producing microorganisms. To this aim, Different sample were taken from four steps: Stoccaggio sanza vergine, Bioreattori (ACC), Curing, Vaglio rotante. The further screening of lipase activity led to the identification of *Alicyclobacillus acidocaldarius.* In literature it is reported as thermostable enzymes producing (Fan et al., 2014; Mandrich et al., 2006).

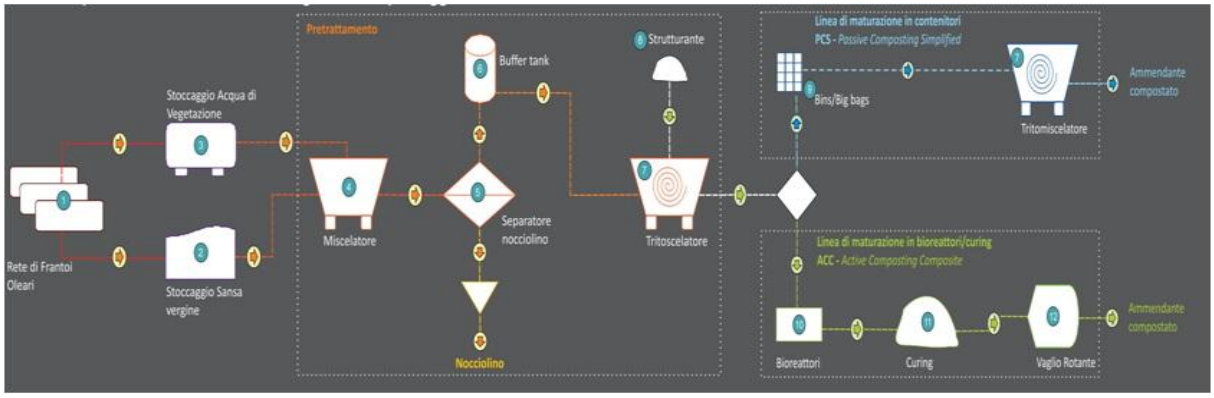


Fig 1 Composting process flowchart in composting centre CESCO.

2. Materials and Methods

2.1 Compost revitalization

Four sample were taken from composting centre CESCO (TIRSAV PLUS project LIFE05 ENV/IT/845). Five grams of each compost sample were suspended in 50 mL in 50 mM Tris/HCl pH 8 and incubated in a 250 mL flask in agitation (150 rpm) at 60°C.

2.2 Solid growth media

Two type of solid growth media were used in order to induce bacteria and yeast/fungi growths. LB agar plates (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, 15 g/L agar) for bacteria. PDA agar plates (dextrose, 20 g/L. potato extract, 4 g/L, agar, 15 g/L agar) for yeast and fungi. All compound were by BD Difco, except NaCl by Vwr.

2.3 Chromogenic/tributyrin plates to detection of lipase activity

Chromogenic plates containing 1% olive oil, 1% phenol red, 1% CaCl2, 1.5% agar were used to visualize lipase activity during the screening. The pH (7.0) was adjusted by adding NaOH 1M. Plates were incubated at 28°C. Using olive oil as substrate and phenol red as pH indicator, the yellowish halo indicate the presence of lipolytic activity. Tributyrin agar plates were used to screen the *P. pastoris* producing clones after the transformations with pJGGαKR/AliLip construct. The screening was carried out in petri dishes using a medium constituted by: 0.5% peptone; 0.3% yeast extract; 0.1% tributyrin; 2% agar, pH 6.0 (Griebeler et al., 2011). Culture plates inoculated with the fungal strains were incubated at 28°C for 120 h. The lipase activity was displayed by clear halo.

2.4 Gene synthesis, and plasmid and strain construction

The AliLip gene, excluding its signal peptide coding region, was designed according to codon usage for *P. pastoris* and synthesised by Geneart Lifetechnology (Germany). Vector pJGGαKR (BioGrammatics) and lipase genes were both hydrolised with *BsaI* and then ligated. The ligation mixtures were transformed into *E. coli* Top10 (*F-mcrA D (mrr-hsdRMS-mcrBC) f80lacZDM15 DlacX74 deoR recA1 araD139 D (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG*), and transformants were selected on LB medium supplemented with 100 µg/mL of ampicillin antibiotic. The pJGGαKR/AliLip vector was linearized with *BsiWI*, and transformed into competent cells of *P. pastoris* BG-10 strain (BioGrammatics) by electroporation in a Bio-Rad Micro-Pulser apparatus, as specified by the manufacturer. Positive clones were selected on MD agar plates supplemented with 0.9 mg/mL of G418, and then

transferred on tributyrin agar plates. The clones with the higher clarification halos were grown in BMGY(13 g/L yeast nitrogen base with ammonium sulfate without amminoacids; 10 g/L yeast extract; 20 g/L peptone; 100 mM potassium phosphate, pH 6.0; $4x10^{-4}$ g/L biotin; 1% glycerol) liquid culture. The BMGY culture were incubated at 28°C under shaking condition.

2.5 Lipase assay

Lipase activity was determined spectrophotometrically using p-nitrophenyl decanoate (Sigma-Aldrich) as substrate. The p-nitrophenyl decanoate was dissolved in isopropanol at a concentration of 10 mM. The assay was carried as follows: 880 µL 50 mM Tris/HCl (pH 8.0), 100 µL of sample and 20 µL of 10mM the p-nitrophenyl decanoate. The activity was assayed by detecting the product, p-nitrophenol, spectrophotometrically (UVIKON 922 UV/Vis Spectrophotometer, BioTek Instruments) at 405 nm (ε_{405}= 3,390 $M^{-1}cm^{-1}$). All measurements were carried out in triplicate.

2.6 Genomic DNA extraction

A colony from agar plate was incubate in 5 mL of PDY in agitation (150 rpm) at 60°C. After an overnight growth the pellet was obtained and suspended in a 1.6 mL of lysis buffer (9 g/L glucose, 3 g/L Tris/HCl, 4.4 g/L EDTA, 0.05 g/L proteinase K) added with 10 mg of lysozime. The sample was mixed by inversion and incubated at 37°C for 10 minutes. 30 µL of 20% sarcosyl was then added and the sample incubated for 5 minutes at 37°C. The sample was extracted twice with phenol/chloroform. The resulting aqueous phase was transferred to a clean tube and 50 µL of 3M NaAc pH6 and 1.5 mL of 96% cold ethanol were added and the genomic DNA was precipitated by centrifugation at 13,000 rpm for 10 minute at 4°C. The resulting pellet was dried for 10 minute at 37°C and resuspended in 200 µL of H_2O.

2.7 Alicyclobacillus acidocaldarius cultivation

Different media were tested to grow *A. acidocaldarius.* PDY (24 g/L potato dextrose, 5g/L yeast extract), PDA (20 g/L dextrose, 4 g/L potato extract), LB (10 g/L tryptone, 5 g/Lyeast extract, and 10 g/L NaCl), YPD (10g/L yeast extract, 20 g/L peptone, 20 g/L glucose), Alymedia (2 g/L yeast extract, 5 g/L glucose, 0.25 g/L calcium chloride, 0.5 g/L magnesium sulphate, 0.2 g/L ammonium sulphate, 3 g/L potassium dihydrogen phosphate, 0.00018 g/L zinc sulphate, 0.00016 g/L copper sulphate, 0.00015 g/L manganese sulphate, 0.000018 g/L cobalt chloride, 0.0001 g/L boric acid, 0.0003 g/L sodium molybdate), PDY + 2% palm oil , PDY + 2% olive oil. All the growths were conducted in agitation (200 rpm) at 60°C.

2.8 Bioinformatics tools

The putative lipase coding-gene sequences present on *A. acidocaldarius* genome (NC_013205.1) and the sequences of the commercial lipases LipaseB (P41365) from *Candida Antarctica,* LIP1 (P20261) from *Candida rugosa*, LIP (P22088) from *Burkholderia cepacia,* lipase (H9MZI2) from *Pseudomonas alcaligens* and LIP (O59952) from *Thermomyces lanuginosus,* were aligned using the Blast software at the National Centre of Biotechnology Information website (Altschul et al., 1997).

2.9 Strains

The *Pichia pastoris* strains used for the heterologous expression was the commercial strain BG-10 by BioGrammatics, Inc.

2.10 Pichia pastoris cultivation

Recombinant cells from solid culture of pJGGαKR/AliLip were inoculated in 50 mL BMGY medium in a 250 mL baffled shaken flask. This preculture was grown for one days at 28° C on a rotary shaker (250 rpm), then a volume of suspension sufficient to reach a final OD_{600} value of 1.0 was used to inoculate 1 liter shaken flasks containing 250 mL of BMGY medium (13 g/L yeast nitrogen base with ammonium sulfate without amminoacids; 10 g/L yeast extract; 20 g/L peptone; 100 mM potassium phosphate, pH 6.0; $4x10^{-4}$ g/L biotin; 1% glycerol). Cells were grown on a rotary shaker (250 rpm) at 28°C.

3. Results and Discussion

3.1 Screening for lipase producing microorganisms

The four samples taken from the composting center CESCO (Fig 2), were resuspended in buffer at pH 8 and incubated in a flask in agitation at 60°C.

Fig 2 Sample from composting center CESCO.

Once a significative optical density was reached, different serial dilutions on plate were carried out in order to obtain individual colonies belonging to all the species present in the four samples. Two type of solid growth media were used in order to induce both bacteria and yeast/fungi growths. Consequentially, a replica plating was made on chromogenic plates to identify the microorganisms able to secrete lipases. Nine clones grown on PDA agar plates showed positive halos (Fig 3). Among these, three colonies were chosen basing on the size of the yellow halo on the plate and their genomic DNA was extracted for strain identification In all the samples the experiments led to the identification of one microorganisms: *Alicyclobacillus acidocaldarius* DSM446.

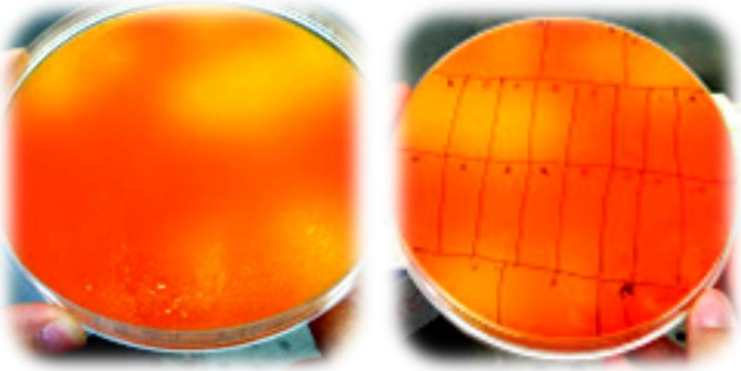


Fig 3 Screening of lipase producing microorganisms on chromogenic plate

Alicyclobacillus acidocaldarius (Darland and Brock 1971) is the type species of the larger of the two genera in the bacillaceae family '*Alicyclobacillaceae*' (Mavromatis et al., 2010). *Alicyclobacillus* spp. are Gram-positive, thermo-acidophilic, and spore-forming bacteria that can cause spoilage in pasteurized and heat-treated fruit juices/beverages, evident by the presence of a distinct medicinal or antiseptic off-odor attributed to guaiacol (CHANG et al., 2015; Chang and Kang, 2004; Yokota et al., 2007). In literature is reported the recombinant production (Agafonov et al., 2005) and 3D structure construction (De Simone et al., 2000) of the esterase EST2 from *A. acidolcadarius.* However there are few studies about lipases from this microorganism. In addition the complete genome sequence of *A. acidocaldarius* DSM446 are on database and on the genome are annotated five putative lipase and four putative phospholipase. In this framework was interesting to study this bacterium as source of new lipases.

3.2 Production of lipases from A. acidocaldarius

Several medium compositions were tested in order to improve the extracellular lipase production in *A. acidocaldarius*. The comparison among the media showed that the highest lipase production was obtained in rich medium as PDY, probably because in this condition the bacteria reached the highest optical density. Once chosen the best medium, the effect of the addition of oil with different composition was tested. The experiments showed that the presence of both oils negatively affected lipase secretion.

Table 1 Scheme of tests of lipase production in different media.

Media	U/L	Hour
LB	200	60
PDA	300	60
Alymedia	50	60
YPD	50	60
PDY	700	60
PDY+2% olive oil	350	60
PDY+2% palm oil	400	60

3.3 Lipase recombinant production

Considering the low production of secretes lipases from *A. acidocaldarius,* an heterologous production approach was applied. With the aim to select the encoding gene (s) for the recombinant production, multiple alignments with some commercial lipases common used in industry were carried out. These enzymes show the features, as thermostability, stability in organic solvent and substrate specificity for long chain esters, required by industry. In light of this, these enzymes were good template to choose the sequence (s) for the recombinant expression. A multiple alignment among the commercial lipases and the putative lipase coding-genes on *A. acidocaldarius* genome was carried out. About four lipases used as template (table 2), the best fit was obtained with the sequence C8WXL8, however the percentage of

coverage of sequence are too low, and its predicted function is phosphoesterase. The best fit with a 45% of coverage and 56% of identity of sequence was obtained between the sequence C8WTM7 and a lipase from *T. lanuginosus*. Thus, this sequence was chosen for the further recombinant expression. The selected lipase coding sequence from *A. acidocaldarius* was synthesised and opitimizing the codon usage to the recombinant host selected, *P. pastoris.* The cDNA was cloned into pJGGαKR expression vector under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (pGAP) promoter. This strategy allows to overcome the problem of the use of methanol (it is a toxic substance and easily causes fire hazard) in the conventional recombinant system under the control of inducible alcohol oxidase (pAOX) promoter (Shen et al., 2016). The gene was fused to the coding sequence of the α-factor secretion signal. Wilde type *P. pastoris* showed a yellow band on chromogenic plates with phenol red, for this reason the screening of the best-producer clone was performed on tributyrin agar plates. The extracellular lipase activity of each clone was judged by the dimension of clear halo around the recombinant yeasts (Fig 4) and ten clones were chosen.

Table 2 Scheme of best fit among commercial lipases and putative lipase coding-genes on *A. acidocaldarius* genome. The sequences with highest score for each alignment are reported.

Alicyclobacillus coding-genes / Commercial lipase	C8WXL8		C8WXL8		C8WXL8		C8WXL8		C8WTM7	
	Query cover	ident	Query cover	ident	Query cover	ident	Query cover	ident	Query cover	ident
LipaseB *(C. antartica)*	35%	43%	--	--	--	--	--	--	--	--
LIP1 *(C. rugosa)*	--	--	35%	28%	--	--	--	--	--	--
LIP *(B. cepacia)*	--	--	--	--	21%	41%	--	--	--	--
Lipase *(P. alcaligens)*	--	--	--	--	--	--	26%	36%	--	--
LIP *(T.lanuginosus)*	--	--	--	--	--	--	--	--	45%	56%

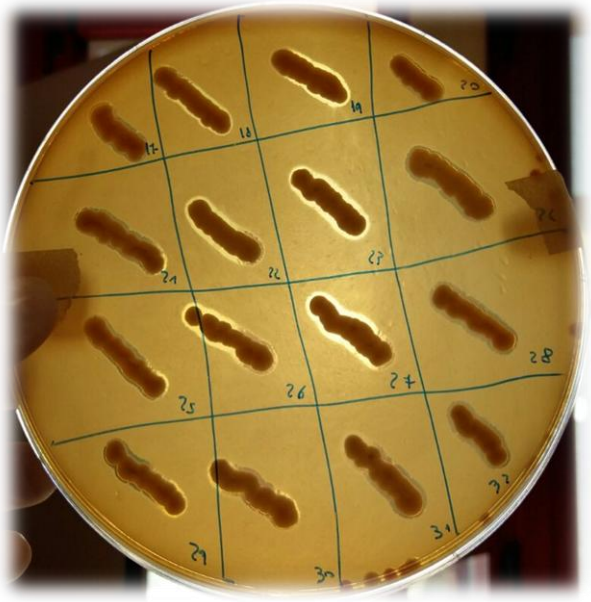


Fig 4 Tributyrin agar plate with AliLip trasformants.

The further screening performed in liquid culture allowed to select one clone to be used for the heterologous production. *AliLip* strain showed a low lipase production (300 U/L), whereas in *A. acidocaldarius* growth was 700 U/L. The expression of an esterase (EST2) from *A. acidocaldarius (Bacillus acidocaldarius)* in *Escherichia coli* (Agafonov et al., 2005) is reported but there are no examples of recombinant expression of *A. acidocaldarius* lipases in eukaryotic hosts. In literature is reported the expression of EST2 (esterase from *A. acidocaldarius*) up to 0.2 g/L using *E.coli* as heterologous host.

3.4 Thermostability of native and recombinant strains

The extracellular lipase activity of both strains, *A. acidocaldarius* and *AliLip,* were tested at two different temperatures (25 and 60°C) with standard assay (section 2.5). The results showed the 100% of the lipase activity in both conditions.

4. Conclusions

In a bioprospecting approach, samples from a composting centre were used as source to isolate microorganisms able to produce thermofilic/thermostable lipases. A lipase producing microorganism was isolated *A. acidocaldarius* DSM446, whose genome is annotated on database. Two criteria were carried out in order to produce lipases of interest. At first, the use of the native strain trough the optimization of the growth medium composition. In this way a maximum of extracellular lipase production of 700 U/L was obtained. In addition, a multiple alignment among the putative lipase coding genes present on the *A. acidocaldarius* genome and some common commercial lipases was carried out. To select gene to be heterologously produced the recombinant expression in *P. pastoris* allowed a maximum of activity of 300 U/L. Both enzymes showed activity at 60 °C confirming the thermostability of enzymes from microorganisms isolated in extreme environment.

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Chapter 2

Edible fungus as a new source of the lipases: Power of the genome mining.

Section 1(submitted to Journal of Biotechnology)

***Pleurotus ostreatus*:**

from the kitchen to the laboratory, a new source of lipases.

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Keywords: *lipase, basidiomycetes, zymogram, Pichia pastoris, p-nitrophenyl ester*, *wool dyeing*.

Abstract
This is the first report about the characterization of lipases by *Pleurotus ostreatus*. The annotated genome on database presents 44 lipase putative coding-genes. In the tested growth conditions at least 4 out of 44 putative lipase coding genes were found expressed with a pattern depending on the culture conditions. The low concentration of secreted lipases persuaded the adoption of a heterologous expression strategy. Two novel identified lipases, PleoLip241 and PleoLip369, were heterologously expressed in the yeast *Pichia pastoris*. Different expression levels were obtained: PleoLip241 reached a maximum of activity of 4000U/L, whereas PleoLip369 reached a maximum of activity of 700 U/L. Despite their sequence similarity, these enzymes exhibited dissimilar stability at all the investigated parameters and different substrate specificity, probably due to diverse substrate accessibility to the binding pocket. The obtained data allow classifying PleoLip241 as belonging to the “true lipase” family. The enzyme was used to remove hydrophobic layer on wool surface in order to improve the dyeability of wool. The encouraging results obtained with lipase treated wool let to forecast PleoLip241 applicability in this field.

1. Introduction

Lipolytic enzymes are grouped into two major families, lipases (EC 3.1.1.3) and carboxylesterases (EC 3.1.1.1). The lipolytic enzymes have a highly conserved catalytic triad, composed of Ser, Asp, and His residues (Aloulou et al., 2006). The enzymes belong to the α/β hydrolase family with a central β-sheet, containing the active serine placed in a loop termed the catalytic elbow (Ollis et al., 1992).
The biological function of lipases is the hydrolysis of triacylglycerols to give free fatty acids, diacylglycerols, monoacylglycerols and glycerol. Lipases also displayed alcoholysis, aminolysis, interesterification, and esterification activity, with rigorous regioselectivity, stereoselectivity, and chemoselectivity. Lipases are produced by animals, plants, and microorganisms including bacteria, yeast, and fungi (El-moniem Abada, 2008). Lipases, especially those of a microbial origin, are widely used for industrial biocatalysts in several processes ranging from food, dairy, pharmaceuticals, agrochemical and detergents to oleo-chemicals, tea industries, cosmetics, leather and in several bioremediation processes. In fact, over the last few years, there has been a progressive increase in the number of publications related to proprieties and industrial applications of lipase-catalyzed reactions (Verma N et al., 2012). In this context it is understandable that reducing production costs at large scale industrial level by employing different microorganisms, and discovering new strains for the production of novel lipases with industrially useful properties is increasingly becoming a new interest area of lipase research (Verma N et al., 2012).
Despite the fact that basidiomycetes fungi are preferred sources of extracellular enzymes (Saxena et al., 1999) only few examples of their lipolytic enzymes are present in the literature (Krügener et al., 2009; Zelena et al., 2009) On the other hand, enormous efforts have been made to characterize their lignolytic enzyme systems (Dashtban et al., 2010; Gomes et al., 2009; Mendonça Maciel et al., 2010). Lipases have never been isolated so far from the basidiomycete fungus *Pleurotus ostreatus* even if many putative lipase coding genes have been annotated in its genome [http://genome.jgi-psf.org/PleosPC15_1/PleosPC15_1. home.htmL] (Riley et al., 2014; Wang et al., 2008).
In this work, several culture conditions have been performed with the aim to stimulate lipase production and also to find clues about the expressed lipase coding genes. Among the huge range of the applications of the lipases, the wool treatment was investigated.
The structure conformation of wool presents a lipid layer on surface. It represents a hydrophobic barrier for the dye uptake (Ledder, 1986). Alcoholic alkaline solution or chlorine treatments are already used to improve the dyeing tissue. However these methods can modify the tissue's feature as elasticity, softness and flexibility, and in addition their use led to an increase in environmental pollution(Kantouch et al., 2005). The lipase treatment can be taken as eco-friendly alternative. The great advantage of the use of the lipases in the wool treatment is to confine the enzymatic activity on the lipid layer on the surface that represents a low percentage of the wool. In this way it is possible to improve the uptake of dye without changing the features of the wool. In literature there are few studies about the use of lipases to improve wool dyeing(Kantouch et al., 2005; Shahin and El-Khatib, 2016). In this framework, it is interesting to test the lipase activity from *P. ostreatus* in the treatment of the wool.

2. Materials and methods

2.1 Fungal strains and culture conditions

The basidiomycetes fungus used in this study was *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC MYA-2306) from ATCC, the Global Bioresource Centre. *P. ostreatus* GenBank accession number is **KL198004**. The *Pichia pastoris* strain used for heterologous expression was the commercial strain BG-10 by BioGrammatics Ltd.

All *P. ostreatus* pre-cultures were prepared as follows: 250 Erlenmeyer flasks containing 75mL basal medium PDY broth (24 g/L potato dextrose (Difco) and 5g/L yeast extract (Difco)). Flasks were maintained in continuous agitation at 125 rpm and 28°C in complete darkness. After 5 days, the entire culture was homogenized by Waring Blender 7011HS® (3 flashes of 3‟ at maximum rpm) and inoculated, in a 1:10 ratio in PDY broth added with olive oil (0.1 ÷ 1 %), Olive Mill Wastewater (1 ÷ 10 %) glycerol (0.1 ÷ 1 g/L), and glucose (5 ÷ 10 g/L).

Recombinant *P. pastoris* cells from solid culture of pJGGαKR/PleoLip241 and pJGGαKR/PleoLip369 were inoculated in 50 mL BMGY medium (13 g/L yeast nitrogen base with ammonium sulfate without amminoacids; 10 gL^{-1} yeast extract; 20 g/L peptone; 100 mM potassium phosphate, pH 6.0; $4x10^{-4}$ g/L biotin; 1% glycerol) in a 250 mL baffled shaken flask. This preculture was grown for one day at 28° C on a rotary shaker (250 rpm), then a volume of suspension sufficient to reach a final OD_{600} value of 1.0 was used to inoculate 1 liter shaken flasks containing 250 mL of BMGY medium. Cells were grown on a rotary shaker (250 rpm) at 28°C.

Others media formulations were tested to improve lipase production. YP (1% Yeast extract, and 2% bacteriological peptone) was used with the adding of different carbon sources: 1% glucose, 1% glycerol, 2% oleic acid, 2% palm oil.

2.2 Chromogenic/tributyrin plates for detection of lipase activity

Chromogenic plates containing 1% olive oil, 1% phenol red, 1% $CaCl_2$, 1.5% agar were used to detect lipase activity secreted by *P. ostreatus*. The pH (7.0) was adjusted by adding NaOH 1M. Plates were incubated at 28°C. Using olive oil as substrate and phenol red as pH indicator, the yellowish halo indicated the presence of lipolytic activity.

Tributyrin agar plates were used to identify the high-producing *P. pastoris* clones after the transformations with pJGGαKR /PleoLip241 and pJGGαKR /PleoLip369 constructs. The screening was carried out in petri dishes using a medium constituted by: 0.5%peptone; 0.3% yeast extract; 0.1% tributyrin; 2% agar, pH 6.0 (Griebeler et al., 2011). Plates were incubated at 28°C for 120 h. The lipase activity was displayed by the appearance of a clear halo.

2.3 Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) was performed at alkaline pH under non-denaturing conditions. The separating and stacking gels contained, respectively, 9% and 4% acrylamide and 50 mM Tris–HCl (pH 9.5) and 18 mM Tris–HCl (pH 7.5) as buffers. The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8.4). After electrophoresis the gel was rinsed in dH_2O and in 20 mM Tris–HCl (pH 8). Visualization of the bands was achieved by overlapping the chromogenic agar preparation (described in 2.2) to the gel.

Denaturing polyacrylamide gel electrophoresis was carried out using standard techniques (He, 2011). SDS–PAGE 15% Tris–glycine gels were run underdenaturing conditions and proteins were stained with Coomassie brilliant blue G-250. The molecular weight standard used was PageRuler™ Plus Prestained Protein Ladder (200–10 kDa) from ThermoFisher Scientific.

2.4 Protein identification

Bands corresponding to active proteins were excised from the gel and washed first with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubating with 10 mM dithiothreitol (DTT) for 45 min at 56°C. Cysteines were alkylated by incubation in 5mM iodoacetamide for 15 min at room temperature in the dark. The gel slices were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestion was carried out with trypsin (12.5 ng/µL) in 50mM ammonium bicarbonate buffer, pH 8.5. Gel particles were incubated at 4°C for 2h. The buffer solution was then removed and a new aliquot of buffer solution was added for 18h at 37°C. Peptides were then extracted by washing the gel particles with 0.1% (v/v) formic acid in 50% (v/v) acetonitrile at room temperature and lyophilized. Peptide mixtures were analyzed by LC–MS/MS, using a HPLC–Chip LC system (Agilent 1200) connected to a Q-TOF 6520 (Agilent Technologies). Lyophilized samples were resuspended in 10 µL of 0.1% (v/v) formic acid. After loading, the peptide mixtures were concentrated and washed at 4 µL/min in a 40 nL enrichment column with 0.2% (v/v) formic acid in 2% (v/v) acetonitrile. Fractionation was carried out on a C-18 reverse phase column (75µm×43mm) at a flow rate of 0.4µL/min with a linear gradient of eluent B (95% v/v acetonitrile and 0.2% v/v formic acid) in eluent A (2% v/v acetonitrile and 0.1% v/v formic acid) from 7% to 80% in 51 min. Mass spectrometry analyses were performed using data dependent acquisition MS scans (mass range 300–2400m/z), followed by MS/MS scans (mass range 100–2000m/z) of the 4 most intense ions of a chromatographic peak. Raw data from LC–MS/MS were converted to m/z data, and searched against the PleosPC15 database available at the Joint Genome Institute′s website (http://genome.jgi-psf.org/PleosPC15_2) using the licensed version of Mascot 2.1 (Matrix Science).

2.5 Bioinformatics analysis

Protein sequences were aligned with those available in the GenBank database (Benson et al., 2005) using the Blast software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997). The 3D structure models were built with Phyre2 program (Kelley et al., 2015). N-glycosylation sites were identified with NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Phylogenetic analysis was carried out on PleoLip241 and PleoLip369. Sequences were aligned with ClustalW using default settings for multiple sequence alignments. Based on this alignment, a Neighbour Joining tree was constructed using MEGA 7.0 software (Tamura et al., 2007).

2.6 Gene synthesis, and plasmid construction

The PleoLip241 gene, excluding its signal peptide coding region, was designed according to codon usage for *P. pastoris* and synthesised by Geneart Lifetechnology (Germany).

Vector pJGGαKR (BioGrammatics) and lipase genes were digested with *Bsa*I and then ligated. The ligation mixtures were transformed into *E. coli* Top10 *(*F-*mcr*A Δ *(mrr-hsd*RMS-*mcr*BC*)* ϕ80*lac*ZDM*15* Δ*lac*X74 *recA1 ara*D139 Δ *(ara-leu)* 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G), and transformants were selected on LB medium supplemented with 100 µg mL^{-1} of ampicillin antibiotic.

The pJGGαKR/PleoLip241 and the pJGGαKR/PleoLip369 vectors were linearized with *Bsi*WI, and transformed into competent cells of *P. pastoris* BG-10 strain (BioGrammatics Ltd) by electroporation in a Bio-Rad Micro-Pulser apparatus, as specified by the manufacturer. Positive clones were selected on MD agar plates

supplemented with 0.9 mg/mL of G418, transferred in BMGY liquid cultures and incubated at 28°C under shaking condition.

2.7 Lipase assay

Lipase activity was determined spectrophotometrically (UVIKON 922 UV/Vis Spectrophotometer, BioTek Instruments) using p-nitrophenyl decanoate as substrate. The p-nitrophenyl decanoate was dissolved in isopropanol at a concentration of 10 mM. The assay was carried as follows: 880 µL 50 mM Tris-HCl (pH 8.0), 100 µL of sample and 20 µL of 10mM the p-nitrophenyl decanoate. The activity was assayed by detecting the released product, p-nitrophenol, at 405 nm (ε405= 3,390 M−1cm−1).

2.8 Protein purification

After the growth, the cells were harvested by centrifugation at 8000g and 4°C for 15 min. The sample was concentrated and dialysed on T-Series TFF Cassettes system (PALL Corporation). The samples dialysed in 50 mM Tris/HCl pH 7 were loaded on CM Sepharose FF column previously equilibrated in the same buffer. The proteins were eluted with a NaCl gradient (0 to 1 M NaCl) in the same buffer.

2.9 Effect of pH on lipase activity and stability

The optimal pH of lipase activity was determined using *p*-nitrophenyl decanoate in the range of 2 to 12 by using different pH buffer solutions (McIlvaine buffer, pH 2÷ 8; 50 mM Tris/HCl, pH 8÷10; sodium carbonate, pH 10÷12) at room temperature (25°C). Stability at pH was assessed by incubating the enzyme at the same pHs.

2.10 Effect of temperature on lipase activity and stability

The optimal temperature of the enzyme activity was evaluated at temperature ranging from 30° to 60°C in 50 mM Tris/HCl buffer, pH 8. Lipase thermostability was determined by incubating the enzyme in the same temperature range.

2.11 Substrate specificity

p-nitrophenyl esters (Sigma-Aldrich) containing acyl chain with different length (Acetate, Butyrate, Valearate, Octanoate, Decanoate, Dodecanoate, Myristate, Palmitate, Stereate) were used to determine the substrate specificity. The assays were carried out in standard conditions and the two lipases were normalised in terms of their activity towards standard substrate (PNP-Decanoate) (50 mM Tris-HCl pH 8, 25°C). All the measurements were made in triplicate.

2.12 Kinetic parameters determination

Kinetic parameters were obtained at pH 8 using *p*-nitrophenyl esters in the range 0.001÷ 1 mM. All kinetic parameters were determined by a non-linear regression curve using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com".

2.13 Wool dyeing

For each sample, 20 mg of pure new wool was treated with 3U of PleoLip241 in 50 mM Tris/HCl pH 8. The reaction mixtures were incubated 2 hours at 40°C in shaking conditions (100 rpm).

After treatment, wool was dyed using 10 mM of the commercial dye Direct Blue 71 (Sigma). Dyeing was performed at 60°C for 1 hour in shaking conditions (100 rpm). The untreated wool was used as control. After the dyeing, the wool was squeezed and the absorbance spectrum of the liquid bath was analysed using an UVIKON 922 UV/Vis Spectrophotometer (BioTek Instruments). The dye remaining in the solution was expressed as percentage ratio between $Abs_{594\ nm}$ of the lipase treated wool and that of the untreated wool.

3. Results and discussion

3.1 Lipase production in P. ostreatus.

Although *P. ostreatus* presents on its genome 44 putative lipase coding-genes (http://genome.jgi-psf.org/PleosPC15_2), no lipase from this fungus has been characterised so far. With the aim to stimulate lipase production from *P. ostreatus* and characterise the extracellular enzymes, the fungus was grown in the presence of different carbon sources (olive oil, Olive Mill Wastewater (OMW), glycerol and glucose) (Table S1)., The different conditions were quickly analysed by a chromogenic screening on plate, using olive oil as substrate (Singh et al., 2006), and the condition/s leading to the secretion of true lipases were selected. All carbon sources, except for glycerol led to the secretion of active lipases. Time course analysis of extracellular lipolytic enzyme production was conducted in selected conditions (Fig. 1). Production profiles in the presence of 5% v/v OMW and 1% glucose were comparable reaching a maximum of lipase activity of about 30 U/L in five days, comparable with the data in literature. Indeed, Singh and co-authors(Singh et al., 2014) reported a range from 25.9 (57 U/L) to 146.5 U/mg (322.3 U/L) for *Schizophyllum commune* ISTL04 lipase production. Both conditions were analysed through native PAGE with the aim to verify the number of different lipases secreted. The pattern of active protein bands obtained in the two growth conditions is showed in Fig. 2A. In the presence of OMW only an active lipase band was detectable, while two active lipases were detected in the presence of glucose. With the aim to correlate lipolytic enzyme(s) with encoding gene(s), the active proteins were excised from the gel, hydrolysed with tripsin and the obtained peptides subjected to protein identification by MS/MS ion search on a MASCOT server against the annotated *P. ostreatus* genome. Identified proteins are reported in Fig. 2B. It is worth to note that in these two adopted conditions at least 4 out of 44 putative lipase coding genes are expressed with a pattern depending on the culture conditions. The low concentration of secreted lipases hindered both a detailed characterisation of the proteins, and their future industrial applications. For this reason, a heterologous expression approach was adopted. Two of the four identified proteins (PleoLip241 and PleoLip369), found expressed in both conditions, were chosen for the recombinant expression.

3.2 In silico analysis

An analysis of the promoters of the four expressed lipases, P*leoLip241, PleoLip369, PleoLip103* and *PleoLip104, was performed.* Promoter regions extending around 1500-bp upstream of the ATG was searched for the putative response elements fatty acid responsive elements (FARE, CCTCGG) (Feng et al., 2011), heat shock elements (HSE, NGAAN) (Mager and De Kruijff, 1995), NIT2 binding site (TATCT) (Marzluf, 1997), putative response elements PRE (ATATC and TGGGT motifs; Soden and Dobson, 2003), , CGTCA-motif (CGTCA, Fink et al., 1988), O2-site (GATAA; Nakagawa et al., 2001), TGACG-motif (TGACG; Wingender et al., 2001), Cre-A-binding site (GCGGGG; Litvintseva and Henson, 2002), and stress-responsive elements (STRE, CCCCT; Treger et al., 1998). Several putative response elements were identified differentially distributed along the promoter sequences (Fig. 3). STRE, NIT2 and O2-site elements were found in both sequences chosen for the recombinant expression, *PleoLip241* and *PleoLip369*. In the latter was also identified free fatty acid element (FARE). In addition, *PleoLip369, PleoLip103* and *PleoLip104* showed a high number of HSE elements.

As far the selected enzymes, these are closely related to microbial lipases. PleoLip241 shows the highest identity with lipase from *Pleurotus eryngii* (97.4 %), whereas PleoLip369 with a lipase from *Hypsizygus marmoreus* (53 %).
A multiple alignment with lipases whose 3D structures were available and with lipases showing the highest identity with the new Pleo-lipases allowed to identify the residues of the catalytic triad (Ser, Asp, His) and of the conserved pentapeptide (G-X-S-X-G). In the case of PleoLip241 the conserved residues of active site was composed by Ser^{273}, Asp^{403}, His^{520}; with GQSAG as pentapeptide. In the case of PleoLip369 the catalytic triad is Ser^{266}, Asp^{394}, His^{512} and the conserved pentapeptide GESAG (Fig. S1). The estimated molecular weight was 67.2 kDa for PleoLip241 and 66 kDa for PleoLip369. Four potential N-glycosylation sites were identified in PleoLip241 (Asn-132, Asn-360, Asn-396, Asn-521) and six in PleoLip369 (Asn-24, Asn-31, Asn-128, Asn-342, Asn-366, Asn-390).
The modelled structure of both enzymes displayed the common α/β hydrolase fold of lipases and carboxyesterases (Fig 4A). Protein models were overlapped and their comparison highlighted the presence of an additional loop obstructing the substrate pocket accessibility in PleoLip369. The phylogenetic tree for the aminoacid sequences of PleoLip241 and PleoLip369 and other similar proteins is shown in Figure 4B. Based on this Neighbor Joining (NJ) tree, PleoLip241 and PleoLip369 fall in different clusters and might display different properties.

3.3 Recombinant production of PleoLip 241 and PleoLip369
PleoLip 241 and PleoLip369 were expressed in the yeast P. pastoris under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (pGAP) promoter. Both proteins were found to be expressed in active form and secreted in the extracellular broth, although a significant difference in their production level was observed. Several culture conditions were tested, and the best results were obtained growing the yeast in in BMGY at 28°C for both proteins. PleoLip241 production reached a maximum of 4000 U/L, whereas a maximum of 700 U/L was obtained for PleoLip369 after 9 days growth. However, both values are interesting if compared with the recombinant expression of other basidiomycete lipases reported in literature; Krugener and co-authors (Krügener et al., 2009) performed a recombinant expression in *Escherichia coli* of LIP 2 (lipase from *Pleurotus sapidus*) with a production of 30-31.4 U/mg (111-116 U/L). Chu and co-authors(Chu et al., 2008) also performed a recombinant expression in *E. coli* of the lipase from *Antrodia cinnamomea* a production of 37.2 µg/mg towards trilinolein. In addition, Sanchez-Carbente and co-authors(Sánchez-Carbente et al., 2017) isolated a lipase from *Bjerkandera adusta.* The further recombinant expression in *P. pastoris* led to a production of 39.9 U/L
PleoLip 241 and PleoLip369 enzymes were purified through a cationic exchange chromatography. SDS-PAGE of the active fractions indicated that both proteins were purified to homogenity (Fig. 5).

3.4 Molecular characterization of PleoLip241 and PleoLip369
3.4.1Effect of pH
Activity of both recombinant enzymes was tested in the pH range from 2 ÷ 12. Both enzymes were active between pH 6 and 9, with a maximum of activity at pH 7 (Fig. 6A). Enzyme stability was assayed in the pH range 6 ÷ 9 (Table 1). PleoLip241 showed a higher stability at all pH values in comparison with PleoLip369. Both proteins exhibited their optimal stability at pH 8.

3.4.2 Effect of temperature

The effect of temperature on the activity of both purified lipases was assessed in the range 30 ÷ 60°C (Fig. 6B). The two lipases exhibited the same behaviour at 30°, 40°C and 50°C. However, while PleoLip241 retained almost the same activity also at 60°C, PleoLip369 activity was almost halved. Lipase stability was investigated in the same temperature range, and also at 4°C to assess protein storage stability (Table 1). Both enzymes were stable at 4° C with a $t_{1/2}$ of about 40 days. Similarly to what observed for pH stability, PleuLip241 was found to be more stable than PleuLip369 at all the tested temperatures.

3.4.3 Effect of metal ions, detergents and organic solvents

The enzymatic activities of both lipases were tested in the presence of several ions, detergent and organic solvents (Table 2). As already observed, PleoLip241 was more stable than PleoLip369 in all the tested conditions.

As for metals, the presence of Cu^{2+} and Fe^{2+} did more negatively affect both enzymes. An enhancement effect by same metals was reported by different authors (Kumar et al., 2005; Murillo et al., 2012), however *P. ostreatus* lipase activities are not enhanced by metals.

In the presence of Tween 20, Tween 80 and SDS, lipase activities of both enzymes were remarkably inhibited. Conversely, Triton X-100, seemed to stabilize and improve both lipase activities. Triton and other detergents are usually added to lipase substrate emulsion to improve the emulsion quality, making the substrate more accessible (Glogauer et al., 2011). On the other hand, examples of lipases inhibited by this detergent are also available in the literature (Su et al., 2016).

3.4.4 Substrate specificity

Substrate specificity of both enzymes was assessed using substrates with different acyl chain length. PleoLip241 preferentially hydrolyzed long chains substrates with the following order C10>C14>C12>C16>C18≥C8, whereas PleoLip369 showed a narrower substrate specificity than PleoLip241 with preference towards C10 (Fig. 7). PleoLip241 specificity allows classifying this enzyme as a true lipase. The different substrate specificity displayed by the two enzymes may be due to diverse substrate accessibility to the binding pocket highlighted by the overlapping of the two protein models (Fig. 4A).

3.4.5 Kinetic parameters

Lipase kinetic parameters were assessed using their corresponding preferred substrates, *i.e.* Decanoate, Dodecanoate, Myristate and Palmitate for PleoLip241, and Octanoate, Decanoate, and Dodecanoate for PleoLip369 (Table 3). Both enzymes displayed very high affinity toward the tested substrates in comparison with other reported lipases (Brabcová et al., 2010; Ghori et al., 2011; Vici et al., 2015; Yan et al., 2016). PleoLip241 displayed a higher affinity towards both C10 and C12 acylic substrates respect to PleoLip369. These results indicate PleoLip241 as an enzyme with a interesting features for industrial applications.

3.5 Effect of Lipase pretreatment on the dyeability of Wool

Considering both the enzyme performances and the level of production, the capability of PleoLip241 to improve the dyeability of wool was tested. The pure new wool was treated with PleoLip241 and Lipolase, and then dyed with the commercial dye Direct Blu 71. The removal of dye from the liquid bath between the untreated and

the lipase treated wool was compared (Table 4). It is worth to note that after lipase treatment a reduced amount of dye was found in the liquid bath respect to the amount of dye present in the dyeing of ten untreated sample. It's possible to correlate this result with an increase of dyeability of wool after treatment with PleoLip241.

4 Conclusions

In this study the lipase production from the fungus *P. ostreatus* was analysed. In the selected conditions at least 4 out of 44 putative lipase coding genes were found expressed with a pattern depending on the culture conditions. The low concentration of secreted lipases hindered both a detailed characterisation of the proteins, and their future industrial applications. For this reason, a heterologous expression approach was adopted. These two novel identified lipases were heterologously expressed in the yeast *P. pastoris*. Despite their sequence similarity, these enzymes exhibited dissimilar stability at all the investigated parameters and different substrate specificity, probably due to diverse substrate accessibility to the binding pocket. The obtained data allow classifying PleoLip241 as belonging to the “true lipase” family. The enzyme was used to remove hydrophobic layer on wool surface in order to improve the dyeability of wool. The encouraging results obtained with lipase treated wool let to forecast PleoLip241 applicability in this field.

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Table 1

pH stability and thermostability of PleoLip241 and PleoLip369. Confidence intervals are given for 95% reliability.

	$t_{½}$								
	pH				**Temperature**				
	6	7	8	9	4°C	30°C	40°C	50°C	60°C
PleoLip241	*4 days*	*21 days*	*30 days*	*4 days*	*45 days*	*30 days*	*30 days*	*1 days*	*1 hours*
PleoLip369	*3 days*	*9 days*	*21 days*	*1 days*	*41 days*	*21 days*	*18 days*	*1 days*	*1 hours*

Table 2

Effect of metal ions, solvents and detergent on PleoLip241 and PleoLip369 activity after incubation of 4 hours at 25°C. Confidence intervals are given for 95% reliability.

	PleoLip241		***PleoLip369***	
Metal ions	**1 mM**	**10 mM**	**1 mM**	**10 mM**
NaCl	102.5	95.9	100	20
$MgCl_2$	92.4	91.4	88	15
$CaCl_2$	100.9	83.8	98	6
ZnCl2	83.2	37.5	86	0
KCl	87.8	83.5	55	7
CuSO4	50	0	35	0
FeSO4	50	20	27	0
MnCl2	100	95	77	18
Solvents	**10%**	**20%**	**10%**	**20%**
Ethanol	69.6	70.3	33.8	--
Glycerol	80.9	68.4	55.3	51.6
Aceton	45.2	40	20.3	--
Methanol	70	75	40	--
Methylacetate	65	50.1	--	--
t-Buthanol	80	65	62	35
Ethyl acetate	63	72	45.3	22.5
Detergents	**1%**		**1%**	
Tween20	0		0	
Tween 80	18.4		0	
Triton X100	135		115	
SDS	0		0	

Table 3

Kinetic parameters of the selected lipases from *P. ostreatus.* Confidence intervals are given for 95% reliability.

PleoLip241			
Substrate	K_M *(µmol/L)*	K_{cat} (s^{-1})	K_{cat}/K_M $(mM^{-1} s^{-1})$
pNP-C10	*30.3 ± 5.2*	*48.5*	*1.6*
pNP-C12	*150 ± 6*	*26.1*	*0.17*
pNP-C14	*85 ± 4*	*20.2*	*0.23*
pNP-C16	*350 ± 38*	*14.2*	*0.04*
PleoLip369			
Substrate	K_M *(µmol/L)*	K_{cat} (s^{-1})	K_{cat}/K_M $(mM^{-1} s^{-1})$
pNP-C8	*510 ± 60*	*1.58*	*0.003*
pNP-C10	*130 ± 50*	*7.19*	*0.055*
pNP-C12	*652 ± 75*	*3.05*	*0.004*

Table 4

Dye remaining in the solution

	Dye remaining in the liquid bath (%)
Non treated wool	86 ± 7.2
Wool treated with PleoLip241	54.9 ± 4.4

Figure Captions

Figure1. Time course activity of extracellular lipase produced by *P.ostreatus* in different culture conditions. All experiments have been conducted in triplicate. Confidence intervals are given for 95% reliability.

Figure2. **(A)** Zymography analysis of the extracellular protein of *P. ostreatus* grown in the presence of OMW and glucose added to PDY broth. Lipase activity was revealed by yellow halo, using olive oil as substrate and phenol red as pH indicator. **(B)** Identified proteins by MS/MS analysis.

Figure3. Distribution of putative cis-acting elements in the promoter regions of *P. ostreatus* lipase genes *PleoLip241*, *PleoLip369, PleoLip103, PleoLip104* around1500-bp upstream of the start codons: TATA box; CAAT box; GC box; (■) FARE, (▮) HSE; (◗) NIT2; (▼) STRE, (⬬) O2-site, (⭘) PRE, (▬) TGACG-motif, (✖) CGTCA-motif.

Figure4. (**a**) Superimposition of 3D structure models of PleoLip241 (cyan) and of PleoLip369 (green). Residues of the catalytic triad are displayed as stick (Serine in red, Histidine in magenta, Glutamic acid in blue). The close-up highlights the loop (shown in blue) obstructing substrate pocket in PleoLip369. The corresponding region in PleoLip241 is shown in orange. Images were elaborated with *PyMol*. (**b**) Neighbour Joining tree of lipase amino-acid sequences. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons (pair wise deletion option). Phylogenetic analyses were conducted in MEGA4. *P. ostreatus* lipases are highlighted.

Figure5. SDS-PAGE of recombinant lipases. Lane 1: Protein ladder; Lane 2: PleoLip369 crude extract; Lane 3: PleoLip241 crude extract; Lane 4: purified PleoLip369; Lane 5: purified PleoLip241.

Figure6. Relative activity of PleoLip241 and PleoLip369 at different pH values (**a**) and temperatures (**b**). The highest value of activity is considered as 100 % of activity. Confidence intervals are given for 95% reliability.

Figure7. Specific activity of PleoLip241 and PleoLip369 towards PNP-esters. Confidence intervals are given for 95% reliability.

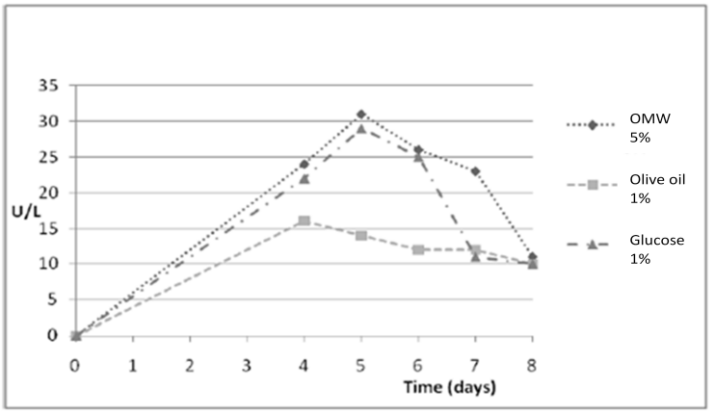
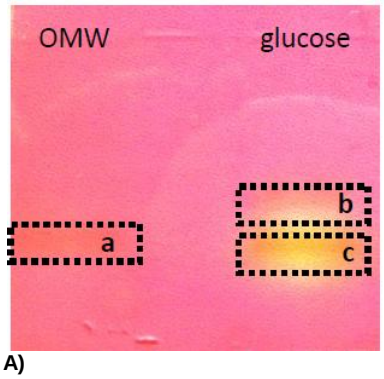


Fig 1



A)

Band B

Band A

Band C

Protein ID	Nominal Mass	Indentities (%)	Description	*P. ostreatus* protein
1040369	18322	59	α/β hydrolase	PleoLip104
170071	57706	50	Hypotetical protein	
1091241	59574	88	α/β hydrolase	PleoLip241
1060369	58926	53	α/β hydrolase	PleoLip369
1032023	47754	61	α/β hydrolase	PleoLip103

B)

Fig 2

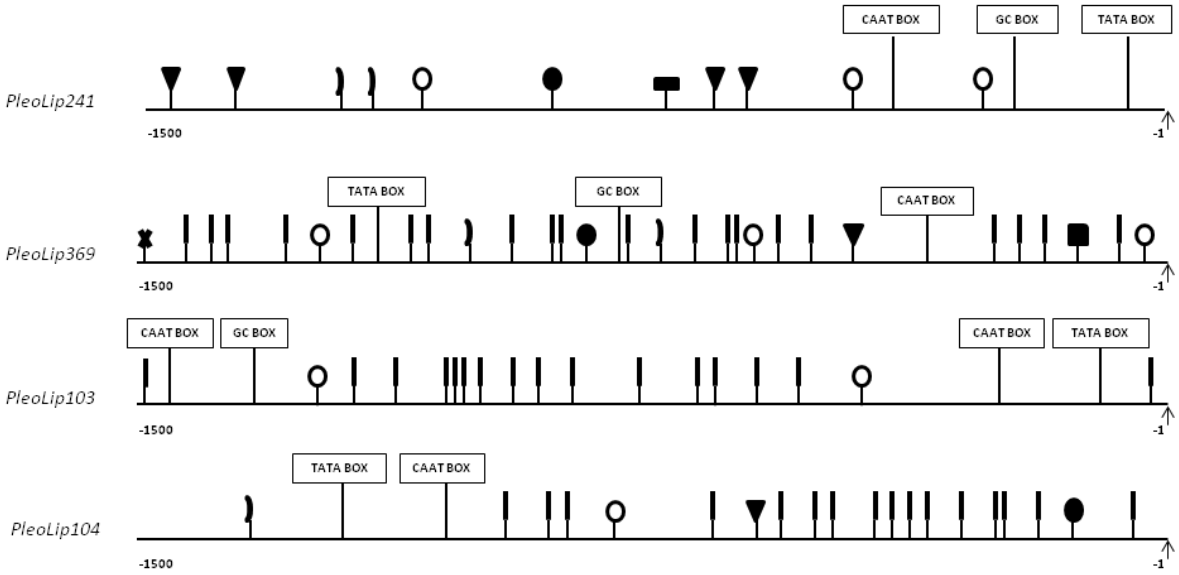
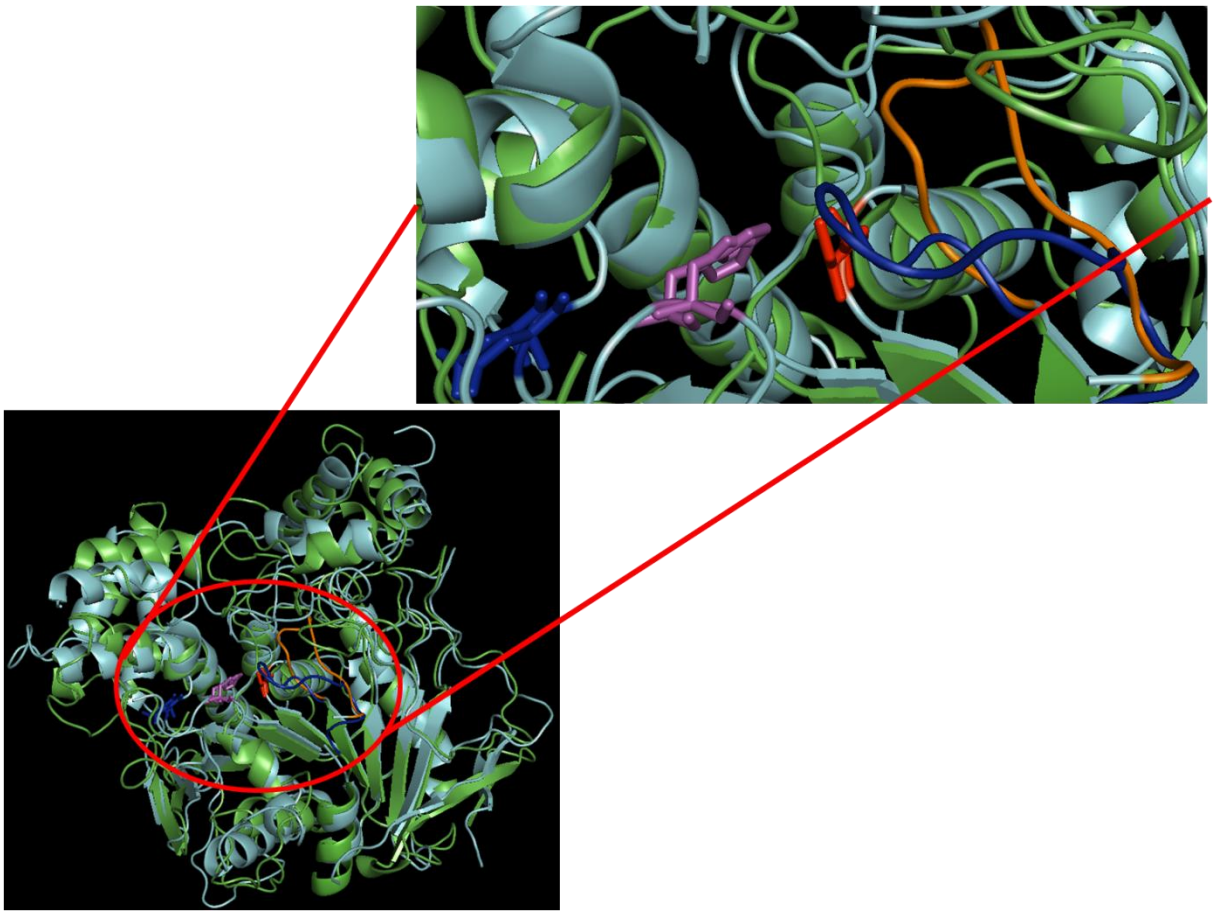


Fig 3

A



B

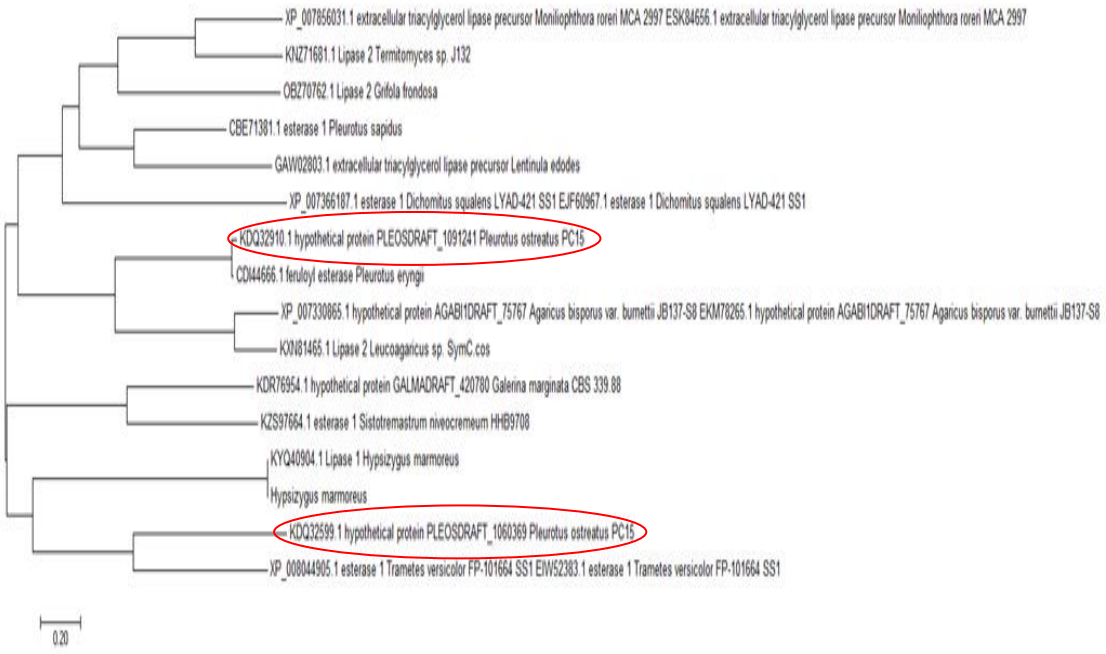

XP_007856031.1 extracellular triacylglycerol lipase precursor Moniliophthora roreri MCA 2997 ESK84656.1 extracellular triacylglycerol lipase precursor Moniliophthora roreri MCA 2997
KNZ71681.1 Lipase 2 Termitomyces sp. J132
OBZ70762.1 Lipase 2 Grifola frondosa
CBE71381.1 esterase 1 Pleurotus sapidus
GAW02803.1 extracellular triacylglycerol lipase precursor Lentinula edodes
XP_007366187.1 esterase 1 Dichomitus squalens LYAD-421 SS1 EJF60967.1 esterase 1 Dichomitus squalens LYAD-421 SS1
KDQ32910.1 hypothetical protein PLEOSDRAFT_1091241 Pleurotus ostreatus PC15
CDI44666.1 feruloyl esterase Pleurotus eryngii
XP_007330865.1 hypothetical protein AGABI1DRAFT_75767 Agaricus bisporus var. burnettii JB137-S8 EKM78265.1 hypothetical protein AGABI1DRAFT_75767 Agaricus bisporus var. burnettii JB137-S8
KXI81465.1 Lipase 2 Leucoagaricus sp. SymC.cos
KDR76954.1 hypothetical protein GALMADRAFT_420780 Galerina marginata CBS 339.88
KZS97664.1 esterase 1 Sistotremastrum niveocremeum HHB9708
KYQ40904.1 Lipase 1 Hypsizygus marmoreus
Hypsizygus marmoreus
KDQ32599.1 hypothetical protein PLEOSDRAFT_1060369 Pleurotus ostreatus PC15
XP_008044905.1 esterase 1 Trametes versicolor FP-101664 SS1 EIW52383.1 esterase 1 Trametes versicolor FP-101664 SS1
0.20

Fig 4

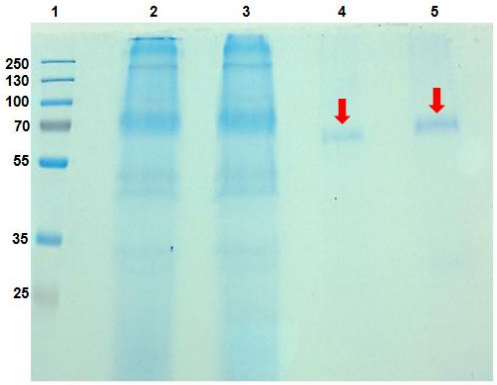
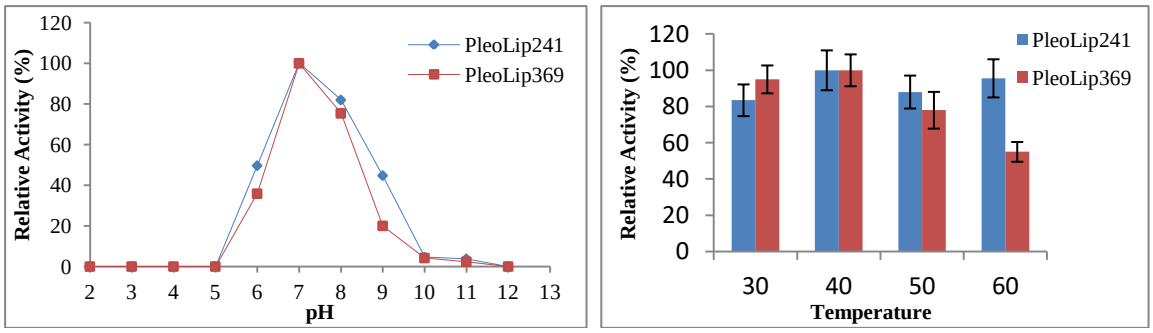


Fig 5



A **B**

Fig 6

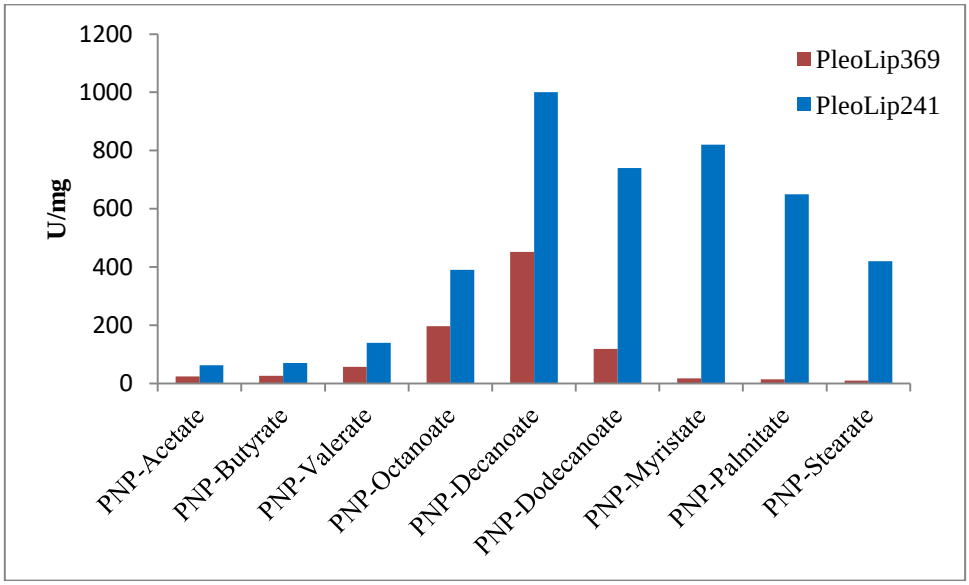
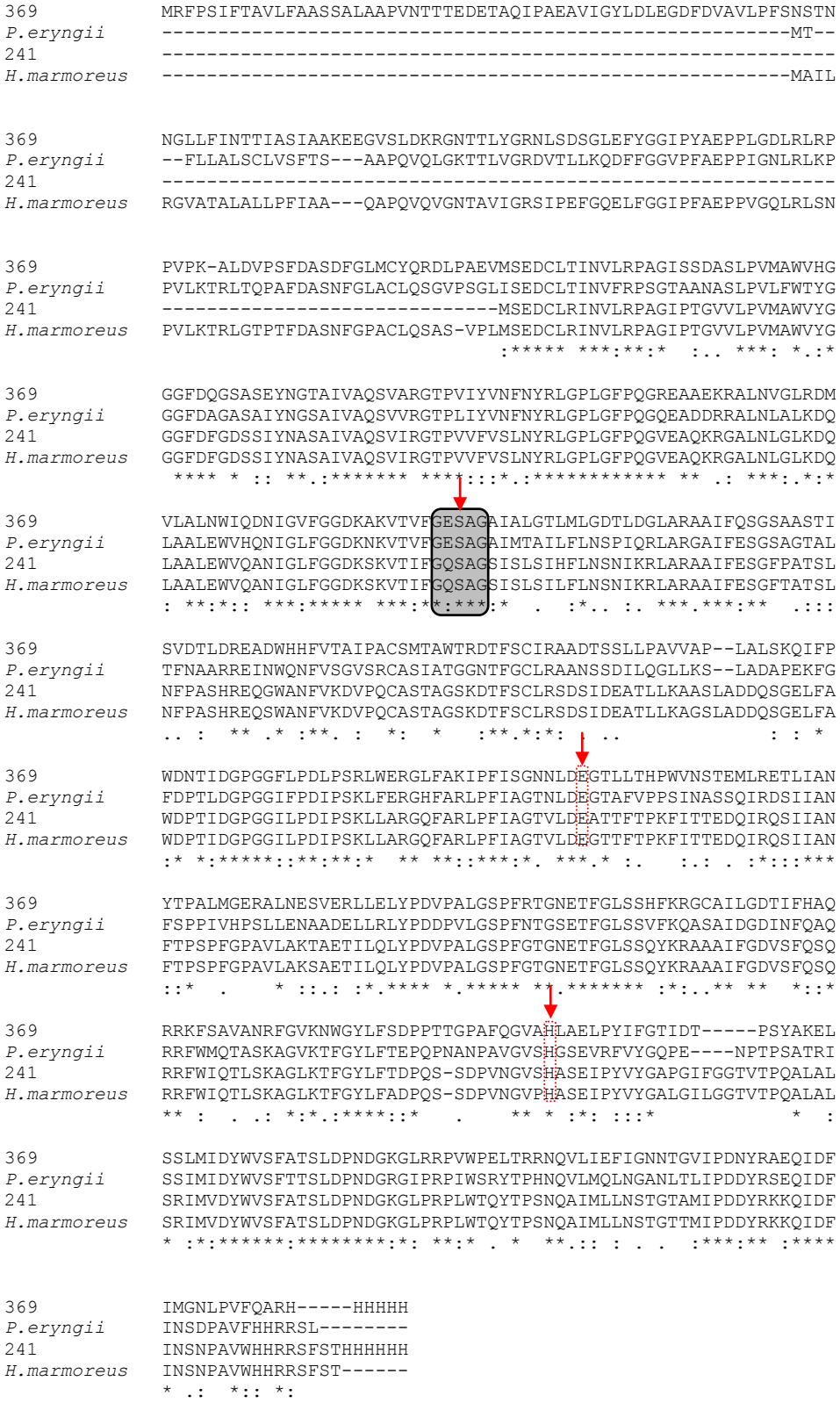


Fig 7

Growth media	Activity halo
PDY (basal condition)	-
PDY + 0.1%$_{v/v}$ olive oil	-
PDY + 0.5%$_{v/v}$ olive oil	-
PDY + 1%$_{v/v}$ olive oil	✓
PDY + 1%$_{v/v}$ OMV	-
PDY + 5%$_{v/v}$ OMV	✓
PDY + 10%$_{v/v}$ OMV	✓
PDY + 0.1%$_{v/v}$ glycerol	-
PDY + 0.5%$_{v/v}$ glycerol	-
PDY + 1%$_{v/v}$ glycerol	-
PDY + 0.5%$_{w/v}$ glucose	-
PDY + 0.7%$_{w/v}$ glucose	-
PDY + 1%$_{w/v}$ glucose	✓

Supplementary table 1

Growth media conditions for extracellular lipase induction. The conditions that induce the production of extracellular lipase after five growth days are reported**.** All experiments have been conducted in triplicate.



Supplementary Figure 1.

Multiple alignment among between lipase condign sequences from *P. ostreatus* and sequences of lipases from *Hypsizygus marmoreus* and from *Pleurotus eryngi*i. In the grey box the conserved pentapeptide is displayed. The red arrows indicate the three aminoacids of catalytic triade.

Conclusions

This PhD project was focused on the isolation, identification and characterization of new lipases for industrial applications. Two different approaches were employed in order to isolate new lipase producing microorganisms: culture and genome mining.
The culture mining approach was applied to two different “habitats”, a natural marine environment and a sample from composting of oil mill wastes.
The bioprospecting approach from the Pozzuoli harbour led to the isolation of a new *G. candidum* strain, *G. candidum* GTP able to reach an extracellular lipase production of 9,000 U/L. Secreted lipases were characterized and compared with mycelium-associated lipases from the same strain.
When samples from composting processes of oil mill wastes were screened for lipase producing microorganisms, *A. acidocaldarius* strain producing up to 700 U/L was isolated. A putative lipase coding sequence from its genome was selected on the basis of its similarity with industrial lipases and was heterologously expressed in the yeast *P. pastoris.*
When the fungus *P. ostreatus* was cultured in conditions to stimulate production of lipases, the expression of 4 lipolytic enzymes was obtained. Two of these lipases were heterologously expressed in *P. pastoris* and fully characterized.
Three main lipase producers were selected, the bacterium *A. acidocaldarius* and two fungus, *G. candidum* GTP and *P. ostreatus.* Native and recombinant production of lipases from these microorganisms allowed to determine some functional features.
The secreted lipases from *G. candidum* GTP and PleoLip241 from *P. ostreatus* belonging to the “true lipase family” seem to be ideal candidates for further analyses. Preliminary experiments show a potential use of extracellular lipases from *G. candidum* GTP as additive in commercial detergent, whereas PleoLip241was used to improve the dyeability of wool.

SUMMER SCHOOL

ESSIB 2015: 2nd European Summer School on Industrial Biotechnology
Subject of study: Design of Biocatalysts: concepts, Methods, Application.
The University of Stuttgart, Institute of Technical Biochemistry, Stuttgart, Germany

RESEARCH ACTIVITY IN FOREIGN LABORATORY

From May 5st to September 5th my research activity was carried out in Prof. Thierry Tron's laboratory at Aix-Marseille University, Marseille, France.

Ci sono soltanto due possibili conclusioni:

Se il risultato conferma l'ipotesi, allora hai appena fatto una misura.

Se il risultato è contrario alle ipotesi, allora hai fatto una scoperta.

Enrico Fermi