NEW BIOSYSTEMS: FROM APPLIED RESEARCH TO TECHNOLOGY TRANSFER

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Alle mie nipotine

The accumulation of data is not science any more than a pile of bricks is a house.

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Summary

In the frame of white biotechnology, enzymes are attracting an enormous attention not only because of their potential but also for the recent social and economical interest in green chemistry. Microorganisms constitute a huge reserve of genetic diversity, representing an important resource reservoir to undertake bioprospecting for new biocatalysts (such as lipases and laccases) with applications spanning across all major industries. Bioprospecting is defined as “the exploration of biodiversity for commercially valuable genetic and biochemical resources”. Bioprospecting efforts are currently potentiated by the integration of high-throughput approaches, where robotized technologies are employed to systematically screen through the biodiversity from specific environments. Unimaginable habitats, and in particular the marine one represent interesting source of new bio-systems. Bioprospecting can be conducted by culture-dependent (a traditional approach based on cultivation of natural isolates that can be screened for activities of interest) or culture-independent methods (metagenomic approach). Considering the interest towards lipases and laccases in many industrial applications, this study has been focused on the identification of new biocatalysts using different bioprospecting strategy. In detail this study allowed: *i)* the isolation of a new lipolytic organism (*Geotrichum candidum*) from the polluted fisherman’s mole in Putueoli, trough an enrichment culture approach, and the optimization of lipase activity production, formulating the most inductive growth medium; *ii)* the recombinant expression in the yeast *Pichia pastoris* of a putative lipase annotated in the genome of the fungus *Pleurotus ostreatus* showing the potentiality to be a bifuctional enzyme; *iii)* the recombinant production of a bacterial laccase from the arctic *Pseudoalteromonas haloplanktis* bacterium in the soluble intracellular fraction of *Escherichia coli* and its application for the oxidation of dye precursors (resorcinol and 2,5 DABSA), *iv)* the identification of 40 lipase positive clones from a marine metagenomic library of 30,000 fosmidic clones. Moreover, because of the interest in marine bioprospecting, a comparative philogenetic analysis of marine biome in two close sites has been performed. Obtained results showed, surprisingly, a clear divergence in the two biome.

Riassunto

Le *white biotecnologies* o biotecnologie industriali intendono sostituire processi chimici di sintesi con processi a basso impatto ambientale basati sull'uso di biosistemi - intesi sia come (micro)organismi che come enzimi - per una "Chimica verde". L'impiego degli enzimi a livello industriale è in netto aumento, come rivelato dal loro mercato in espansione, stimato intorno ai 4.411,6 milioni di dollari nel 2013, e con una crescita annuale prevista del 8,3% tra il 2014 e il 2020. La sempre maggiore richiesta di enzimi per processi industriali stimola la ricerca di nuovi biocatalizzatori attraverso *bioprospecting,* ovvero l'esplorazione della biodiversità da campioni ambientali, al fine di isolare prodotti ad elevato valore aggiunto. Il *bioprospecting* sta avendo sempre maggiore successo grazie allo sviluppo di approcci *high-throughput* per la ricerca di nuovi enzimi. Il *bioprospecting* può essere condotto secondo due diverse strategie, entrambe efficacemente adottate per la selezione di enzimi di interesse industriale. Un approccio classico che prevede l'esplorazione di microorganismi coltivabili, e un approccio basato invece sull'utilizzo di tecniche genomiche e di sequenziamento massivo per lo studio di comunità microbiche di difficile coltivazione (metagenomica) (figura1).

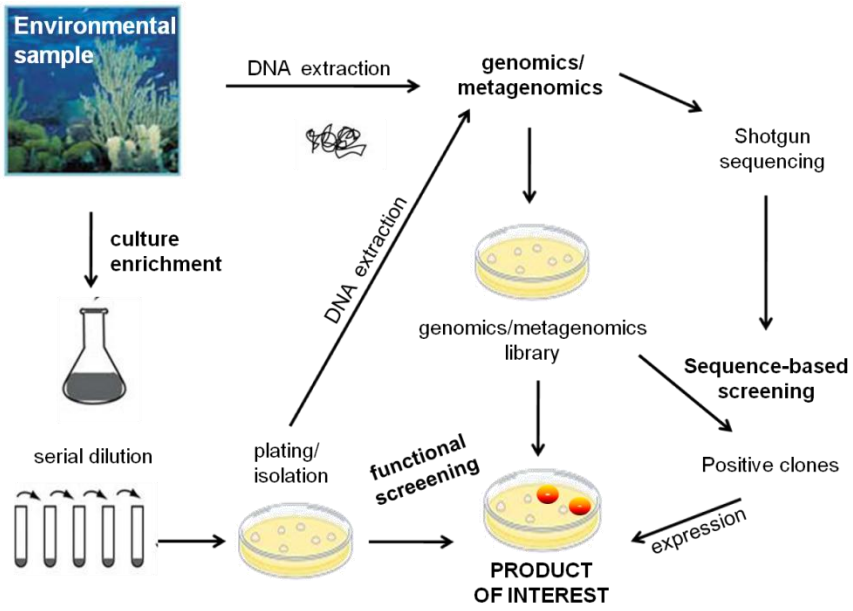

Figura1: Rappresentazione schematica degli approcci di bioprospecting di campioni ambientali.

Il presente lavoro di tesi è stato volto all'identificazione di nuovi biocatalizzatori (lipasi e laccasi) isolati e/o selezionati secondo strategie differenti. Lipasi e laccasi rappresentano due classi di enzimi molto interessanti per le loro potenzialità applicative.

Le **lipasi** sono enzimi ad attività carbossilesterasica che catalizzano sia l'idrolisi di trigliceridi in acidi grassi liberi e glicerolo, che la reazione inversa di sintesi. Le lipasi di origine microbica costituiscono un importante gruppo di enzimi in quanto mostrano una spiccata selettività per il substrato (chemoselettività, regioselettività, stereo selettività) e sono in grado di catalizzare reazioni in fase eterogenea. Tali caratteristiche permettono l'utilizzo delle lipasi come biocatalizzatori in molteplici applicazioni industriali, soprattutto nel campo alimentare, farmaceutico e dei detergenti.

Le **laccasi** sono enzimi ubiquitari ad attività fenolo-ossidasica, che presentano diverse funzioni biologiche. Grazie alla loro capacità di ossidare un'ampia gamma di substrati aromatici trovano applicazione in diversi settori industriali, che vanno da quello della produzione della carta, a quello tessile, alimentare, farmaceutico, cosmetico. Sebbene la maggior parte delle laccasi attualmente studiate e integrate in flussi di processi industriali siano di origine fungina, recentemente anche le laccasi batteriche stanno suscitando notevole interesse, soprattutto grazie alla loro peculiare stabilità.
Dato l' interesse nei confronti di biosistemi derivanti da ambienti marini, è stata anche condotta un'analisi filogenetica della biodiversità presente in tale habitat. In particolare, le seguenti tematiche sono stati affrontate e sviluppate.

1) **Isolamento e identificazione di lipasi da ambiente marino**

Gli ecosistemi marini sono ancora ampiamente inesplorati, rappresentando quindi una fonte interessante per l'isolamento di nuovi biocatalizzatori. In particolare, il bioma che popola lo strato superficiale dell'ambiente marino, esposto a fluttuazioni di temperatura, luce e salinità, può sviluppare delle caratteristiche peculiari. Inoltre, le zone adiacenti alla costa, e in particolare le aree portuali, sono soggette a contaminazioni da inquinanti, quali idrocarburi e oli di varia origine. La presenza di tali contaminanti rende queste aree ambienti promettenti per l'isolamento di (micro)organismi che producono attività enzimatiche per la degradazione di oli.

Risultati
Nel presente studio è stato utilizzato un approccio di *bioprospecting* coltura dipendente al fine di isolare nuovi (micro)organismi lipolitici dalla "Darsena vecchia" di Pozzuoli (mar Mediterraneo), un ambiente marino contaminato e caratterizzato da un basso ricircolo di acqua.
Un primo approccio di arricchimento selettivo e screening su piastra cromogenica usando olio di oliva come substrato, ha permesso di isolare 12 microorganismi produttori di lipasi. Criteri più stringenti, dimensione e tempo di comparsa dell'alone di attività, hanno permesso di selezionare i migliori produttori. Due delle dodici colonie sono state morfologicamente caratterizzate e genotipicamente identificate. Una delle due colonie caratterizzate, non è una coltura pura, infatti un batterio e un lievito sono stati visualizzati e identificati rispettivamente come appartenenti ai genere *Microbacteria* sp e *Pichia fermentans*. Nonostante diversi tentativi non è stato possibile separare il batterio dal lievito nella coltura mista e quindi definire il responsabile della produzione di attività lipasica. L'altro clone è stato invece identificato come *Geotrichum candidum.* Succesivi studi di ottimizzazione delle condizioni di crescita per massimizzare la produzione sono stati condotti su questo ceppo. Nelle condizioni ottimizzate si è ottenuta una produzione di lipasi extracellulari di circa 6000 U/L in sei giorni di crescita.

2) **Identificazione ed espressione ricombinante di una nuova lipasi da *Pleurotus ostreatus***

Nell'era post-genomica, un gran numero di sequenze geniche e proteiche è disponibile e può essere utilizzato per la ricerca di nuovi biocatalizzatori. Di fatto i genomi di svariati funghi sono stati sequenziati e annotati in banche dati.

P. ostreatus è un fungo basidiomicete, con alto potenziale in applicazioni biotecnologiche il cui genoma è stato completamente sequenziato e annotato. Questo fungo è stato ampiamente studiato per la produzioni di diverse classi enzimatiche, ma nessuno studio è stato condotto per la produzione di lipasi, sebbene nel genoma di *P. ostreatus* siano annotati 70 geni codificanti putative lipasi. Questo lavoro di dottorato rappresenta, pertanto, il primo studio volto ad isolare lipasi da tale fungo.

Risultati
È stato studiato l'effetto di differenti induttori a diverse concentrazioni (glucosio, glicerolo, olio di oliva e acque di scarto della produzione di olio (OMW)) sulla produzione di lipasi extracellulari da *P.ostreatus*. Tutti gli induttori ad eccezione del glicerolo inducono la produzione di lipasi extracellulari, con un effetto concentrazione dipendente. I migliori livelli di produzione sono stati ottenuti in presenza di glucosio e OMW. Le proteine extracellulari sono state frazionate per elettroforesi e rilevate per attività. Le corrispondenti bande sono state escisse, idrolizzate e i peptidi sottoposti a spettrometria di massa tandem, ed i risultati processati utilizzando software specifici (Mascot).
Nelle condizioni in esame sono state identificate cinque delle 70 putative lipasi annotate, due delle quali (PleoLip241 e PleoLip369) sono state identificate in entrambe le condizioni di crescita. Un'analisi filogenetica di queste due proteine è stata condotta per meglio comprenderne l'affiliazione. PleoLip241 mostra un'alta identità di sequenza (97%) con una feruloil-esterasi espressa da *Pleurotus sapidus.* Per tale motivo, si è ipotizzato che PleoLip241 possa essere un enzima bifunzionale, ed è stato quindi selezionato per successivi esperimenti.
Il gene codificante PleoLip241 è stato sintetizzato ed espresso per via ricombinante nel lievito *Pichia pastoris,* con un incremento della produzione di oltre 20 volte rispetto alla produzione nativa.

3) **Espressione di una nuova laccasi batterica artica per la sintesi di coloranti.**

I coloranti utilizzati in diversi settori industriali sono, attualmente, sintetizzati attraverso processi chimici. Grazie alle biotecnologie, si sta tentando di abbandonare la sintesi chimica grazie sviluppo di metodi eco-sostenibili basati su biocatalizztori che evitano l'impiego di condizioni operative estreme abbattendo i tempi di sintesi, oltre che i costi per lo smaltimento degli scarti.
Le laccasi, grazie alla loro capacità di ossidare un'ampia gamma di substrati aromatici, possono essere utilizzate per la biosintesi di nuove molecole coloranti catalizzando l'ossidazione di fenoli e composti aromatici con la formazione di polimeri colorati. Attualmente, diverse laccasi di origine fungina sono già state utilizzate per tale produzione. Nel presente studio una laccasi batterica è stata espressa per via eterologa e le sue capacità ossidative nei confronti di precursori di coloranti sono state valutate.

Risultati
L'analisi *in silico* del genoma di *Pseudoalteromonas haloplanktis*, un batterio artico gram negativo, ha permesso di identificare una sequenza di DNA codificante per una putativa laccasi. Tale sequenza è stata sintetizzata, ed espressa eterologamente nel batterio mesofilo *Escherichia coli*. Nelle opportune condizioni di crescita e induzione

l'attività laccasica è stata efficientemente sovra-espressa in forma solubile nella frazione intracellulare. La proteina arricchita per trattamento al calore ha mostrato una buona capacità ossidativa nei confronti del 2,5 DABSA e del resorcinolo, due precursori per la sintesi di coloranti.

4) **DNA metagenomico dalla superficie del mar Mediterraneo: una fonte di biocatalizzatori.**

L'ecosistema microbico marino è caratterizzato da una profonda diversità, che spesso rimane inesplorata, in quanto il 99% dei microorganismi presenti in tale habitat non è coltivabile e gli enzimi da essi prodotti non sono quindi accessibili. La meta-genomica rappresenta una soluzione a questo problema, permettendo di analizzare tutto il DNA e di identificare bio-molecole che resterebbero altrimenti non identificabili. Nell'ambito di questo progetto di dottorato uno screening del DNA metagenomico estratto dalle specie batteriche presenti in un campione prelevato in due diversi punti nello strato di acqua superficiale del porto di Napoli (mar Mediterraneo) è stato condotto al fine di isolare nuovi enzimi lipolitici e laccasici.

Risultati
È stata costruita una library metagenomica costituita da 30,000 cloni fosmidici in *Escherichia coli*. Ciascun clone contiene un frammento di DNA meta genomico di circa 40 Kb, con una quantità totale di materiale genetico espresso di 1200 Mb. Lo *screening* funzionale, utilizzando la tributirrina come substrato per l'attività lipolitica, ha permesso la selezione di 40 cloni positivi, con una *hit rate* (probabilità di trovare la funzione di interesse nella library) di 0,05%. Non è stata invece rilevata attività laccasica ne' attraverso screening funzionale ne' attraverso screening basato sulla somiglianza di sequenza.

5) **Analisi filogenetica delle comunità microbiche presenti in due siti geograficamente vicini nel mar Mediterraneo.**

La biodiversità microbica presente in un habitat naturale può essere analizzata sfruttando i progressi nelle tecniche di sequenziamento del DNA. Questo tipo di analisi richiede l'utilizzo di programmi bioinformatici capaci di analizzare l'enorme quantità di dati ottenuti dal sequenziamento massivo. QIIME è un programma *open-source* che permette di analizzare e comparare più biomi contemporaneamente. L'ambiente marino rappresenta una risorsa di diversità biologica e in particolare il microstrato della superficie marina è un ambiente con proprietà peculiari e poco caratterizzato. Considerando che il presente progetto di dottorato ha, in più casi preso in esame biosistemi derivanti da quest'ambiente, si è ritenuto interessante effettuare un'analisi filogenetica comparativa dei microorganismi presenti in due siti geograficamente vicini nel mar Mediterraneo.

Risultati
Campioni di acqua marina sono stati prelevati dallo strato superficiale del mar Mediterraneo nel porto commerciale di Napoli in due siti geograficamente vicini (definiti C ed U) e da questi due campioni si è estratto il DNA batterico. Le comunità batteriche e archeali associate ai due diversi siti presi in esame sono state paragonate attraverso l'analisi di pirosequenziamento delle regioni 16S. Un dataset di 200.000 sequenze è stato ottenuto è successivamente processato usando il

software QIIME, rimuovendo le sequenze chimera e imponendo una similarità al 97% delle unità tassonomiche operative (OTU), (tutte le sequenze aventi tra loro una similarità maggiore o uguale al 97% sono raccolte in una singola OTU). Le OTU ottenute per ogni campione sono riportate in tabella.

Tabella 1: Analisi delle letture del DNA 16S derivante dal pirosequenziamento del DNA estratto dall'acqua di mare (nei due siti in triplicate).

Campione	Sequence	N° OTUs (97%)	Archeal Phyla	Bacterial Phyla	Chao Index	Shannon Index
C1	9282	154	1	12	1972	8.01
C2	20823	232	1	12	4880	9.09
C3	17587	188	1	15	2311	8.11
U1	19855	258	2	21	5680	9.52
U2	29468	309	2	19	3921	8.35
U3	7523	174	2	14	2152	7.97

Tutte le sequenze ottenute sono classificate in due domini: batteri ed *archea* con un abbondanza relativa del 99,8% e dello 0,2% in entrambi I campioni. Nei campioni prelevati nel sito U, due phyla archeali sono presenti: *Euryarchaeota* and *Thaumarchaeota.* Quest'ultimo è stato ritrovato solo nel sito U, rappresentando la maggiore differenza nei due siti. Sebbene non era atteso ritrovare un enorme diversità nei due micro biomi, i risultati sperimentali e in particolare l'analisi Unifrac, che serve a calcolare la distanza tra comunità di organismi, mostrano chiaramente che i biomi sono diversi nonostante la vicinanza geografica.

Conclusioni

In conclusione il seguente progetto di dottorato ha portato:

1) All'identificazione di un nuovo ceppo di *G. candidum,* proveniente da ambiente marino capace di produrre attività lipasica extracellulare.
2) All'espressione in forma attiva, per la prima volta, di una lipasi da *P. ostreatus*.
3) All'espressione ricombinante di una laccasi batterica artica, stabile alle alte temperature e con capacità ossidativa nei confronti di precursori di coloranti.
4) Alla costruzione di una *library* metagenomica con una *hit rate* dello 0,05% per la presenza di lipasi.
5) Ad un'analisi filogenetica comparativa di biomi marini da località geograficamente vicine.

General Introduction

1. White biotechnology and enzyme market

Biotechnology offers an increasing potential for the production of goods to meet various human needs. In enzyme technology – a sub-field of white biotechnology – new processes have been and are being developed to manufacture both bulk and high added-value products utilizing enzymes as biocatalysts in different fields such as in food industry, for the production of fine chemicals, and pharmaceuticals. Enzymes are attracting an enormous attention not only because of their potential but also for the recent social and economical interest in green chemistry.
As a fact, the chemical industry often uses environmentally problematic solvents and toxic compounds, leading to toxic wastes that are difficult to handle. Enzymatic processes are, on the contrary, more sustainable than the chemical ones, because they lead to a considerable reduction in waste and energy consumption (Lotti and Alberghina, 2007) which in turn reduces processing costs. With the increased awareness of environment and cost issues, the field of industrial enzymes is now experiencing major R&D initiative, resulting in both the development of a number of new products and in improvement in the process and performance of several existing products. Today, nearly 4,000 enzymes are known, and of these, about 200 are commercialized. Until the 1960s, the total sales of enzymes were only a few million dollars annually, but the market has, since then, grown spectacularly (Godfrey and West 1996; Wilke 1999). Thanks to the improved understanding of production biochemistry, fermentation processes, and recovery methods, an increasing number of enzymes can be produced affordably. Moreover, the advances in the application of enzymes have greatly expanded demand. As a fact, the global market for enzymes was USD 4,411.6 million in 2013 and is expected to increase to USD 7,652.0 million by 2020, growing at a CAGR (compounded annual growth rate) of 8.3% from 2014 to 2020 according to a recent study by Grand View Research, Inc. The growing demand of enzyme in food and beverage applications is expected to be a key factor contributing to this increase in the market. To date North America is the largest regional market for enzymes with market revenue of USD 1,648.6 million in 2013, while Asia Pacific is expected to be the fastest growing market at an estimated CAGR of 9.4% from 2014 to 2020 (http://www.grandviewresearch.com/industry-analysis/enzymes-industry) (Figure 1).

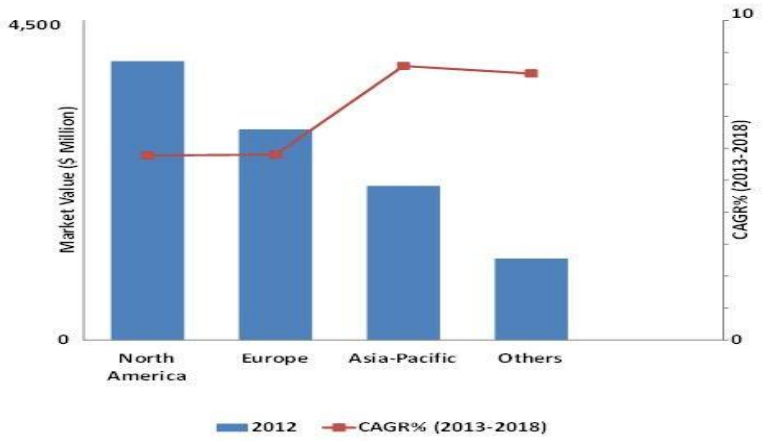

Figure 1: Market Values in the 2012 for enzyme in different geographic areas and CAGR (compounded annual growth rate) between 2013 and 2018.

The major applications for industrial enzymes include dairy processing, food and beverages, cleaning agents manufacturing, animal feed, bio-fuel productions,

textiles, leather, paper processing. Food and beverages is one of the major applications of industrial enzyme due to their versatile application as well as stringent government rule with regards to the utilization of synthetic food ingredients.

2. Bioprospecting of novel biocatalysts

Microorganisms have been mutating and evolving on Earth for billions of years, and currently constitute a huge reserve of genetic diversity. As a consequence, microorganisms represent an important source of bio-molecules and enzymes with applications across all major industries. Due to their diverse evolutionary backgrounds, microorganisms can act as a resource reservoir to undertake bioprospecting for new biocatalysts.

Bioprospecting, as defined by the Convention on Biological Diversity (CDB) Secretariat, is “the exploration of biodiversity for commercially valuable genetic and biochemical resources”. Unimaginable habitats ranging from hot springs to polar ice, salt lakes to deep-sea vents, mountain tops to lake bottoms, sponges to plant tissues represent interesting source of new bio-systems.

Bioprospecting efforts are currently potentiated by the integration of high-throughput approaches, where robotized technologies are employed to screen systematically through the biodiversity from specific environments.

Bioprospecting generally consists of four steps:

• Step 1: on-site collection of samples,
• Step 2: isolation and characterisation,
• Step 3: screening for potential uses,
• Step 4: product development and commercialisation, including patenting, trials, sales and marketing. 3:

As showed in Figure 2, bioprospecting of microorganisms from environmental samples can be conducted by culture-dependent or independent methods (metagenomic approach) (Dionisi *et al.*, 2012; Vester *et al.*, 2015).

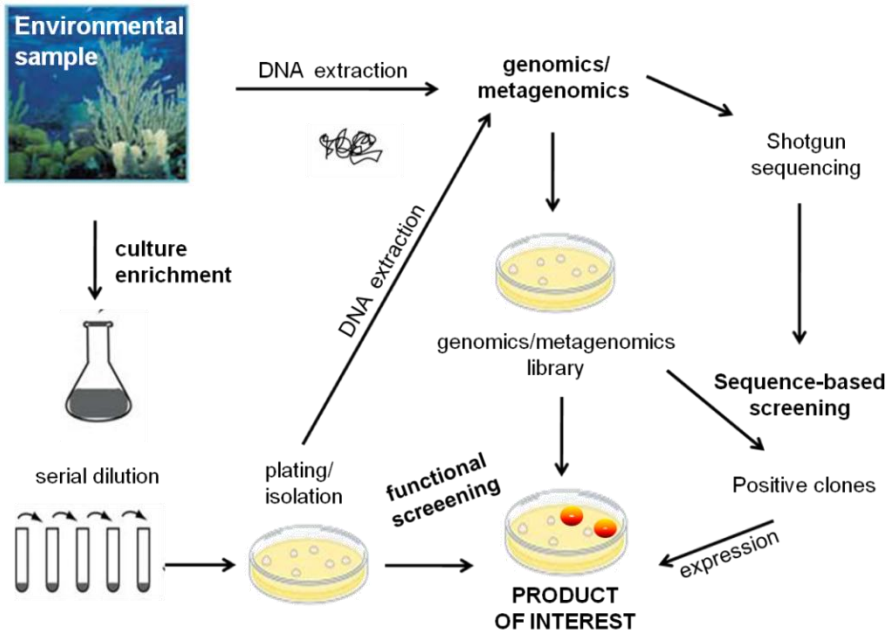

Figure 2: Schematic representation of microbial bioprospecting approaches (Dionisi *et al.*, 2012).

Culture-dependent method

For almost a century, culture-dependent method has been applied and optimized to isolate biosystems from environment. This traditional approach is based on cultivation of natural isolates that can be screened for activities of interest, like enzymatic activities. The culture-dependent approach is carried out using synthetic media supplemented with various nutrients, sometimes miming the natural growth conditions.

The potential for the discovery of novel enzymes from environment microbes is supported by the fact that many enzymatic activities have to date been identified from cultured microbes. Moreover most of industrially relevant enzymes have been isolated from the cultivable fraction of microbes from diverse terrestrial and marine environments. Despite that, culture-dependent methods present several disadvantages, because microbes can only be cultivated if their metabolic and physiological requirements can be reproduced *in vitro* (Nadkarni *et al.*, 2009), and artificial media typically allows growth of only a small fraction of these organisms. Apart from selectively allowing growth of some species and suppressing growth of others, the community composition of the cultivable fraction is distorted during culturing because replication times vary, with fast-growing species efficiently outcompeting others (Nocker *et al.*, 2007).

Culture–independ method

Considering that about 1% of bacteria from terrestrial ecosystems and about 0.1% of bacteria from marine habitats are currently cultivable (Kennedy *et al.*, 2008; Simon and Daniel, 2009; Barone *et al.*, 2014), it is necessary to use culture-independent method to fully access and exploit the microbial biodiversity within various habitats. Metagenomic overcomes the need for isolation or cultivation of microorganisms. Metagenomic is defined as the direct genetic analysis of total genomic DNA contained within an environmental sample The deployment of "next generation DNA sequencing technologies" in many centers has provided greatly enhanced capabilities for sequencing large meta-datasets. Analyses of these data sets opened a window into the enormous taxonomic and functional diversity of environmental microbial communities. Metagenomic has proven to be a powerful tool for comparing and for exploring the ecology and metabolic profiling of complex environmental microbial communities, as well as for identifying novel biomolecules by using libraries constructed from isolated nucleic acids (Simon *et al.,* 2011).

The analysis of metagenomic libraries involves two main strategies: sequence-based and function-based approach (Guazzaroni *et al.*, 2015).

Sequence-based metagenomic is based on direct sequencing of metagenmic DNA, either with or without cloning prior to sequencing and then subjecting the sequences to bioinformatics analyses comparing obtained sequence data with sequences deposited in databases (Kunin *et al*., 2008, Sleator *et al*., 2008). This methodology has been enhanced by the development of high throughput sequencing apparatus and bioinformatics tools. The application of this approach involves the design of polymerase chain reaction (PCR) primers for the target sequences that are derived from conserved regions of known protein families. The main drawback of this method is its dependence on some pre-existing knowledge that limits the possibility for identifying new protein families (Ferrer *et al.,* 2009).

Functional metagenomic is based on screening DNA library clones directly for a phenotype. So the function-based screening is extremely powerful for identifying new enzyme with new biocatalytic activities. In functional metagenomic an environmental

sample (seawater, marine sediments, marine invertebrate, soil) is collected and the total DNA is extracted. The isolated DNA is used to generate a metagenomic library usig a suitable cloning vector. This vectors (cosmids, fosmids, BACs), are then transferred to a suitable host. *Escherichia coli*, *Agrobacterium tumefaciens*, *Pseudomonas putida and Ralstonia metallidurans* (Craig *et al.*, 2010) as well as *Sulfolobus solfataricus, Thermus thermophilus* and *Streptomyces spp*. (Simon and Daniel 2011) have been assessed as suitable expression hosts. The individual clones are then screened for enzymatic activities or other bioactivities. The advances in high-throughput screening with robotic systems make this method an extremely effective way for isolating novel enzymes and biomolecules from otherwise inaccessible microbes (Wong *et al.*, 2010). However, an important disadvantage is that significant differences in the levels of expression exist between taxonomic groups present within the metagenomic DNA sample (Kennedy *et al.*, 2008).

Marine bioprospecting

The marine environment is one of the natural locations of interest for enzyme bioprospecting (Abida *et al.*, 2013). Oceans represent 70% of Earth's surface (Zhao *et al.*, 2011), where the amount of microorganisms is estimated to be approximately $3.67*10^{30}$. It has been estimated that bacterial densities can amount up to 10^6 per millilitre of seawater (Azam, 1998). Assuming 3,000 genes per single genome, and that 40% of these genes have catalytic activity, there could be up to $1.2*10^9$ putative reactions in that sample (Dinsdale *et al.*, 2008; Vieites *et al.*, 2009). Marine environment contains many different habitats, and microorganisms from intertidal zones must tolerate rapid and repeated fluctuations in environmental conditions like temperature, light, salinity. Enzymes produced by marine microorganisms often show higher stability in operational conditions (i.e., relatively high ionic strength, organic solvents) and different types of functional properties when compared with enzymes isolated by conventional sources (Ferrer *et al*. 2005; Trincone 2011; Dionisi *et al*. 2012). Marine enzymes may therefore represent ideal biocatalysts for industrial applications.

3.Lipases

General features

Lipases (EC 3.1.1.3), leading biocatalysts contributing to the multibillion dollar enzyme market, are triacylglycerol acylhydrolases that catalyze both hydrolysis and synthesis of esters. The most proper lipase substrates are triglycerides made of long-chain fatty acids with over 10 carbon atoms, whereas esterases (EC 3.1.1.1) act on soluble short-chain acylglycerols and simple esters. Lipases are produced by animals, plants, and microorganisms (Abada, 2008).

Structure and Mechanism of Lipases

Microbial lipases are reported to be monomeric proteins, having molecular weight in the range of 19-60 KDa. They have a primary structure highly variable. While considering the three-dimensional structures, most lipases share the same structural architecture, known as the α/β hydrolase fold.The active site contains a catalytic triad specific to serine-hydrolases, comprising of a nucleophilic serine, a histidine, and a catalytic acidic residue that can be either aspartate or glutamate (Aloulou *et al.*, 2006). The active site serine residue is located in a highly conserved Gly-X-Ser-X-Ser pentapeptide (Joseph *et al.*, 2008) located in a 'nucleophile elbow' (Jaeger and

Reetz, 1998). Most “true” lipases have a particular structure, designated as "lid", located on the protein surface, close to the active site (Kourist *et al.*, 2010). The lid is an amphiphilic peptide, as revealed by three-dimensional structure of lipases, which is mainly hydrophobic on the side directed towards the active site, and hydrophilic on its external face. The lid displays a variable position depending on the physicochemical environment of the enzyme (Cygler and Schrag, 1997; Ericsson *et al.*, 2008; Miled *et al.,* 2003). The presence of a proper hydrophobic solvent or a solvent interface produces the so called phenomenon of ‘interfacial activation’ which is stabilized by hydrophilic interactions with the adjacent enzyme surface, allowing free access of the substrate and the solvent to the active site of the enzyme (Gao *et al.*, 2011). The phenomenon of ‘interfacial activation’ was used for long time to distinguish a lipase from other lipolytic enzymes. However, not all lipases show this phenomenon. As a fact, lipase B from *Candida antarctica* displays no interfacial activation due to the absence of a lid. A short helix (α5) from CAL-B structure was assumed to act as a lid due to its flexibility. (Martinelle *et al.*,1995; Trodler and Pleis 2008).

Lipases mediated reactions

Lipases catalyze the hydrolysis of ester-carboxylate bonds releasing fatty acids and organic alcohols at organic-aqueous interface (Pereira *et al.*, 2003; Kamimura *et al.*, 1999; Merçon *et al.*, 1997). However, the reverse reaction of esterification or even various transesterification (acidolysis, alcoholysis and interesterification) reactions can occur in the absence of water (Freire and Castilho 2008).
The main reaction catalysed by lipases are:

Hydrolysis

$$RCOOR' + H_2O \rightarrow RCOOH + R'OH$$

Synthesis. Reactions under this category can be further separated into the following sub-categories:

a) *Esterification*

$$RCOOH + R^1OH \rightarrow RCOOR^1 + H_2O$$

b) *Interesterification*

$$R^1COOR^2 + R^3COOR^4 \rightarrow R^1COOR^4 + R^3COOR^2$$

c) *Alcoholysis*

$$R^1COOR^2 + R^3OH \rightarrow R^1COOR^3 + R^2OH$$

d) Acidolysis

$$R^1COOR^2 + R^3COOH \rightarrow R^3COOR^2 + R^1COOH$$

Lipases can be divided according to their specificities as follows (Villeneuve, 2003):

- **Substrate specificity** lipases able to catalyze the hydrolysis not only of triacylglycerols (TAGs), but also of monoacylglycerols, and di-acylglycerols and even phospholipids in the case of phospholipases
- **Regioselective** lipases show the preference to break one direction of chemical bond. It is subdivided into the following types: *i)* the non-regio-specific lipases that catalyze the complete hydrolysis of triacylglycerols into fatty acids and glycerol in a random way releasing fatty acids from all three positions; *ii)* the **region-specific** (or specific 1.3 lipases) lipases that only hydrolyze triacylglycerols at the C1 and C3 glycerol bonds producing 2-monoacylglycerols

and 1.2-or 2.3-diacylglycerols; *iii)* the **selective** lipases that act only toward some type or some group of fatty acids. They hydrolyze fatty acid esters located at any triacylglycerol position.

- **Enantioselective** lipases showing the ability to discriminate enantiomers in a racemic mixture. The enantio specificity of lipases can vary according to the substrate and this variation can be connected to the chemical nature of the ester (Barros *et al.*, 2010).

The ability of lipases to catalyze reactions with great efficiency, stability, and versatility makes these enzymes highly attractive from a commercial point of view.

Application and Commercial lipases

Microbial lipases represent a versatile and widely used class of enzymes in biotechnological applications, organic chemistry and pharmaceuticals (Contesini *et al.*, 2010; Guncheva and Zhiryakova, 2011; Li and Zong, 2010; Momsia and Momsia 2013; Sharma and Kanvar, 2014). Lipases are used in these applications thanks to their high substrate specificity or high chemo-, regio- and stereoselectivity (Gunstone *et al.*, 1999; Patel *et al.*, 2008), their stability in organic solvents, and in a wide range of pH and temperatures (Doukyu and Ogino, 2010).

Table 1: Some commercial lipases.

Source	Application	Commercial name	Producing company	Action
Penicillium camembertii	Refining of oils and fats	Lipase G	Amano Enzyme	Hydrolysis
Thermomyces lanuginosus	Baking industry	Lipopan®	Nova Nordisk	Hydrolysis and oxygen uptake
Mucor miehei	Oils and fats industry	Lipozyme®	Nova Nordisk	Interesterification
Rhizomucor miehei	Dairy industry	Palatase	Nova Nordisk	Hydrolysis
Thermomyces lanuginosus	Pet food industry	Novozyme® 871	Nova Nordisk	Emulsification
Pseudomonas mendocina	Detergents	Lumafast®	Genencor International	Hydrolysis
Pseudomonas alcaligenes	Detergents	Lipomax®	Genencor International	Hydrolysis

Lipolytic enzymes play an important role in many industrial fields (Table 1):

In **food industry** lipases are employed during the elaboration of bread, cheese, and other foods to improve their shelf-life and their rheological properties. Microbial lipases can be used to produce high added value products, such as cocoa butter equivalents, human milk fat substitutes trough interesterification reactions. Transesterification can also be used to modify the properties of triacylglycerol mixtures and to produce fats with optimum melting characteristics, free of trans fatty acids, with an increased nutritional value, and suitable for feeding (Hita *et al.,* 2009; Kim *et al*., 2010; Anobom *et al.*, 2014). Moreover lipases are also used to remove fat from meat and fish product. Novo Nordisk markets a range of enzymes for food processing industries: Lipopan®, Lipozyme®, Novazyme®.

In **detergent industry** lipolytic enzymes are widely used to remove fat-containing stains including those derived from fried fat, salad oils, butter, fat-based sauces, soups, human sebum, and lipstick. They are also used in the synthesis of surfactants for soaps, shampoos and dairy products (Hasan *et al.*, 2006; Horchani *et al.*, 2009;

Niyozima and More 2015; Pandey *et al.*, 1999; Romdhane *et al.*, 2010; Schmid and Verger, 1998). The first commercial lipase developed for detergency was launched by Novozymes, formerly Novo Nordisk Lipolase® originated from the fungus *T. lanuginosus* and expressed in *Aspergillus oryzae*. Furthermore other commercial lipases were developed to be used in detergent industry like Genencor International Lumafast® and Lipomax produced from *Pseudomonas* spp. (Anobom *et al.*, 2014).

In **leather industry** lipases can remove fats and grease from skins and hides. Fat removal generates a higher-quality product presenting a more uniform colour and a cleaner appearance than the one manufactured by traditional methods (Anobom, *et al.*, 2014; Bora *et al.*, 2013). NovoLime, a protease/lipase mixture for enzyme-assisted liming of hides and skins, and NovoCorAD, an acid lipase for degreasing of hides and skins, are some commercially available lipases for leather industry.

In **organic chemistry** lipase are exploited to produce substances whose elaboration by classical chemical methods is difficult or expensive. For example, they are used in pharmaceutical and agrochemical industries for the modification or synthesis of antibiotics, anti-inflammatory compounds, pesticides and for the production of enantiopure compounds or the resolution of racemic mixtures (Hasan *et al.,* 2006; Lin *et al*., 2011; Pandey *et al*., 1999; Reetz, 2002).

In **paper making industry** lipolytic enzymes are used to remove pitch, the lipid fraction of wood that interferes with the elaboration of paper pulp. They also help in the removal of lipid stains during paper recycling and in avoiding the formation of sticky materials (Dubé *et al.*, 2008; Guncheva and Zhiryakova, 2011; Sayari *et al.,* 2001; Hasan *et al.*, 2006). Novo Nordisk Resinase A®, produced from the fungus *Candida cylindracea* controls sticky problem for papermakers (Farrell *et al.*, 1997).

In **cosmetics and perfumeries industry** lipases have potential applications because they show activities in surfactants and in aroma production (Metzger, 2006). As an example, retinoids (Vitamin A and derivatives), prepared by catalytic reaction of immobilized lipases, are of great commercial potential in cosmetics and pharmaceuticals, such as skin care products (Ansorge-Schumacher and Thum 2013).

Lipases find also many **other applications**. They are used as component of biosensors, in biodiesel production, in textile processing, in hard-surface cleaning, in the synthesis of polymers, biodegradable plastics, in the management of waste and toxic compound (Hasan *et al*., 2006; Horchani *et al*., 2010; Kademi *et al*., 2006; Monsalve *et al*., 2010; Tan *et al*., 2010, Verma *et al*., 2012, Demarche *et al.*, 2011; Gea *et al.*, 2007).

4.Laccases

General features

Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are multicopper oxidases. Laccase have various biological functions, such as degradation of complex polymers (lignin, humic acid), lignification, detoxification, pathogenicity, morphogenesis, sporulation, polymerization of melanin and spore coat resistance (Strong and Claus, 2011).

Laccases are widely distributed in nature; they were first discovered in 1883 in the sap of the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883), later these enzymes have been identified in fungi, higher plants, insects and only recently in bacteria (Octavio *et al.*, 2006). Among them, fungal laccases are the most studied and characterized enzymes, and have been currently used in biotechnological

applications (Brijwani *et al.,* 2010; Pezzella *et al.*, 2015). However, the presence of laccases also from bacterial sources has persuaded researchers to isolate and characterize other members of this family. To date, only few bacterial laccases have been characterized, such as laccases from *E.coli*, *Bacillus halodurans, Bacillus subtilis, Thermus thermophilus and Bacillus licheniformis* (Sharma *et al.,* 2007; Zeeb *et al.,* 2014).

Structure and Mechanism of Laccases

Fungal laccases are extra-cellular monomeric globular glycoproteins of approximately 60–70 kDa. They are generally glycosylated, with an extent of glycosylation ranging between 10 and 25% and only in a few cases higher than 30% (Giardina *et al.*, 2010). Bacterial laccases are, instead, assumed to be mostly intracellular, periplasmic or spore-bound as shown in *Azospirillum lipoferum* and *B. subtilis* (Givaudan *et al.*, 1993; Martins *et al.,* 2002).

The substrate oxidation by laccases is a one-electron reaction. Laccases have multiple copper centers transferring electrons from the reducing substrate to molecular oxygen. Subsequently, the electrons are passed through the trinuclear cluster of the reactive site, where molecular oxygen is reduced and water is released. The oxidation of the substrate generates typically unstable reactive radicals which undergo non-enzymatic reactions. As a result of the radical production, cross-linking of monomers, as well as degradation of polymers and cleavage of rings is possible (Zeeb *et al.,* 2014).

Laccase catalysis takes place with reduction of oxygen to water and with concomitant oxidation of a wide range of aromatic compounds which includes polyphenols (Bourbonnais and Paice, 1990), methoxy-substituted monophenols, and aromatic amines (Bourboinnais *et al.*, 1995). The oxidation generates oxygen-centered radicals that can be converted to quinone in a second enzyme catalyzed reaction. Reactivity of laccases can be expanded also towards nonphenolic lignin subunits by the inclusion of mediators, like 1-hydro-xybenzotriazole (HOBT), N-hydro-xyphthalimide (NHPI), and 2,2-azinobis-3-ethylthiazoline-6-sulfonat (ABTS) (Gochev and Krastanov, 2007).

Laccases from a large number of fungi have been studied. These enzymes offer great variability in terms of induction mechanisms, degree of polymorphism, levels of expression of different isoenzymes even in the same organism, and physiochemical and catalytic properties.

However, bacterial laccases may have several properties that are not characteristic of fungal enzymes. Firstly, laccases from bacteria can show high thermo resistance, and pH tolerance.

Finally, a novel evolutionary lineage of two-domain laccases has been established. These laccases are different from the well-known monomeric three-domain laccases that are typical for fungi and bacteria. The two-domain laccases, which have only been identified in prokaryotes, have a homotrimeric quaternary structure and form the active site on the interface of each two monomers. Three groups of two-domain laccases were distinguished on the basis of the organization of the copper-binding regions within the protein domains, and representative enzymes of type B and type C two-domain laccases have subsequently been characterized in bacteria, while sequence data suggested the presence of type A two-domain laccases in archaea. For all these reasons, studying bacterial laccases is important from the perspectives of basic science as well as for the development of novel biotechnological applications.

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MULTI - AUTHOR REVIEW

How to enjoy laccases

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Abstract An analysis of the scientific literature published in the last 10 years reveals a constant growth of laccase applicative research in several industrial fields followed by the publication of a great number of patents. The Green Chemistry journal devoted the cover of its September 2014 issue to a laccase as greener alternative for chemical oxidation. This indicates that laccase "never-ending story" has found a new promising trend within the constant search for efficient (bio)catalysts able to meet the 12 green chemistry principles. A survey of ancient and cutting-edge uses of laccase in different industrial sectors is offered in this review with the aim both to underline their potential and to provide inspiration for new ones. Applications in textile and food fields have been deeply described, as well as examples concerning polymer synthesis and laccase-catalysed grafting. Recent applications in phar-maceutical and cosmetic industry have also been reviewed.

Keywords: Green chemistry Industrial applications Textile Food Polymer Pharmaceutical Cosmetics

Introduction

First uses of laccases in industry date back to 1990s [1, 2]. Since then, a careful study of reactions catalysed in vivo by fungal laccases has led to the discovery of an ever-increasing number of biotechnological applications reach-ing a peak in the last 10 years.

Laccase-catalyzed oxidation gives rise to radical species that can evolve towards both synthetic and degradative processes.

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Radical fate can follow three different pathways depending on many factors (Fig. 1). Oxidative coupling (1) is the main mechanism leading to homo- or cross-coupling of molecules resulting into dimers and/or also polymeric species. Such synthetic route translates in vitro into biotechnological applications in dye and polymer synthesis and grafting for surface func-tionalization. On the other hand, depending on their stability and redox reversibility, radicals may undergo rearrangement per se, yielding dead end products (2), or, in the third option (3), act as mediators for further oxidation of non-phenolic compound causing bond cleavage. The latter two kinds of laccase actions can be reproduced in vitro into biotechnological applications such as bleaching technology, and bioremediation.

This review focuses on some emerging trends of laccase uses covering textile, food, pharmaceutical and cosmetic fields. Examples concerning polymer synthesis and lac-case-catalyzed grafting have also been reviewed.

Textile industry

Among the explored industrial fields, laccases have found wide applications in several sectors of textile industry, where these enzymes have been integrated in already existing processes such as fiber bleaching and dyeing and are also emerging as important "green tools" to modify and/ or improve textile properties. Bioremediation of textile wastewaters also occupies a wide slice of laccase applications, although it has not been reviewed in this paragraph.

Fiber biobleaching

Removing of natural pigments, other non-cellulosic matter and impurities from cellulosic material before fiber dyeing and finishing, a process termed bleaching, is a mandatory

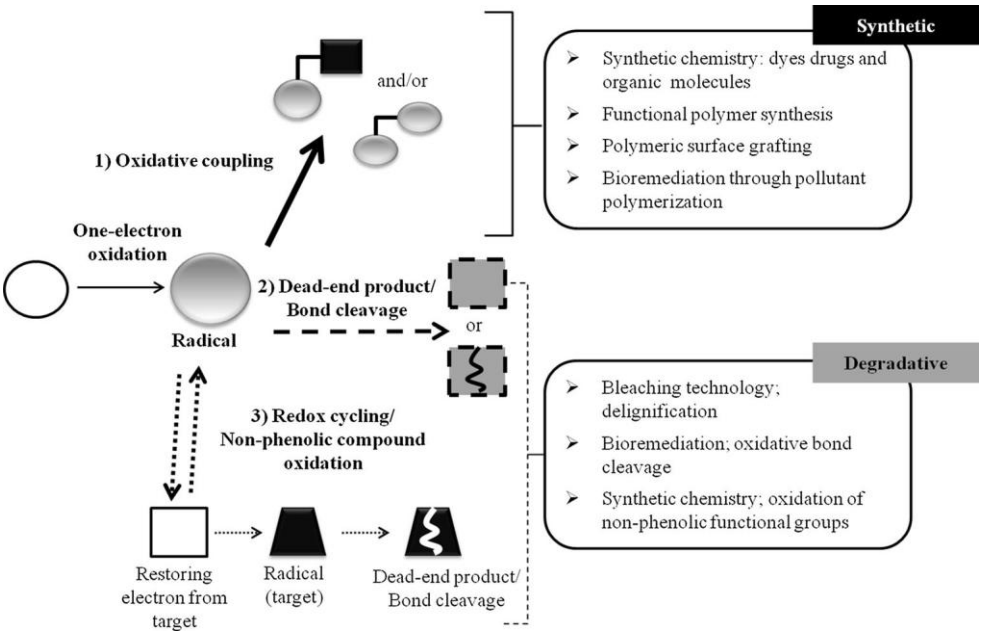

Fig. 1 Laccase-catalyzed oxidation of small organics gives rise to the corresponding radicals that can evolve towards either synthetic or degradative processes, depending on functions performed in vivo by laccases. Adapted from Jeon and coworkers [3, 4] with author permission

step in textile processing. Laccases have emerged as a fiber-preserving alternative to the most commonly used bleaching agent, hydrogen peroxide [5, 6]. When combined with hydrogen peroxide produced by glucose oxidase, laccase treatment of linen fabric resulted into effective whiteness increase and modification of amount and type of lignin surface functional groups [7]. A synergistic effect on oxidation/removal of the natural coloring matter of cotton has been observed coupling laccase with ultrasound treatment, the latter allowing an improved diffusion of the enzyme throughout the textile structure [8]. Enhanced bleaching efficiency by a combination of laccase and ultrasound treatment has been also veriÞed on linen fabrics. In this study, the time/dye uptake isotherms were also enhanced after combined bleaching treatment [9]. More recently, a pilot reactor for laccase-hydrogen peroxide cotton bleaching assisted by ultrasound was scaled up and efficiently applied to fabrics treatment [10]. In addition, Tian and coworkers [11] developed what they called the “green” short-flow process to treat cotton fabric in an energy-saving way. The process, combining hydrogen peroxide with laccase-mediated system (LMS) pretreat-ment allowed a lower dosage of H_2O_2, reduced bleaching temperature and shortened bleaching time.

By oxidizing the indigo dye on denim garments into uncolored compounds, laccase treatment has allowed obtaining the desired wash light effect on blue denim jeans without using harsh oxidative agents such as sodium hypochlorite. The use of laccases for denim processing dates back to 1996, when the first commercial laccase preparation DenilitеI™ was launched on the market by Novozyme. Since then, customized formulation of laccases has been developed to target specific garment wet process conditions, as deeply reviewed by Rodriguez-Couto [12]. Solis-Olba applied an LMS based on ABTS to denim de-coloration, verifying that denim fibers were not damaged during the treatment and also the environmental impact of the process was reduced since denim decoloration residual water can be easily biodegraded [13]. Montazer and Maryan [14] verified that the combination of laccases with cellulases in denim processing helped to improve the lightness and decrease staining on both back of garment and on white pocket. An example of effective application of laccase treatment for stone washing effects of denim fabric without using a mediator has also been reported [15].

Functional modification of textile fibers

Laccases have also been used to attach functional mole-cules on textile fibers. Modification of protein fibers, such as wool and cotton, allows improving their properties. Water repellence is often associated to self-cleaning properties, antioxidant and antimicrobial features, which are desired qualities in finishing design.

Grafting of wool with the bi-functional phenolic com-pound nordihydroguaiaretic acid (NDGA) through a laccase-catalyzed reaction in an aqueous ethanol mixture improves wool properties [16]. NDGA grafting on wool provides antioxidant activity and is efficient in protecting wool against the undesirable photoyellowing caused by UV radiation, probably due to the formation of inter/ intramolecular bridges and cross-links among wool

proteins. Shrink resistance, crease recovery angle and tensile strength of the modified samples also increase compared to the untreated ones.

Antibacterial properties toward Bacillus subtilis and Escherichia coli were imparted to flax fibers and fabrics through laccase-assisted modiÞcation with ferulic acid or hydroquinone [17]. Antibacterial properties were also imparted to wool fibers by a laccase (*Trametes* sp.)-mediated grafting of lauryl gallate [18]. Recently, Silva and coworkers [19] exploited the ascomycete *Myceliophthora thermophila* laccase to oxidize flavonoids naturally existing in flax fibers, covalently binding the o-quinones formed to the amino groups of chitosan or/and to catechin. Antibac-terial activity of linen samples grafted with both chitosan and catechin was tested against Staphylococcus aureus and E. coli. The latter showed susceptibility only to chitosan-treated samples, whereas S. aureus was equally sensitive to chitosan and catechin actions.

Fiber wettability has also been modified by laccase-assisted grafting of ad hoc selected molecules. Kim and coworkers [20] have reported the synthesis of laccase-catalyzed protein-flavonoid conjugates and following anchoring of conjugates onto cationized fibers. By the anchoring of a-casein-catechin conjugates onto flax fibers, the surface became hydrophilic, with a lower contact angle (48L). Wool water-repellent property was modified through an enzymatic coating of wool with lauryl gallate [18].

The exploitation of LMS for the anti-shrinking treatment of wool has been patented [21] and also investigated by Lantto and coauthors [22]. Although molecular reasons causing this effect have not been examined in depth, it seems probable that laccase action would promote inter-molecular cross-links of wool fibers, thereby decreasing elasticity and thus reducing the tendency to shrinkage. More recently, Zhang and coworkers [23] have exploited a high-redox potential laccase from Aspergillus to prepare conductive cotton by laccase-catalyzed in situ polymeri-zation of conductive polyaniline (PANI). Electrical properties of cotton, such as antistatic property and elec-tromagnetic shielding effect, were significantly enhanced, highlighting the potential for using common textile mate-rials in flexible electronic devices.

Textile dyeing

The use of laccases to synthesize colorants in situ has come out as a “green” way for textile dyeing at mild process conditions. Laccase-mediated coupling and grafting reac-tions have also been used for coloring various textiles [17, 24-29]. In the Þrst attempt of cellulose textiles dyeing by Hadzhiyska and coworkers [25], cotton cellulose was dyed in situ by a polymeric dye generated by oxidative coupling of colorless 2,5-diaminobenzenesulfonic acid (DABSA) and 1-hydroxyphenol (catechol) with laccase-treated Þbers, obtaining up to 70 % of dye fixation. However, it has been reported that all fabrics showed high wash fastness but low light and friction resistance. Schro¬der and coworkers [17] used a response surface methodology (RSM) approach to determine the best conditions for laccase-induced coating of flax fibers and fabrics by a *Trametes hirsuta* laccase. Different phenols have been analyzed for their potential as monomers for enzyme-catalyzed polymerization: all the methoxyphenols showed different coloration with weak fastness properties. Kim and coworkers [26, 28] have reported the utilization of the natural flavonoids present in the cotton as anchors to attach other phenolic compounds to the fiber surface. A T. hirsuta laccase was used to cat-alyze the oxidation of flavonoids in solution producing quinines, further polymerized and grafted onto surface of the cotton, providing a yellow to brown coloration, depending on the external flavonoids used and on the reaction conditions. The natural flavonoids present in the cotton were found to play an important role on the grafting reaction, improving dyeing and color fastness. The washing and friction fastness test of flavonoids colorized cotton showed good results confirming the feasibility of this new and promising coloration technique. An important improvement in the wet rubbing fastness of the dyed sample has been observed by Blanco and coworkers [29]. In their work, there is a covalent fixation of the in situ generated polymeric pigment. Aromatic amine moieties of DABSA introduced onto tosylated cotton were coupled and copolymerized with a catechol into colored product cova-lently fixed on the fabric upon oxidation with laccase. The controlled amination of cellulose in a first step, and the subsequent coloration allowed for up to 95 % pigment Þxation on the fabric. Tzanov and coworkers [24] have also proved the ability of laccases for wool dyeing. They used a dye bath prepared with a dye precursor (DABSA), dye modifiers (catechol and resorcinol) and laccase, in the absence of any dyeing auxiliaries, carrying out the reaction at pH and temperature values safe to the wool material. Furthermore, they have shown that by prolonging the contact time between wool, enzyme, precursor and modifier, deeper colors were obtained in contrast to the conventional process, where deeper colors are attained by increasing the amount of dye.

In situ laccase-assisted overdyeing using catechol and catechin as dye precursors has been reported by Guimares [30]. Laccase-generated polymers gave rise to new color-ation states from dark brown to green-yellow and replaced dyes in the overdyeing process. Through this process, overdyeing of denim was successfully achieved with acceptable levels in terms of durability. Also leather was colored by laccase action using dihydroxynaphthalenes as substrates [27]. The colored products are lightfast and

bound to the natural collagen by a covalent tanning manner.

New routes for dye synthesis

Textile dyes occupy an important fraction of chemical industry market. Although consumption of dyes by the textile industry already accounts for two-thirds of the total dyestuff market, recent world dyes and organic pigments industry have estimated a further 3.5 % annual growth for the period 2013-2018 [31]. Due to their toxicity and the harsh operative conditions required for their synthesis, this growth of demand has been accompanied by stringent legislation regarding removal of dyes from industrial effluents and has encouraged the development of eco-friendly processes for their disposal as well as for their synthesis. In this scenario, laccases have shown to play a pivotal and double role: being efficient biocatalysts for biodegradation of synthetic dyes and attractive enzymes for coupling reactions leading to the production of new colored products. Laccase-mediated remediation of textile waste-waters has not been reviewed in this paragraph.

Laccases are known to synthesize many natural compounds in nature, through oxidative coupling reactions. This ability can be translated in vitro into powerful syn-thetic routes towards new colored natural products. Small colorless aromatic compounds such as phenols, amino-phenols, diamines are oxidized by laccase to aryloxy radicals, which may undergo further non-enzymatic reac-tions leading to formation of colored products [32]. Experimental evidence has shown that laccase is able to catalyze the formation of colored products (from yellow/ brown to red and blue) by oxidation of benzene derivatives containing at least two substituents (comprising amino, hydroxyl, and methoxy groups) [32, 33]. Thus, derivati-zation of various phenolic and non-phenolic substrates may represent a strategy to expand the range of these potential precursors. Presence of mediators has been shown to pro-mote color formation, such as in the case of trimerization of indole with formation of a yellow compound in the presence of 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) [34]. Also, inorganic polyoxometalates have been found to act as better mediators than the organic one, 1-hydroxy-benzotriazole (HBT) in the laccase-catalyzed synthesis of polycatechols, assuring better flax coloration, color fixation and resistance [35]. A variety of secondary amines char-acterized by biologically activities and intense colors were obtained during C-N coupling of 3-methylcatechol with different primary linear, branched-chained and cyclic amines, mediated by both native and recombinant laccases [36]. Phenoxazinones are also another class of compounds with excellent coloring properties, non-toxic and obtain-able through laccase-catalyzed oxidation of many aminophenolic compounds [37-39]. Azo-dyes, by far the most important class of commercial dyes, have been syn-thesized by oxidative coupling reaction between different substrates [40, 41]. In an interesting example, Enaud and coworkers [42, 43] synthesized a novel azo-dye containing two sulfonated anthraquinonic chromophores by a laccase immobilized on an inexpensive carrier. The acid dye obtained is neither cytotoxic nor mutagenic demonstrating good dying properties in polyamide fibers.

Food industry

Food industry benefits from laccase application in different sectors and for multiple purposes: from modification of food sensory parameters and texture to improvement of products shelf-life and determination of certain compounds in beverages. As a fact, many laccase substrates, mostly phenols, thiol-containing proteins and unsaturated fatty acids are fundamental components of various foods and beverages, thus their modification may lead to new func-tionalities, quality improvement and cost reduction [44].

Baking industry

Application of laccases in baking process has been first reported by Labat et al. [45] describing laccase ability to cross-link arabinoxylans (AX) matrix in dough through dimerization of the esterified ferulic acid, causing increased strength, stability, and reduced stickiness of dough, and thus resulting into its improved machinability. In addition, an increased volume and improved crumb structure and softness of the backed products have been observed. Enhancement of maximum resistance and decreased extensibility has been obtained by laccase treatment of both flour and gluten doughs [46]. The hard-ening effect was higher in flour dough, where laccase action is prevalent on AX fraction, with respect to gluten dough, where laccases react with gluten protein matrix with less efficiency. Similar results have been obtained on gluten-free oat flour, where laccase treatment was shown to not affect oat globulins [47]. Prolonged incubation with laccase caused softening phenomena, probably due to radical-catalyzed break-down of the cross-linked AX net-work [46]. Firmness of fresh oat bread and tightness of wheat dough were found to be increased after laccase treatment, as a consequence of the reduction of water-extractable arabinoxylan (WEAX) content following AX cross-linking [48]. When combined with xylanases, laccase treatment further improved textures of both oat and oat- wheat breads [48]. Coupled laccase and protease supple-mentation, combined with endo-b glucanase side activity of the laccase preparation, also improved bread-making

performances. In this case, the effect of hydrolytic activi-ties prevailed over laccase promoted protein cross-linking, by reducing the formation of globular aggregates and positively affecting flour pasting properties [49].

Food sensory parameters

Consumer's perception of the quality of a food product is strictly influenced by its exterior aspect and odor. Laccases have been applied to the improvement of food sensory parameters by acting on processes affecting physicochemical deterioration of food products, or enhancing their flavor and taste properties.

Tsuchiya *et al.* [50] have patented the use of a recombinant laccase and cholorogenic acid to control the malodor of cysteine in different products, including foodstuff. They have shown that enzymatically treated cysteines have a reduced H_2S-related smell with respect to untreated ones.

Laccase-catalyzed deoxygenation of vegetable oils has been shown to prevent oxidation of linoleic and linolenic acids responsible for generation of undesirable volatile compounds [51]. The authors have also demonstrated that addition of flavoring agents, such as anthocyanin, a spice, and other phenolic compounds available as food ingredi-ents has a very positive influence on the deoxygenation rate by acting as laccase substrates too.

A method for enhancing color in tea-based foodstuff by the means of Pleurotus laccases has also been patented [52]. The oxidation step converted the colorless catechins in the leaves to a complex mixture of yellow and orange to dark brown substances, such as theaflavins and thearubigins. Also, cacao of superior taste and flavor has been obtained by treatment of cacao nib by Coriolus versicolor laccase [53]. Reduction of tannin content in cocoa pod husk after laccase treatment has been found to improve its nutritive value when used as ingredient for animal feeds [54].

Wine and beer stabilization

Wine stabilization is one of the main processes demanding laccases in food industry. Polyphenols in wine play a major role in determining aroma, color and taste of wine, which are strictly related to the particular phenolic composition. However, the same compounds are also the main actors involved in madeirization process causing turbidity, color intensification and aroma and flavor alteration. Laccase treatment aimed at reducing polyphenol content has been reported as an effective method to preserve wine quality, since the use of enzymes would selectively remove target-speciÞc polyphenols without undesirably altering wine's organoleptic characteristics. Laccases fit well with process requirement, due to their stability in acidic medium and reversible inhibition with sulÞte [55]. Although the efficiency in polyphenol removal has been demonstrated, many authors underlined the need to add supplementary steps to wine production process, such as sulfur treatment, clarification and Þltration to remove the oxidized products produced by laccase [56]. Other authors have also confirmed the potential of laccases in prefermentative treatment when coupled with conventional clarifiers such as proteins and polyvinylpyrrolidone (PVPP) [56]. Differential selectivity in phenol removal has been highlighted by Minussi *et al.* [57] in the treatment of red and white wines. Red wine treatment reduced phenolic compounds but mainly affected those responsible for the must antioxidant properties. On the other hand, treatment of white wines has shown higher reduction in total phenols than in the total antioxidant potential, resulting into reducing processing costs and increased storability of white wines.

Another negative aspect connected to wine storage is the characteristic cork off-flavor imparted to wine in contact with cork stoppers, which is related to phenols naturally present in cork and/or produced by microbial degradation. The use of laccase for the treatment of cork stoppers for wine bottles has been patented as an effective method to reduce the cork taint by phenols oxidation [58].

Similarly, the addition of laccase to fermented beer has been shown to improve its storage stability by scavenging the oxygen, which is responsible for the development of catty taint in bottled beer through reaction with fatty acids, amino acids and proteins [59]. Laccase treatment reduces the excess of naturally occurring proanthocyanidines and polyphenols that cause protein precipitation and therefore haze formation in beer [59].

Fruit juice clarification

Analogously to what happens during wine storage, phenols naturally occurring in fruit juices undergo interaction with proteins resulting into formation of haze or sediments. In addition, juice browning also imputable to both enzymatic and chemical oxidation phenomena involving phenols turns out into a final product which is less appealing to the consumers. Several authors have proposed the use of lac-cases as stabilizing agents, due to their ability to oxidize most of phenols in juices. Contradictory results have been published. Sammartino et al. [60] demonstrated that enzymatically treated apple juice was less stable than the one stabilized by conventional methods. Also, laccase treatment increased susceptibility to browning during storage [61, 62]. On the other hand, effective phenol removal and improved juice stabilization have been reported [56]. When laccase treatment was followed by a filtration step, juice quality could be highly improved [63, 64]. Neifar *et al.* [65] coupled a laccase treatment of

pomegranate juice optimized by RSM with ultrafiltration, obtaining a reduction of polyphenol content and antioxi-dant capacity, but a concomitant increase of clarity of about 30 % versus untreated juice. More recently, Gassara-Chatti [66] has utilized a formulation of ligninolytic enzymes from Phanerochaete chrysosporium encapsulated in polyacrylamide hydrogel for clarification of mixed juice of berry and pomegranate, resulting in a significant poly-phenolic reduction and clarity amelioration with respect to treatment with free enzymes.

Value-added food products

Laccases have been applied to enzymatic gelation of sugar beet pectin (SBP), a food ingredient capable of generating gels after oxidative cross-linking of ferulic acid [67]. Such kinds of gels are of particular interest in food industry due to their thermo-irreversibility, allowing a foodstuff to be heated without losing its gel structure. When added to three different food products, black currant juice, milk, and chopped heat-treated meat emulsion, gelation occurred in all the cases, although in the first two some unwanted side effects occurred [67]. Laccase-induced gelation of SBPs has been found to be highly related to molecular weight, acetylation and methylation degree of different pectin preparations [68]. Moreover, gelling rate and mechanical properties of laccase-induced gels have been proved to be greatly dependent on the amount of laccase activity used: higher enzymatic activity promoted the formation of softer gel due to the short reaction time available for the right arrangement of molecules. On the contrary, lower activity levels assured the formation of correct and permanent cross-links and led to a more rigid gel [68].

Another interesting application demanding laccases concerns the improvement of the stability of globular protein-coated lipid droplets to environmental stresses. As a fact, such kinds of emulsifiers are widely utilized in food industries, although their application is restricted to a limited range of matrixes because of their sensitivity to changes in pH, ionic strength and temperature. Laccases have been found to increase emulsion stability of oil droplets coated by multilayered biopolymer interfaces by promoting cross-linking among b-lactoglobulins in the primary interfacial layer as well as among beet pectin molecules constituting the secondary adsorbed layer. Also protein-polysaccharide cross-links between layers could be responsible for the observed increased emulsion stability to salt in a wider pH range [69]. Laccases also catalyzed the cross-linking of interfacial layers in multilayered oil-in water emulsions obtained by electrostatic deposition of pectin onto a fish gelatin interfacial membrane [70]. Cross-linking occurred exclusively in the layers and not between droplets, since no aggregates were formed. Resulting emulsions were characterized by enhanced stability in a broad pH range (3.5-10). In another example, oil-in water emulsions coated by laccase-mediated cross-linked sugar beet pectin were characterized by smaller and well-dispersed droplets improving their long-term stability [71].

Ma et al. [72] used laccase for the cross-linking of whey protein isolates (WPI) to improve their emulsiÞcation properties. Chemically modified and enzymatically treated WPI has been shown to enhance storage stability of WPI-stabilized emulsions. More recently, a mixed solution of WPI, SBP and laccase was microemulsified as nanodroplets in an organic phase [73]. Caffeine was encapsulated into the nanodroplets as a model compound to test their applicability as vehicles for drugs and/or nutraceuticals. Although stable conjugated particles of spherical shape and nanoscalar size were obtained, the authors recommended this method for encapsulation of non-oxidative nutraceuticals and biomaterials since antioxidants such as caffeine may counteract with the oxidizing action of laccase during cross-linking of biopolymers.

Laccase-mediated cross-linking of food proteins has also been used to develop added-value products in the food industry [71, 74], as well as to influence protein digest-ibility and reduce their allergenicity [75, 76]. b-Lactoglobulin (BLG), accounting for approximately 10-15 % of total milk proteins, is an important nutrient of dairy products and, at the same time, one of the major milk allergens causing serious health risk in cow's milk-allergic patients [77]. A Trametes versicolor laccase has been used to cross-link BLG phenolic groups in the presence of a sour cherry extract as a natural source of phenolic mediators [76]. Enzymatic processing by cross-linking may be used to tailor BLG properties, by improving its safety and facilitating its digestibility, while conserving beneficial health effects, such as the radical-scavenging activity of peptides released during enzymatic digestion.

Compound detection in beverages

Different biosensors based on laccases have been developed to measure polyphenols in food products, such as wine, beer and tea. Effective potential of a biosensor based on immo-bilized laccase has been first verified by Ghindilis *et al.* [78] for detection of tannin in tea of different brands. Since then, a lot of reports concerning the development of laccase-based biosensors have been published in the scientific literature. Laccases from different *Trametes* species were immobilized on carbon nanotube screen-printed electrodes for determi-nation of polyphenols index in wines [79]. Reported data have shown that biosensor performance is dependent on the laccase source used. Ibarra-Escutia *et al.* [80] optimized an amperometric biosensor based on a *T. versicolor* laccase for monitoring phenol compounds in tea infusion. This

biosensor displays an excellent stability in the operative conditions and exhibits good performances in terms of response time and sensibility, allowing accurate sample analysis without any pretreatment step. Also, a disposable biosensor was developed for phenol detection in tea infu-sions [81]. Finally, a bioelectronic tongue based on lipid nanostructured layers containing tyrosinases and laccases has been recently developed by Medina-Plaza and coauthors [82]. The enzymes have been incorporated into a biomimetic environment provided by a Langmuir-Blodgett film of arachidic acid. Lutetium bisphthalocyanine (LuPc2) has also been introduced in the films to act as electron mediator. This multisensory system discriminates phenols according to the number of phenolic groups attached to the structure as well as grapes of different varieties according to their phenolic content. A combination of factors contributes to the high performances of this biosensor: the high functionality of the enzyme obtained using a biomimetic immobilization, the signal enhancement caused by the LuPc2 mediator, the improvement in the selectivity induced by the enzymes and the complementarity of the enzymatic activities.

Laccase-mediated polymer synthesis

In the frame of industrial application, laccase ability to synthesize polymers is of outstanding importance. Indeed, polymeric materials are essential for everyday life, being present in many and different fields, such as food, textile, electronics, communications, transportations, pharmacy, and medicine [83]. Even if current polymer production schemes are highly optimized, the huge quantity of annually produced polymers demands a greener technology, particularly in the perspective of toxic formaldehyde reduction. Hence, it is not surprising that also enzymes are being engaged for polymer synthesis [83], being laccases and peroxidases dominating this field [84]. In a polymerization reaction, laccase achieves its natural work with the initial formation of radicals from a typical substrate (aromatic with hydroxyl group or groups). Then, radicals can undergo polymerization reactions towards homopolymeric materials [85]. It is also possible to create new heteromolecular hybrid molecules coupling a laccase substrate and a non-laccase substrate (variable reaction partner) [85] (Fig. 1). Radical polymerization lacks any control on the structure and morphology of the final product, thus if a deÞned molecule is pursued, it is necessary to find oppor-tune reaction conditions, as reviewed by Hollmann and Arends [84]. Short reaction time, defined molar concen-trations [86], presence of templates [87], substrate engineering [88, 89], and use of cosolvent [90, 91] are some of the more frequently applied strategies to obtain defined polymers.

Despite the proved capability, short-term implementa-tion of enzyme-mediated polymer synthesis on industrial scale is still hampered by economic issues. However, the high efforts put in this field let foresee a fast revolution in polymer chemistry in the next decade.

Polymer synthesis by laccases has already been reviewed by different authors [83-85, 92, 93]; the aim of this paragraph is to underline those applications we believe more promising or more ready for a real industrial exploitation.

Flavonoids

Flavonoids are benzo-c-pyrone derivatives consisting of phenolic and pyrane rings [94] endowed with biological and pharmacological effects, including antioxidant, anti-mutagenic, anti-carcinogenic, anti-viral and anti-inflam-matory properties [95]. High molecular weight polyphenols have been reported to exhibit enhanced biological proper-ties with longer circulation time in vivo [96]. As a fact, in the last decade it has been demonstrated that oxidative coupling of flavonoids generates polymers with increased antioxidant activity. Kurisawa and coworkers [97] have employed a Myceliophthora laccase to catalyze the oxi-dative polymerization of catechina flavonoid found in green tea and wine in a mixture of a polar organic solvent and buffer. Polymer yield and composition were improved testing different solvent composition. When compared with monomeric catechin, the synthesized polymer exhibited significantly improved superoxide scavenging activity and xanthine oxidase (XO) inhibitory activity. Jadahav and Singhal [98] used a laccase conjugated to Arabic gum to perform catechin polymerization. Free laccase produced water-insoluble cross-linked oligomer, whereas conjugated laccase produced water-soluble linear oligomer endowed with high antioxidant activity and reducing power. An improved antioxidant activity if compared with the corre-sponding monomer has also been observed in the polymerization of quercetin and kaempferol with a laccase from Ustilago maydis [99]. More recently, the oxidative grafting of flavonoids to modify/impart polymer properties is gaining increased attention, as described in other sections.

Artificial urushi

“Urushi” is a Japanese traditional coating material show-ing excellent toughness, brilliance and solvent resistance for a long period in comparison with synthetic coatings. It is a natural resinous sap obtained by the Rhus vernicifera tree through the cross-linking of urushiol monomers [100]. The urushiol structure is a catechol derivative, with unsaturated hydrocarbon chain consisting of monoenes,

dienes, and trienes at the 3-, or 4-position of catechol. In nature, the film forming of urushiols proceeds via laccase catalysis under air. It can be regarded as the only example of a practical natural paint that utilizes in vitro enzymatic catalysis for hardening [100]. New urushiol analogs were developed to overcome the difficulty to chemically synthesize natural urushiols [101, 102]. "Artificial urushi" may be defined as the cross-linked film prepared by in vitro laccase-catalyzed oxidation of new urushiol analogs, in which the unsaturated group is connected with the phenolic group through an ester linkage. The artificial urushi prepared by laccase shows hardness and brilliance comparable to those of natural urushi [100-102].

Conducting polymers

Conducting polymers, such as polyaniline (PANI), poly-thiophene (PEDOT), and polypyrrole (PP), have attracted a great attention in the last years.

PANI is regarded as one of the most important con-ducting polymers. Due to its good electrical and optical properties, as well as its high environmental stability, it can be used as active component of organic lightweight bat-teries, microelectronics, optical display, for anticorrosive protection, in bioanalysis, etc. [83]. PANI is commonly synthesized by chemical or electrochemical oxidation of aniline monomer under very harsh conditions and yielding a product with a poor solubility in common solvents [83]. Among all the forms of PANI only the emeraldine salt is conductive, and it is usually obtained from the emeraldine base via protonation of its imine sites in acidic conditions (doping) [103]. Problems concerning reaction conditions and PANI solubility can be overcome thanks to biotechnology, e.g., PANI can be enzymatically synthesized by oxidoreductases (glucose oxidase, peroxidase, laccase) in the presence of templates. Many studies deal with the use of peroxidases, but more recently also laccases are increasingly being used, thanks to their ability to work using only air (dioxygen) as cofactor [83]. When PANI polymerization is conducted in the absence of templates, extensive branching is observed and this is not desirable for conducting materials. Besides sulfonated polystyrene (SPS), the first used template in laccase-mediated PANI synthesis [104], many other templates have been used, such as other negatively charged polymers [105, 106], nega-tively charged micelles [23, 107-110], and negatively charged vesicles [111]. Shumakovich and coworkers [106] have performed a comparison of chemical and enzymatic methods of aniline polymerization. The high-redox poten-tial T. hirsuta laccase was used in the synthesis of polyaniline in the presence of poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPS) as template. PANI nanoparticles obtained by the two methods are morphologically distinctive. In addition, the mechanism of enzymatic aniline polymerization differs from that of the chemical synthesis. The latter proceeds with a significant induction period, which is not observed in the laccase-catalyzed reaction. With the aim to repeatedly use the enzyme, Vasileva and colleagues [105] used an immobi-lized laccase on CM cellulose to perform PANI synthesis in the presence of PAMS as template. The conductance of the interpolymer complex synthesized using the immobilized laccase is close to the value of PANI synthesized using the free enzyme, and comparable with the value of the chemically obtained PANI. During recent years, great interest has been given to the synthesis of chiral conducting polymers because they are very promising for practical application in different fields, e.g., for creating surface-modified electrodes, electrochemical asymmetric synthesis, chiral chromatography, and chiral membrane separating technology [112]. A fungal laccase from *T. hirsuta* was used for the synthesis of chiral polyaniline in the presence of optically active compounds, either in a homogeneous way (in the bulk solution) and heterogeneous one (on the surface of glass slides, in situ synthesis). The optical activity of the enzymatically synthesized polymer in the doped and dedoped forms was conÞrmed using Circular Dichroism spectroscopy. When the low redox potential laccase from *R. vernicifera* was used for PANI polymerization, the presence of mediator was required to increase the rate of the reaction [110]. Conducting polyaniline synthesized by the LMS method is produced with a higher yield and displays five times higher conductivity than that obtained in the absence of mediator.

3,4-ethylenedioxythiophene (EDOT) has attracted sig-nificant attention in recent years since EDOT polymers (PEDOT) have demonstrated potential applications in organic field effect transistors, organic light emitting diodes and photovoltaic cells [113]. Due to high redox potential of the monomer, EDOT cannot be oxidized in the presence of only laccases, thus it is necessary to use a redox mediator. A T. hirsuta laccase was used in the presence of potassium octocyanomolybdate(4^+) as high potential redox mediator to catalyze the reaction of EDOT oxidation and to perform synthesis of PEDOT using PAMPS as template. UV-vis and FTIR spectra conÞrmed the formation of water dispersible conducting polymer [113].

Polypyrrole (PP) is a biocompatible conducting polymer endowed with a high conductivity, stability in the con-ducting state, and interesting redox and electromechanical features [114]. An LMS based on a *T. versicolor* laccase and ABTS was used for the synthesis of PP [115]. The presence of ABTS increased the rate of the reaction and allowed to produce oligomers with higher molecular weight with respect to those obtained without mediator.

The conductivity of these polymers, both with and without ABTS, is similar to that of PP prepared electrochemically or chemically.

New materials

The recent synthesis of a novel photoluminescent material [116] is herein cited as a symptomatic case of viable future chances given by laccase-mediated polymer synthesis. A *T. versicolor* laccase was used for the oxidative polymeriza-tion of 4-fluoroguaiacol in the presence of 30 vol % organic co-solvent [108]. The novel synthesized material displays fluorescence with emissions in blue, green and red, thus it may be used as a component in optoelectronic devices. Obtained results have also led to demonstrate that the coupling of the repeated units proceeds by defluorination to attain oxy-phenol propagation with coupling by the C4 phenyl carbon.

Polymer grafting

Polymers with new and/or improved properties can be obtained not only by new synthetic routes, but also modi-fying existing polymers with selected molecules, using the so-called grafting technology [31, 93, 117]. Coupling of low-molecular weight molecules to polymers can be achieved through functional groups located at the polymer terminal, in the main chain or in the side chain [83]. This enzymatic modification of existing materials, yielding products with modified properties, has an immense potential in different industrial sectors, such as food, textile, pulp and paper, wood, and also cosmetic to a lesser extent. In this paragraph, selected cases of polymer modification of lignocellulosic materials will be reviewed, since examples of grafting in other fields are described in the corresponding sections (“Functional modification of textile fibers”, “Textile dyeing”, “Baking industry”, “Value-added food products”, “Pharmaceutical and cosmetic industries”).

Lignocellulosic materials

Laccase-mediated polymer modification covers different aspects in the pulp and paper industry. The potential of laccase-assisted biografting of phenolic acids for improv-ing strength properties of kraft paper made from high kappa pulps is discussed in several papers [112-124]. The main focus of Chandra studies has been the grafting of phenolic acids to high-lignin content softwood kraft fibers mediated by a Trametes villosa laccase [118-122]. Results indicated that improvements of hydrogen bonding between fibers and creation of phenoxy radical cross-links within the sheets increased paper strength [122]. Recently, Witayakran and Ragauskas [123] have investigated the possibility of grafting various amino acids onto high-lignin content pulps obtaining an increasing of carboxyl group content and paper properties. The ability to use laccases to selectively graft amino acids to lignin-rich pulp fibers provides a new and unique fiber modification technology which will have many future opportunities. In most cases, treatment of flax and sisal pulp with laccases (from *Pyc-noporus cinnabarinus* and *T. villosa*), with or without a mediator, in the presence of different phenolic compounds, improved strength-related properties (particularly wet ten-sile strength) in the resulting paper [125-128].

Recently, there has been an increase in innovative research aimed at developing functional packaging mate-rial with antimicrobial properties [129, 130]. Several bioactive phenolic compounds (acids, essential oil com-ponents and dopamine) were grafted onto the surface of unbleached kraft liner fibers using a laccase from *T. pubescens* [129]. Obtained results have shown that the handsheet papers obtained by laccase antibacterial surface process (LASP) display a greater efficacy against gram-positive and gram-negative bacteria, if compared with paper treated only with monomeric phenol derivatives. Antimicrobial activity was proved to be function of grafted structure, time of the treatment and concentration of phenol derivatives. Among the tested essential oil compounds, isoeugenol is the most effective: isoeugenol/LASP, besides killing S. aureus, displays a bacteriostatic effect on the more resistant spore forming gram-positive Bacillus sub-tilis. A P. cinnabarinus laccase was used to initiate the grafting of three different antimicrobial phenol structures (syringaldehyde, acetosyringone and p-coumaric acid) onto unbleached flax fibers with a high cellulose content [130]. Grafted pulps presented high antimicrobial activity against three bacteria analyzed (*S. aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*).

Wood (*Picea abies*) veneers and pulp were treated with tannins, as natural antibacterial phenols, in the presence or absence of commercial *M. thermophila* laccase with the aim to impart antibacterial properties to the substrates [131]. The treatment in the presence of laccase significantly improved the antibacterial resistance of veneers and paper made from tannin-treated pulp against *S. aureus*, while a more modest protective effect was observed against *E. coli*. Authors have also provided mechanistic evidence of the binding of catechin and gallic acid onto putative lignin monomers. Catechin was bonded to the phenolic molecules mainly through 5-5 linkages, while bonding to monomers with sinapyl units was mainly through 4-O-5 linkages. Bridging molecules such as 4-hydroxy-3-meth-oxybenzylurea, compatible with melamine resin, were attached to fiber lignin by means of laccase with the aim of boosting the disinfecting properties of phenol resin utilized

for the impregnated kraft paper of certain environments where high hygienic requirements exist, such as hospitals and kitchens [132].

In an effort to use green chemistry technology to increase wood surface hydrophobicity, laccase-mediated grafting of pulps has been successfully tested. Fluorophenols and alkylamines were coupled, mediated by a *T. hirsuta* laccase, to complex lignin models to increase hydrophobicity of wood veneers [133-135]. The proposed mechanism has shown that the fluorophenols were bonded to sinapyl units via 4-O-5 linkages, while coupling to guaiacyl units occurred through 5-5 linkages. The alkyl amines were bonded onto simple phenolics and lignin models through the $-NH_2$ thereby establishing a -C-O-N and a –C-N bond. The advantage of laccase-mediated covalent binding of molecules onto wood surface is that the grafted molecules are not readily displaced and released into the environment.

Several approaches of in situ laccase-created radicals in wood chips have been investigated as a way of producing binderless (artificial adhesive-free) wood such as Medium Density Fiberboards (MDFs) and Particle Boards (PBs), commonly used for interior building and furnishing [136].

Many attempts have been made at increasing bonding between the fibers or other constituent particles by their chemical modification. The activation of lignin for bonding can be carried out by oxidative enzymes [137-139]. Par-ticularly, the auto-adhesion of beechwood (Fagus sylvatica) fibers was enhanced by a pretreatment of the fibers with a T. villosa laccase (Novozymes A/S) [139]. The mechanism of enzymatic catalyzed bonding is linked to the generation of stable radicals in lignin by oxidation. Fiberboards made from laccase-treated fibers have a high wet strength compared to boards made from untreated fibers. Furthermore, the surfaces of laccase-treated fibers have shown a markedly increased hydrophobicity, as well as a change in the chemical composition, indicating that lignin extractives precipitated on the fiber surfaces. Another approach to substitute synthetic resins in wood composite production is a two-component system in which laccase-treated technical lignin, such as kraft lignin or lignosulfonate, functions as the adhesive. This method has been applied to the production of PB [140-142], even if the use of water-soluble lignosulfonates generates products displaying poor dimensional stability [141]. A variant process has also been described where a laccase-oxidized lignin-based adhesive, with 1 % methylene diphenyl diis-ocyanate, resulted in PBs with doubled tensile strength and reduced swelling in water [143].

Other examples

Membrane fouling is a serious problem in membrane fi l-tration due to adsorption of components from the feed [144]. Among other factors, also the (surface) material of the membrane affects adsorption. Poly(ether sulfone) (PES) is a popular hydrophobic material for membranes. A (more) hydrophilic membrane surface may reduce (or even prevent) adsorption [145]. A laccase-mediated grafting of PES with 4-hydroxybenzoic acid and gallic acid in con-trolled reaction conditions has proved to have clear potential for reduction of fouling by proteins, polysaccha-rides, and polyphenols, probably related to the more hydrophilic surface [146].

Pharmaceutical and cosmetic industries

Laccases can catalyze the synthesis of various therapeutics, such as antibiotics, antimicrobials and anti-cancer drugs. The usefulness of laccases for the amination of new anti-biotics has been demonstrated. Many potential aromatic substituents of penicillins and cephalosporins are natural laccase substrates. Mikolasch and coworkers synthesized different novel b-lactam antibiotics (e.g., cephalosporins, penicillins and carbacephems) through laccase-catalyzed amination of amino b-lactams and 2,5-dihydroxyphenyl-acetic acid, 2,5-dihydroxybenzoic acid or catechols [147-150]. Novel cyclosporin analogs can be obtained through a laccase-mediated oxidation [151].

Recently, the laccase-catalyzed synthesis of anhydro-vinblastine, mitomycin and structurally similar anti-cancer compounds has been reported. Anhydrovinblastine, useful in the leukemia treatment, was synthesized in a coupling process of catharine and vindoline [152, 153]. It has been demonstrated that laccases from *M. thermophila* and *P. cinnabarinus* synthesize 5-alkylamino- and 2,5-bis (alkyl-amino)-(1-4)-benzoquinones, structurally similar to mitomycin, a chemotherapeutic agent used to treat differ-ent cancers [154]. Synthesis of other anti-cancer molecules, such as aminonaphthoquinones and *p*-benzoquinone, has been, respectively, obtained in a monoamination and di-amination laccase-catalyzed reaction [155, 156]. Moreover, laccases from different organisms exhibit cytotoxic or antiproliferative effects against the tumor cells, mainly MCF7 and Hep G2 [157-161].

Laccases from *Tricholoma giganteum*, *Lepiota ventr-isospora*, *Lentinus edodes*, *Pleurotus cornucopiae*, *Coprinus comatus*, *Lentinus tigrinus*, *Hericium coralloides*, *Agaricus placomyces*, and *Abortiporus biennis* have been reported as inhibitors against HIV-1 reverse transcriptase activity [162-169]. Another laccase has been reported to possess the capability of fighting aceruloplasminemia, restoring the iron homeostasis [170].

Additionally, laccase application has been reported in the generation of iodine [171-174], a well-known anti-fungal compound, generally used as disinfectant. Laccases

are also able to catalyze the formation of other antimi-crobial compounds, such as the cinnabarinic acid [175], or to have antibacterial and antifungal activity themselves. Recently, Grover and coworkers have developed a laccase-nanotube-based paint, showing a bactericidal and sporicidal activity [176].

Furthermore, an increasing interest has been focused on laccase application in the cosmetics as a biocatalyst in the hair dye synthesis and in the formulation of personal hygiene products, including deodorant, toothpaste, mouthwash, detergent and soap. Laccase-based hair dying is an emerging field of research. Traditional chemical-based hair coloring products are often irritant, difficult to handle and not safe. Hydrogen peroxide (H_2O_2) and phenylenediamines are the most used chemicals in hair dying. They are allergenic and carcinogenic [177, 178] and can, respectively, cause severe hair damage [179, 180]. Laccase can act as an oxidizing agent, substituting H_2O_2. For this reason, laccase-based hair dyes are less irritant [181, 182]. Recent works have shown that laccase-catalyzed polymerization of natural phenols is applicable to the development of new cosmetic pigments [181]. A laccase from *T. versicolor* and natural plant-derived phenolic compounds have been used to produce a colorful array of eco-friendly dyes [183]. Jeon and coworkers [184] have studied laccase-catalyzed in situ polymerization of natural phenols to form products for hair dyeing. Three kinds of two-monomer combinations for gray hair dyeing were developed, namely, gallic acid/syringic acid, catechin/cat-echol and ferulic acid/syringic acid, generating brown, black and red colorations, respectively. Laccase polymer-ization of natural phenols can provide stable colored polymers, since color changes in all dyed hairs tested were negligible, even after repeated shampooing with detergent excess. The alkaline laccase secreted by *Flammulina velutipes* has been tested for hair coloring [185]. Lately, Chen and coworkers have proposed the laccase from the acti-nomycete Thermobifida fusca as an excellent thermo-alkali-stable laccase for hair coloring [186]. Fang et al. have demonstrated that the bacterial laccase Lac15D displays a constant activity toward dye precursors and their combinations under alkaline conditions, suggesting its application in hair coloring [187].

In the cosmetic field, laccases are also used for skin lightening [188, 189]. Laccases may not only find utility in the oxidative reaction, production of pigments and mela-nins, but also in the reduction of melanin-derived blemishes [190]. In addition, laccases can oxidize various

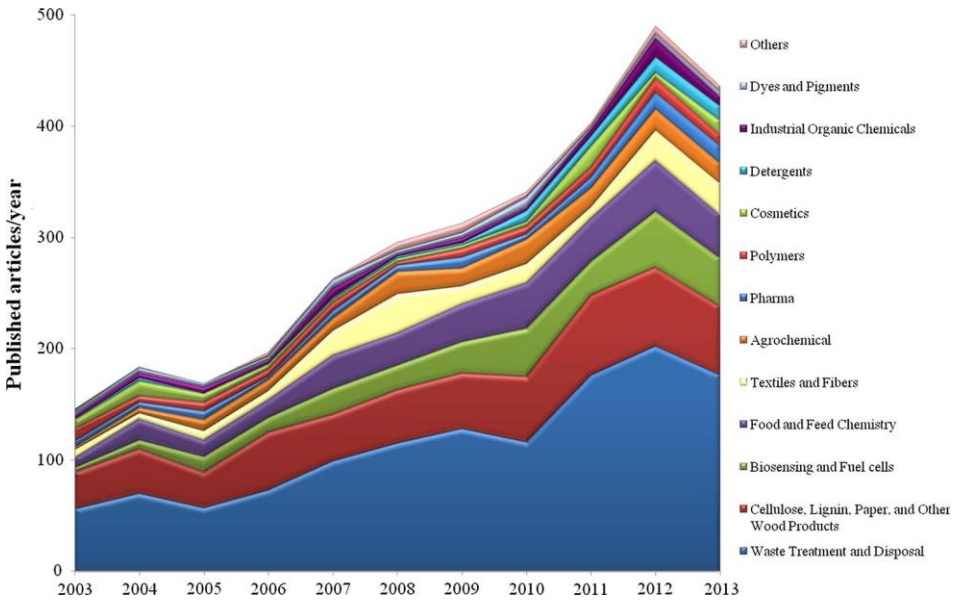

Fig. 2 Data are from Chemical Abstracts Service/SciFinder Scholar based on the combined CAS and Medline databases with duplicated removed. Source articles include journal articles and are referred to the decade 2003-2013. Categories used for classification of journal articles are based on the following CAS section title: Waste treatment and disposal; water, cellulose, lignin, paper, and other wood products; electrochemical, radiational, and thermal energy technology; electro-chemistry; food and feed chemistry; textile and fibers; fertilizers, soils, and plant nutrition; agrochemical bioregulators; pharmaceuticals; pharmacology; essential oils and cosmetics; toxicology;surface active agents and detergents; heterocyclic compounds (more than one hetero atom); heterocyclic compounds (one hetero atom); industrial organic chemicals, leather, fats, and waxes; coatings, inks, and related products; dyes, organic pigments, fluorescent brighteners, and pho-tographic sensitizers; industrial carbohydrates; surface chemistry and colloids; terpenes and terpenoids; plastics fabrication and uses; plastics manufacture and processing; chemistry of synthetic high polymers; biomolecules and their synthetic analogs; alkaloids; amino acids, peptides, and proteins

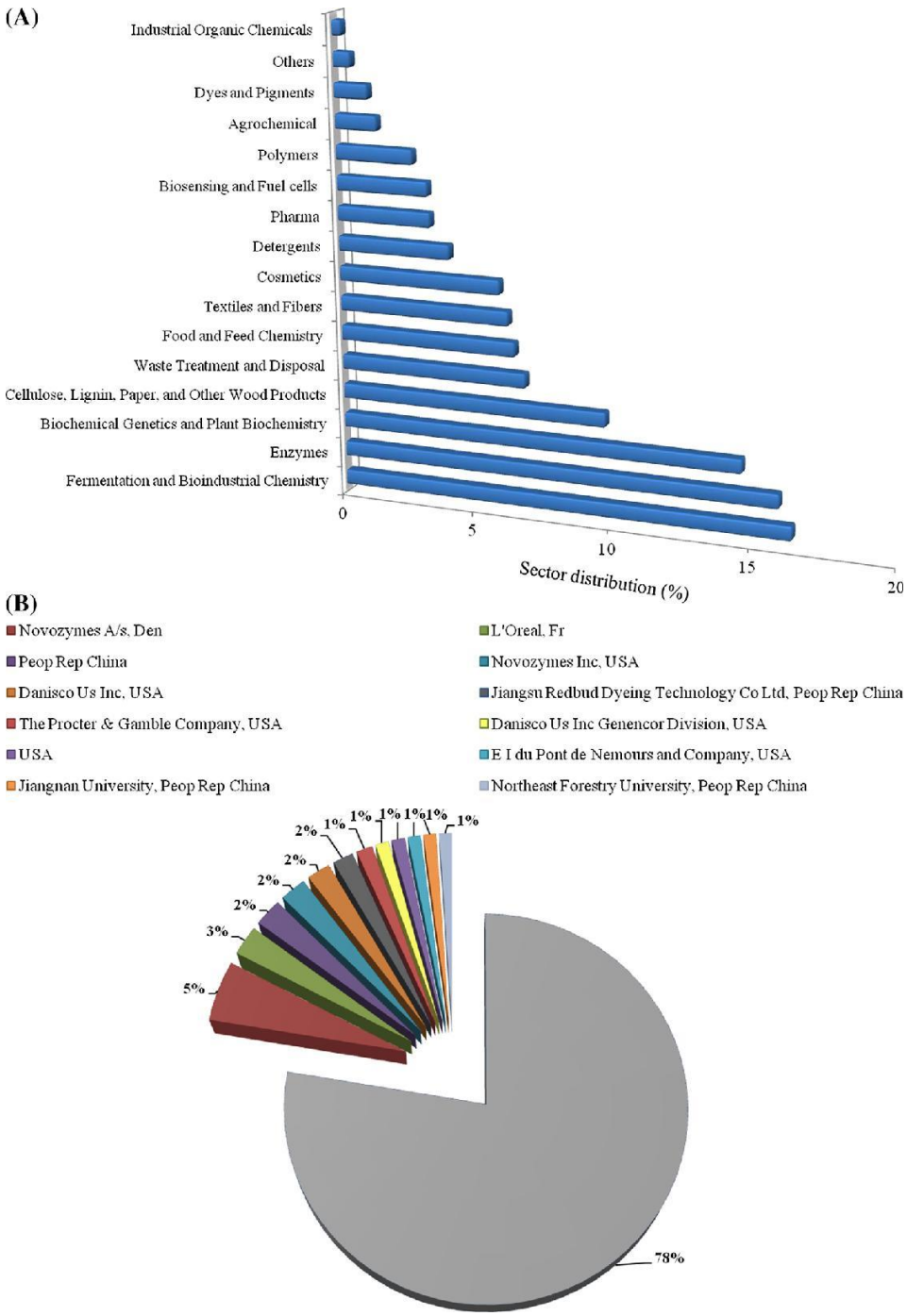

Fig. 3 a Distribution of patents deposited in the decade 2003-2013, according to classification into CA section titles. Data are from Chemical Abstracts Service/SciFinder Scholar based on the combined CAS and Medline databases with duplicated removed.

b Distribution of patents deposited in the decade 2003-2013 among the owners (companies and research institutions). The gray slice corresponds to owners possessing less than 1 % of total published patents

thiols and other sulfur-containing compounds, responsible of malodor or even kill the microbes that generate offen-sive molecules [191]. For this ability, these enzymes have been introduced in deodorant for personal hygiene [192], oral care products [193] and soaps [194].

The importance of these oxidoreductases as efficient catalysts in cosmetic field is underlined by the increasing number of patents registered in the last years [195-209].

Conclusion

Figure 2 summarizes and classifies the scientific literature published in the last 10 years about implementation of laccases in several industrial fields. The trend is positive for most of the analyzed sectors, with applications in waste water treatment occupying the widest slice of published articles. Articles concerning modification of natural

polymers such as lignin and cellulose with related applications in wood and paper industries also represent an important portion of the applicative research on laccases. Other emerging sectors are represented by laccase exploitation in biosensing and fuel cells industry, cosmetics, biopolymer synthesis and in food and textile fields. Also the use of laccases as green catalysts for the synthesis of high-added value organics is emerging as a new, although still poorly exploited, sector of applicative research.

The active research on potential laccase industrial applications has stimulated the publication of a great number of patents in several sectors. Figure 3 reports the relative percentage of patents distribution in different sectors, referred to the decade 2003-2013. The widest slice of patents concerns methods of fermentation and optimization of laccase production, followed by inventions about enzyme modiÞcation aimed at its conjugation and/or immobilization for different uses (enzymes category in Fig. 3a). Analyzing the distribution of patent owners (Fig. 3b), Novozyme (Novo Nordisk, Denmark) owns the most of the totality of patents (5 %), followed by L'Oreal with 3 %. The biggest slice of the pie graph (78 %) is occupied by companies or Research centers possessing less than 1 % of total number of published patents.

Despite this plethora of applications, laccase potential was not fully exploited, due to several issues mainly con-nected with the economy and the efficiency of the enzyme in the operating conditions. Recent efforts to abundantly produce these enzymes, to improve enzyme activity and/or stability through immobilization and protein engineering, are boosting laccase exploitation at industrial level. Since the first commercial product based on laccase enzyme, launched in 1996 by Novozyme (Novo Nordisk, Denmark), widespread companies have been engaged in producing this enzyme in several formulation and for different purposes, mainly for the textile and food industries [2]. Less enzymatic formulations are available for pulp and paper industries, probably for a kind of reserve towards the integration of enzymes in an "ancient" and consolidated process flow, such as paper production. Most of the new companies are located in the Asian continent, where, despite global crises, industry is expanding, probably also due to the less bureaucratic constraints for industrial production and lower salaries of employees in these regions [2]. On the other hand, the increasing demand of specific and eco-friendly alternative biocatalysts has nowadays induced, also in Europe, the creation of new small com-panies offering customized formulations of laccases to target specific process conditions.

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Chapter 1:

Fishing microorganisms from the Puteolanum

“fisherman’s mole”

Abstract

Marine microorganisms provide an interesting source of biocatalysts. Lipases are glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. This study was aimed at isolating microbial lipase producers from superficial backwater in the “Darsena vecchia” of Putueoli. An enrichment culture technique and a screening on olive oil agar plate were applied, leading to the isolation of 12 positive colonies. Among these, the imperfect fungus *Geothricum candidum* has been selected. Production medium was optimized by varying different chemical parameters. The maximum extracellular lipase production (6,000U/L) was observed in the optimized condition after six growth days.

Introduction

Lipases are serine hydrolases that catalyze both the hydrolysis and synthesis of long-chain triacylglycerols. 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 (Momsia and Momsia, 2013). Even if a large number of different lipases has been already discovered, commercialised and developed in many industrial fields (Horchani *et al.*, 2012; Sharma and Kanvar 2014; Anobom *et al.*, 2014), there is yet interest in bioprospecting towards novel lipolytic organisms. Microorganisms able to produce lipolytic activity can be found in different habitats, including oil-contaminated environments (Sirisha *et al.*, 2010; Lee *et al.*, 2015), seeds, wastes of vegetables oils (Thabet *et al.*, 2012), deteriorated foods, dairy products (Abdou, 2003). Among these, oil polluted marine ecosystems represent a large reservoir of organisms with unique biochemical traits. However these ecosystems are yet largely under explored reservoir of biodiversity with respect to industrially useful biocatalysts (Odeyemi, 2013). These ecosystems are exposed to extreme environmental conditions (Kennedy *et al.*, 2011; Lopez-Lopez *et al.*, 2014). In particular, microorganisms from intertidal zone are exposed to rapid and repeated fluctuation in temperature, light and salinity. Survival under these conditions must have necessitated the development of quite novel microbial cellular biochemistry and metabolism, thereby ensuring that biome is likely to possess unique enzyme systems which could possibly match many industrial requirements (Dionisi *et al.*, 2012; McKew *et al.*, 2011). Moreover, the coastal marine water is subject to contamination by organic pollutants like hydrocarbon and oil from a variety of sources (Islam and Tanaka, 2004). The presence of these kinds of contaminants makes this environment a very good source to find microorganisms capable of degrading lipids by producing lipase enzymes.
In this study, a bioprospecting by culture-dependent approach was performed to isolate novel lipase producing microorganisms from polluted coastal marine water in the Mediterranean Sea. The most promising identified strain was characterised and the level of extracellular lipase production was optimised.

Results

Isolation of lipase producing organisms
As reported by many authors, microorganisms able to produce lipolytic activity can be found in different habitats, including soils contaminated by oils, seeds, deteriorated food, wastes of vegetables oils, dairies, compost heaps, cold tips and

hot springs (Andualema and Gessesse 2012, Sharma *et al.*, 2001). In October, 2012 a 1.2 L sea water sample was collected from “Darsena vecchia” of Putueoli in Naples, Italy.
The “Darsena vecchia” of Putueoli, also called Fisherman’s mole (Figure 1), is a small backwater zone with no tidal current. This body of water is enclosed by land with a very small mouth, affording access to Putueoli harbor where a high volume of small boat traffic is present. The depth of this area ranges between 20 cm to 10 m.

Figure 1: Water sampling site in the fisherman’s mole in Putueoli, Naples, Italy.

Since in natural environments mixed population composed of a multitude of different strains and species exists, it is mandatory to set up a proper enrichment culture method.
The principle of enrichment culture is to provide growth conditions favorable for the organisms of interest and as unfavorable as possible for competing organisms. With the aim to isolate extracellular lipase producing microorganisms, enrichment culture method was applied. The water microbial community was first inoculated in two different rich basal media (LB and YEPD) and incubated at two different temperature (15°C and 28°C). Once microbial communities reached a high enough density, an aliquot of these crude cultures was inoculated in more diluted medium and in the presence of olive oil as lipase production inducer. This procedure was repeated several times progressively diluting the nutrient at each step up to use olive oil (1% in water) as the only carbon and energy source in the medium. With this method microorganisms secreting extracellular activities enabling them to profit of olive oil as carbon sources selected. Then a subsequent screening on chromogenic plates to identify microorganisms able to secrete true lipases was performed (Figure 2).

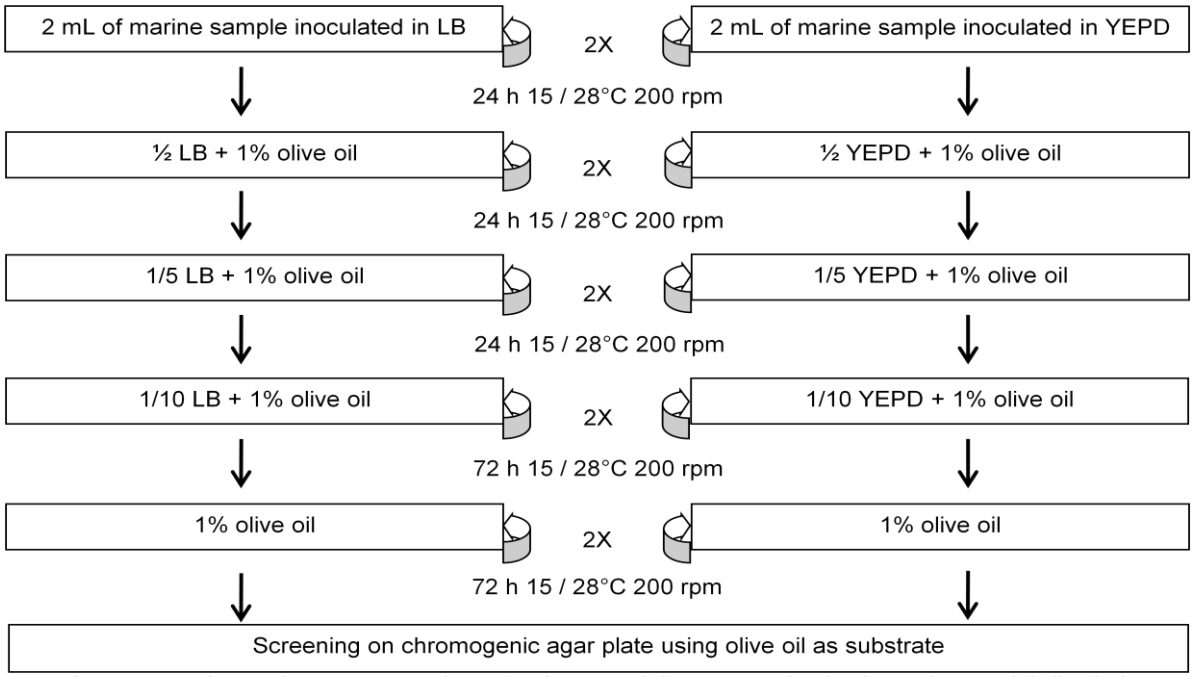

Figure 2: Schematic representation of culture enrichment method adopted to enrich lipolytic microorganisms.

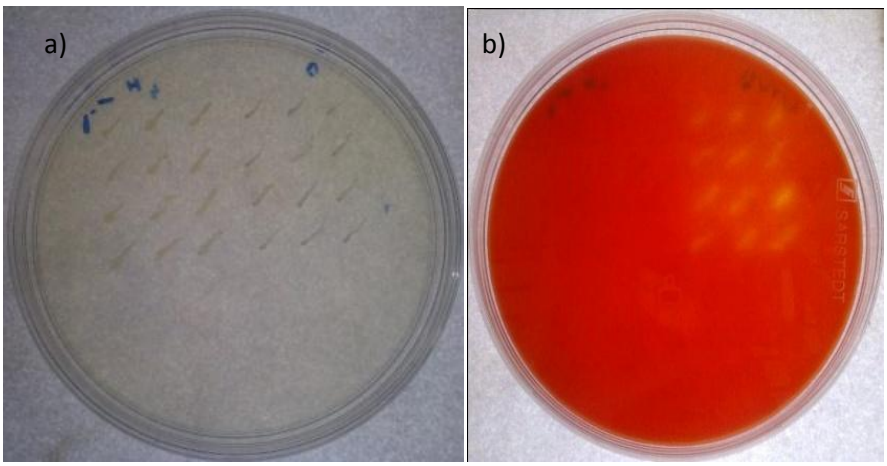

Figure 3: Colony growth on nutrient and chromogenic agar plate.

In detail, the four enriched microbial communities (corresponding to the two starting different media incubated at the two different temperatures) were processed for selection of lipolytic activity secreting microorganisms using a qualitative screening. Among all species grown in the four conditions, only colonies initially grown on YEPD at 28°C are able to produce activity halos on chromogenic phenol red-olive oil plates (Figure 3). With this procedure, 12 discrete "colonies" able to give a color change on the chromogenic agar plate were obtained. Two strains (labeled as C6 and D6) showing the highest lipolytic activity based on halos diameter were selected and picked out for further study. These two strains were grown for 7 days in a minimal liquid media added with olive oil ($1\%_{v/v}$) and lipase activity was monitored. The C6 strain reached a maximum activity of 90 U/L, while the D6 strain reached a maximum activity of 150 U/L after four growth days (Figure 4).

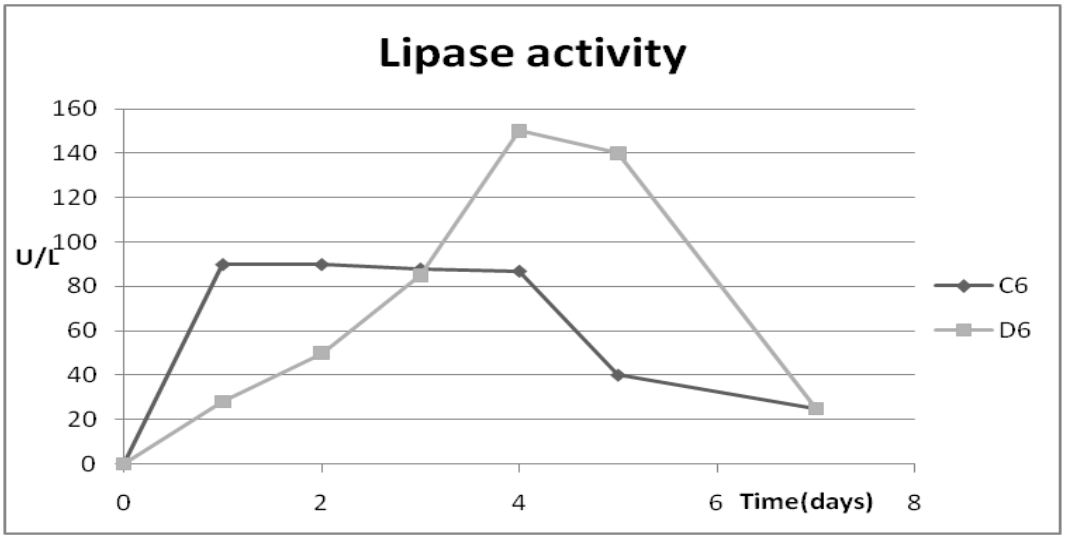

Figure 4: Time course analysis of lipase activity production by the two isolate. Experiments were performed in triplicate. Standard deviation <15%.

Organisms' identification and characterisation

The C6 colonies appear on YEPD media as milk colored to yellowish. They have a rapid growth, reaching maturity in 72 hours. A microscopic analysis revealed that C6 shows an ellipsoidal structure and occurs singly. The major microscopic feature is the lack of arthroconidia. According to the morphological analysis cell, it seems to belong to fungi kingdom. Moreover, image magnification revealed the presence of also small bacteria (Figure 5a-b). Regardless any effort we tried, it was not possible to separate the two microorganisms and to univocally indentify the organism responsible of the produced activity. Molecular method based on the analysis of polymorphism in the non-coding ITS (Internal Transcribed Spacers) DNA region (Sabate *et al.*, 2002; Cadez *et al.*, 2002) and in the 16S rDNA was applied for identification. The nucleotide sequence obtained from the sequencing of the ITS region was compared to sequences deposited in the GenBank nucleotide data library at the National Centre of Biotechnology using the BLAST software. The bacterium and yeast were respectively identified as belonging to *Microbacteria* sp. and *Pichia* sp..

A morphological analysis of the D6 isolate was also performed. The colony rapidly expanding, is white, flat, with cottony aerial mycelium and somewhat fimbriate margin on a rich agar plate medium. 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 appear to belong to fungi kingdom (Figure 5c-5d).

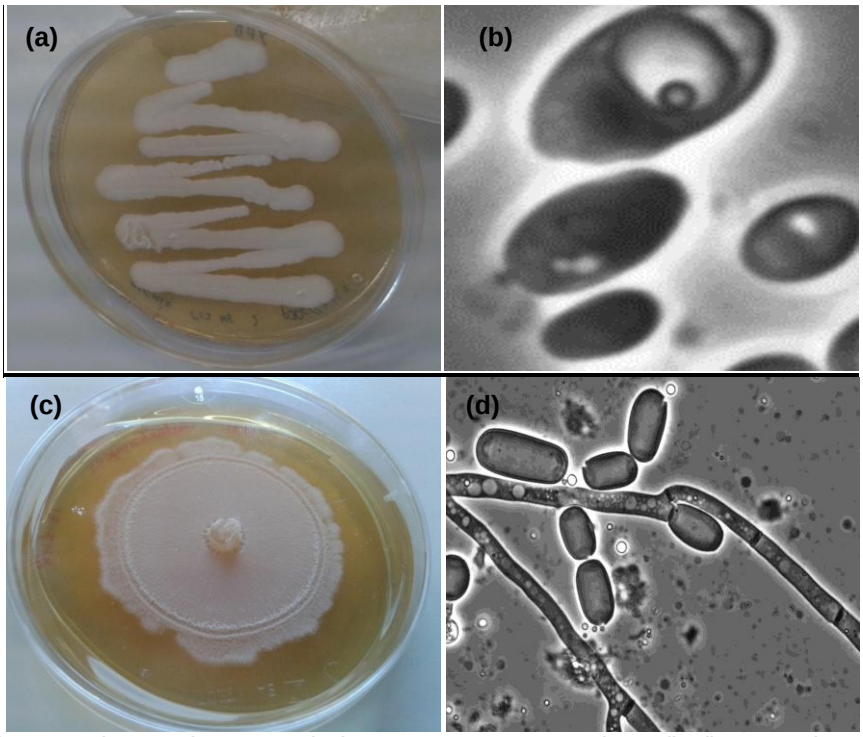

Figure 5: (a-c) C6 and D6 colony morphology on YEPD agar plate, (b-d) C6 and D6 michrography (100-fold amplification).

In addition, this isolate was analysed through sequencing of ITS region and Data bank interrogation. The isolate displays an identity of 97% with *Geotrichum candidum/ Galactomyces geotrichum* strains. The clustering of the new isolate strain with others belonging to *Galactomyces* and *Geotrichum* spp is reported in Figure 6, where it is possible observe that the D6 strain is strictly related to *G. candidum* strain DBMy703.

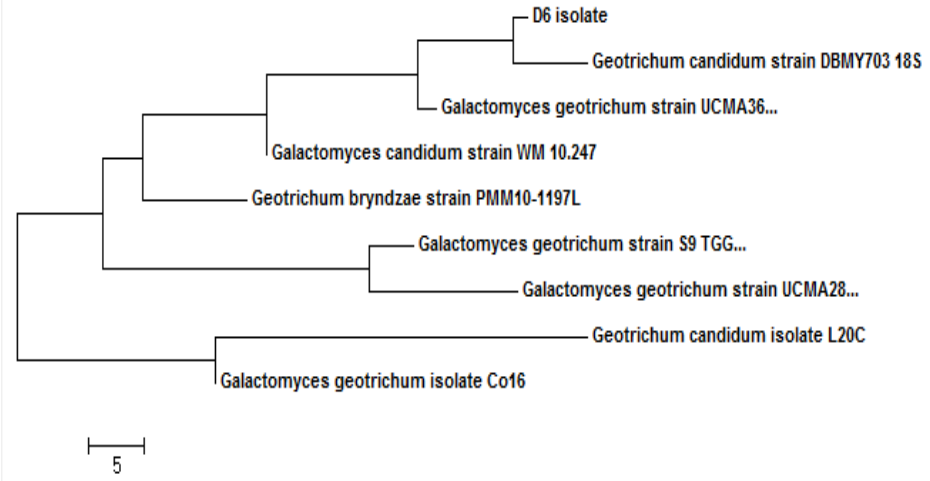

Figure 6: Phylogenetic tree created by neighbor joining clustering method in Mega 4.0 (Tamura *et al.*, 2007).

This is the first report of *G. candidum* isolation from polluted marine environment, since in previous works, *G. candidum* strains have been isolated from dairy products (Marcellino *et al.,* 2001), and soil (Kim *et al.,* 1995).
The D6 strain, identified as a new strain of *G. candidum*, was selected as the lipase producer of interest. Even if other studies on lipolytic activity from this yeast are

already present in the scientific literature, *G. candidum* shows a large genetic variability, thus confirming the interest in characterizing lipase activity production from the selected strain.

Growth medium formulation to optimize lipase production

As reported by many authors lipase production is affected by growth medium composition like carbon and nitrogen sources (Ghanem *et al.*, 2000), presence of activators and inhibitors, temperature, pH, oxygen tension and, in particular, by the presence of a lipidic carbon source, such as oils, fatty acids, glycerol or tweens (Gupta *et al.*, 2004; Treichel *et al.*, 2009).

During the optimization, experiments were performed in sequential step by incorporating at any step all the previously optimized parameters. Triacylglycerols and fatty acids have been reported to induce lipase secretion by various microorganisms. In this study, the effect of the addition of olive and palm oil to the basal growth media was studied. The two tested oils were found to induce lipase production, reaching the same level of enzyme activity. The maximum activity reached was 150 U/L after four growth days (Figure 7). Considering the composition in triacylglycerols of added oils (Table 1), palm oil was selected as inducer. Thus, the effect of $CaCl_2$ addition was studied, and an inductive effect on lipase production from the new isolated *G. candidum* was observed (Figure 7). It has already been reported that calcium can have a stabilizing effect on the lipase enzymes (Mohamed El Khattabi *et al.,* 2003). The effect of nitrogen source was evaluated increasing the concentration of yeast extract in the medium. As can be seen in the Figure 7 the addition of a high concentration of yeast extract to growth medium has a great effect on lipolytic activity production,enabling to reach a maximum activity of 6,000 U/L.

Table 1: Saturated fatty acid composition of olive and palm oils (%) (Zambiazi *et al.*, 2007).

	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{20:0}$	$C_{22:0}$	$C_{24:0}$	**Total**
Olive oil	-	-	10,84	0,14	3,59	0,50	0,15	0,06	15,28
Palm oil	-	1,12	42,7	0,11	4,55	0,39	0,58	0,06	49,45

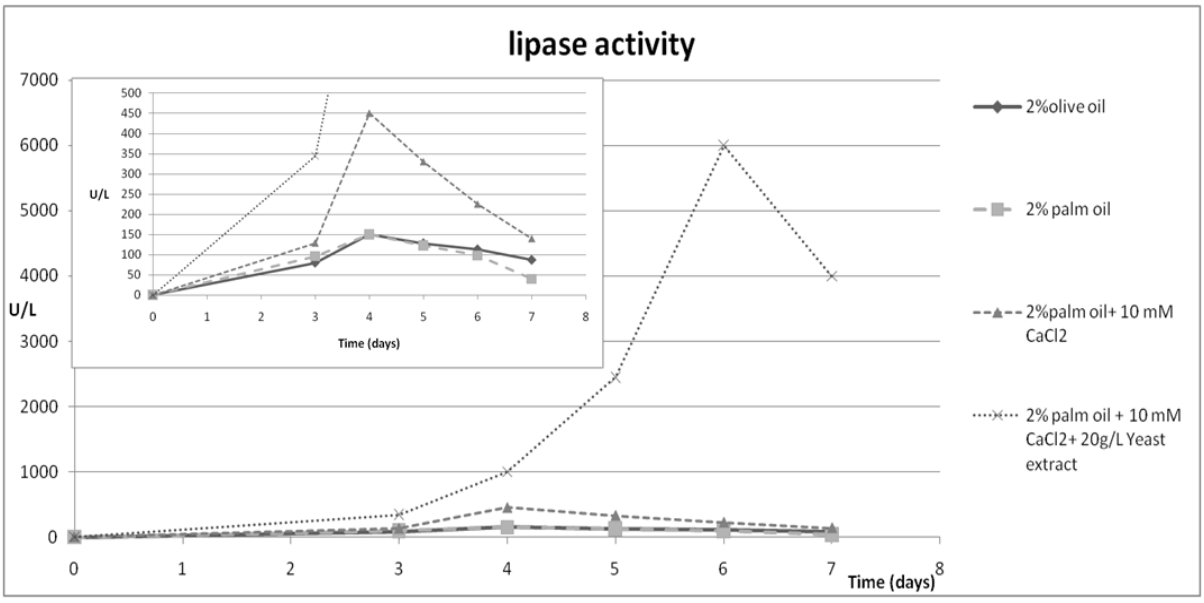

Figure 7: Time course analysis of lipase activity production by *G. candidum* in different growth media. All experiments were performed in triplicate. Standard deviation <15%.

Conclusions

Bioprospecting may be defined as the search for new bio-systems from natural environments. In particular, screening for marine microbial natural products continues to represent an important route for the discovery of novel bioactive chemicals.
Combination of an oil-contaminated seawater, culture enrichment of organisms able to use olive oil as the only energy source and an *ad hoc* screening strategy led to the isolation of 12 microorganisms able to hydrolyze triglycerides present in the olive oil. According to the halo diameters produced on chromogenic plates, two colonies (C6 and D6) have been selected, morphologically characterized and identified as being a coculture of *Microbacteria* sp. and *Pichia* sp., and a pure culture of *G. candidum*. This latter was the most interesting clone secreting lipase activity. The lipolytic activity produced by this yeast like fungus was significantly increased optimizing the growth medium formulation.

Material and method

Water sampling and culture enrichment

A marine water sample of 1.2 L was collected from the "Darsena Vecchia" of Pozzuoli, Naples, Italy. It was filtered using a 0.22 µm pore size membrane. The retained fraction was resuspended in sterile water (16 mL) and used as inoculum. 4mL of suspension was added into a 100 mL Erlenmeyer flask containing 20 mL of growth media: LB and YEPD (Sambrock and Russel, 2001). The cultures were incubated at 15 and 28ºC on a rotary shaker at 200 rpm for 24 h. After that, a 1mL aliquot was inoculated in two times diluted broth in the presence of 10% olive oil. The cultures were incubated at 15 and 28ºC on a rotary shaker at 200 rpm for 24 h. This procedure was sequentially repeated in five and ten times diluted broth. An aliquot from culture in 10 times diluted broth was inocultaded in the presence of 10% olive oil. Each step was repeated two times.

Chromogenic agar plate

Chromogenic plates contain 1% olive oil, 1% phenol red, 1% $CaCl_2$, 1.5% agar. The pH (7.0) was adjusted by adding NaOH 1M. Plates were incubated at 15°C and 28°C. A yellowish halo indicate the presence of lipolytic activity.

Genomic DNA extraction

The genomic DNA was extracted according "Smash and Grab" protocol (Rose *et al.*, 1999). Proteins were removed by Phenol/Chloroform/Isoamyl Alcohol precipitation (Ausubel *et al.*, 1995). The DNA in the top layer was precipitated by adding 100% cold ethanol and centrifuged for 2 min at maximum speed. The DNA pellet was resuspended with 400 µl TE plus 30 µg RNAse A and incubated for 30 min at 37°C. 10 µl of 4M ammonium acetate and 1 mL 100% ethanol were added at the sample and it was incubated in ice for 10 min. The sample was centrifuged 5 min at maximum speed and DNA was resuspended in bidistilled H_2O (30-40 µl). The DNA qualitity was checked through electrophoresis on 0.8% agarose gel.

PCR amplification

PCR was carried out in 50 µL reaction volumes using 100 ng of genomic DNA, 2.0 mM $MgCl_2$, 0.5 µM each primer (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2 5'-GC TGCGTTCTTCATCGATGC-3') 50 mM Tris (pH 8.3), 500 µg/ml of bovine serum albumin, 2.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 250 µM and 0.5 U of *Taq* DNA polymerase. 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., Milan, Italy) and sequenced (Primm srl, Milan, Italy).

BLAST analysis

The sequences were aligned with those 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).

Lipase assay

Lipase activity was determined spectrophotometrically 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 the supernatant previously obtained from the broth culture centifugation and 20 µl of 10mM the *p*-nitrophenyl decanoate (ε_{405}= 3,390 $M^{-1}cm^{-1}$). The activity was assayed by detecting the product, *p*-nitrophenol, spectrophotometrically at 405 nm. One unit of lipase activity was defined in International Units. All measurements were carried out in triplicate.

Growth conditions and Medium composition

Four types of media with varying composition were studied. The production medium A comprised of $1gL^{-1}$ peptone, $2gL^{-1}$ glucose, 0.5 gL^{-1} yeast extract (YE), 2% olive oil. The medium B had 2% palm oil which replaced olive oil in medium A. Medium C had 10 mM $CaCl_2$ added to medium B and medium D had 20 g/L YE added to medium C. All pre-culture was prepared as follow: two 6mm disks of fungal mycelium, excised from agar plates, were transferred to 100 mL Erlenmeyer flasks containing 20mL of basal medium (broth A without olive oil). The flasks were maintained in continuous agitation at 150 rpm and 28°C. The culture culture were carried out in 500mL Erlenmeyer flasks with 90 mL of culture broth and 10 mL of an overnight pre-culture. The flasks were incubated at 28ºC with a constant shaking at 150rpm for 8 days.

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Chapter 2

Pleurotus ostreatus genome mining: searching for new lipases

Abstract

The white-rot fungus *P. ostreatus* has a high potential in biotechnological applications, especially for its oxidoreductive enzymes. This is the first study where digital data of gene and protein sequences from *P. ostreatus* have been analysed to search for lipolytic enzymes. A set of 70 genes was found in the annotated genome of *P. ostreatus* PC15 v2.0 using “lipase” as entry. Considering that, lipase expression is influenced by the presence of inducers in the growth medium, different growth condition have been investigated, selecting the most promising inducers. The extracellular lipases produced in the selected conditions have been identified. Among these, a potentially bifunctional enzyme, called *PleoLip 241*, has been selected and the coding gene has been synthesised and expressed, under the control of a constitutive promoter, in th yeast *P. pastoris*.

Introduction

In the post-genomic era, digital data of gene and protein sequences are abundant. A lot of microbial genome information have been assembled and released in public databases. Based on amminoacidic sequence analyses and comparison, many of these sequences have been classified according to their putative function. The availability of these data make the genome sequence-based approach an efficient method for retrieving novel enzymes.
The Department of Energy Joint Genome Institute (JGI, Walnut Creek, California, USA) is one of the leader institutes in generating genome sequences of plants, fungi, microbes and metagenomes. From 2004, the genomes of many basidiomycetes fungi have been sequenced at the Joint Genome Institute (http://img.jgi.doe.gov/pub/main.cgi/). Among these, the genome of *P. ostreatus* has been sequenced [http://genome.jgi-psf.org/PleosPC15_1/PleosPC15_1. home.htmL]. The complete *P. ostreatus* genome contains 35 Mbp organized in 11 chromosomes, and two different haploid genomes have been individually sequenced (Larraya *et al.*, 2000). *P. ostreatus* (Jacq.: Fr) Kumm. (Dikarya, Basidiomycota, Agaricomycotina, Agaricales) is a white rot fungus, known for its ability to degrade lignocellulosic residues due to its enzymatic oxidative system. The white-rot basidiomycetes *P. ostreatus* deserves attention for many biotechnological applications. Indeed, this fungus has been widely exploited for its abilities in bioremediation processes (Olivieri *et al.*, 2012; Gullotto *et al.*, 2012; Faraco *et al.*, 2009; Karas *et al.*, 2011) and is an invaluable source of oxidases. To date, most of the studies about enzymes in *P. ostreatus* have been focused on ligninolytic enzymes. In literature, there are no studies on lipase or lipolytic enzymes produced by *P. ostreatus* and very few lipolytic activities have been studied in other *Pleurotus* sp. (Linke *et al.*, 2013; Zelena *et al.*, 2009).
In this study, the available genomic and transcriptomics resources were used to search for true lipases from *P. ostreatus* trough an *in silico* analysis approach associated to a screening approach. Five out of seventy different putative lipases have been found expressed and one of this has been selected, and heterologously expressed in *P. pastoris*.

Results

P. ostreatus genome's analysis and screening of growth conditions

A set of 70 genes were found in the annotated genome of *P. ostreatus* PC15 v2.0 when the word “lipase” was used as entry (http://genome.jgi-psf.org/pages/search-for-genes.jsf?organism=PleosPC15_2). Literature data show that the production of lipases is rarely constitutive and generally influenced by growth conditions and by the presence of inducers (Gupta *et al.*, 2004; Sharma *et al.*, 2001; Thakur, 2012). For this reason, different concentrations of olive oil, Olive Mill Wastewater (OMW), glycerol and glucose were added to the broth generally used for *P. ostreatus* growth (Table 1). In order to quickly select the condition/s leading to the secretion of “true” lipases, a screening on chromogenic plate, using olive oil as substrate, was performed (Singh *et al.*, 2006). Thanks to this screening, several conditions allowing the production of an activity halo were identified as "producing" (Table 1). All inducers but glycerol led to the secretion of active lipases. Induction is sensitive to inducers concentration. The screening lead to the identification of 1%$_{v/v}$ olive oil, 5%$_{v/v}$ and 10%$_{v/v}$ OMW, 10g/L glucose as inducers of extracellular lipolytic activity in *P. ostreatus*.

Table 1: Growth media conditions for extracellular lipase induction. ✓ indicate the conditions that induce the production of extracellular lipase after five growth days.

Growth media	Activity halo in five growth days
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% $_{v/v}$ OMW	–
PDY+ 5% $_{v/v}$ OMW	✓
PDY+ 10% $_{v/v}$ OMW	✓
PDY+ 0.1% $_{v/v}$ glycerol	–
PDY+ 0.5% $_{v/v}$ glycerol	–
PDY+ 1% $_{v/v}$ glycerol	–
PDY+ 5g/L glucose	–
PDY+ 7.5g/L glucose	–
PDY+ 10g/L glucose	✓

Time course of lipase production in the presence of selected inducers

OMW and glucose were better inducer than olive oil and time course analysis of extracellular lipolytic enzyme production was conducted during *P. ostreatus*

submerged culture in the presence of the minimum inducer concentrations (Figure1). Production profiles in the presence of 5%$_{v/v}$ OMW and 10g/L glucose are comparable. The maximum lipase activity (about 28 U/L) was reached after five growth days. Both these conditions, giving higher lipase activity production, have been selected for further investigations.

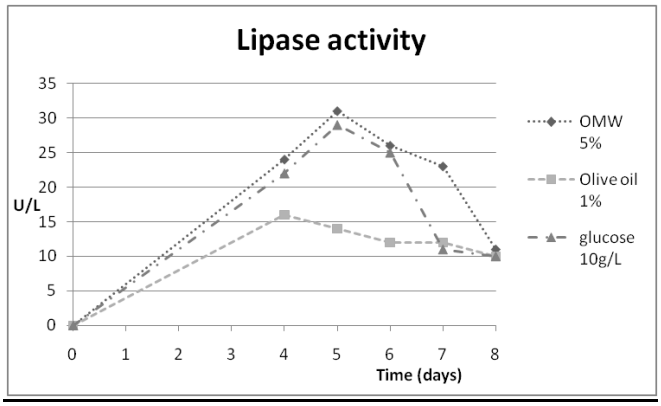

Figure1: Time course activity of extracellular lipase produced by *P.ostreatus* in the presence of different inducer added to PDY broth. All experiments have been conducted in triplicate. Standard deviation< 15%.

Lipase identification

In order to verify the number of different lipases secreted in the selected conditions, supernatant of *P. ostreatus* culture was fractionated on denaturing PAGE and stained for lipase activity. The pattern of activity bands obtained in the two growth conditions is showed in figure 2. In the presence of OMW only a band of activity was detected, while two bands of activity were detected in the presence of glucose as inductor. With the aim to correlate lipolytic enzyme(s) with encoding gene(s), the active proteins were excised from gel, hydrolysed with tripsin and the obtained peptide were subjected to protein identification by MS/MS ion search on a MASCOT server against the annotated *P. ostreatus* genome (http://genome.jgi-psf.org/PleosPC15_2). Identified proteins are reported in Table 2. Two of the proteins (1091241 and 1060369) are expressed in both condition.

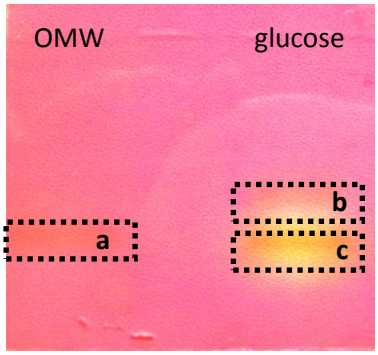

Figure 2: Zymography analysis of the extracellular protein of *P. ostreatus* grown in the presence of OMW and glucose added to PDY broth. Lipase catalytic activity was revealed by using olive oil as substrate and phenol red as pH indicator.

Table 2: Identified proteins by MS/MS analysis.

Band	Protein ID	Nominal mass	Blast	
			Identities	Description
a	**1091241**	59,574	97%	feruloyl esterase [*Pleurotus eryngii*]
	1060369	58,926	53%	feruloyl esterase [*Pleurotus eryngii*]
	1032023	47,754	61%	Carboxylesterase [*Heterobasidion irregulare* TC 32-1]
b	1091241	18,322	60%	Esterase/lipase/thioesterase [*Heterobasidion irregulare* TC 32-1]
	170071	57,706	51%	cephalosporin esterase [*Moniliophthora roreri* MCA 2997]
c	**1091241**	59,574	97%	feruloyl esterase [*Pleurotus eryngii*]
	1060369	58,926	53%	feruloyl esterase [*Pleurotus eryngii*]

A Multiple sequence alignment of the two proteins was performed by CLUSTALW software, and a phylogenetic relationship with other proteins was outlined by MEGA4.0 software (Tamura *et al.*, 2007). The phylogenetic tree for the aminoacid sequences of PleoLip241 and PleoLip369 and other similar proteins is shown in Figure 3.

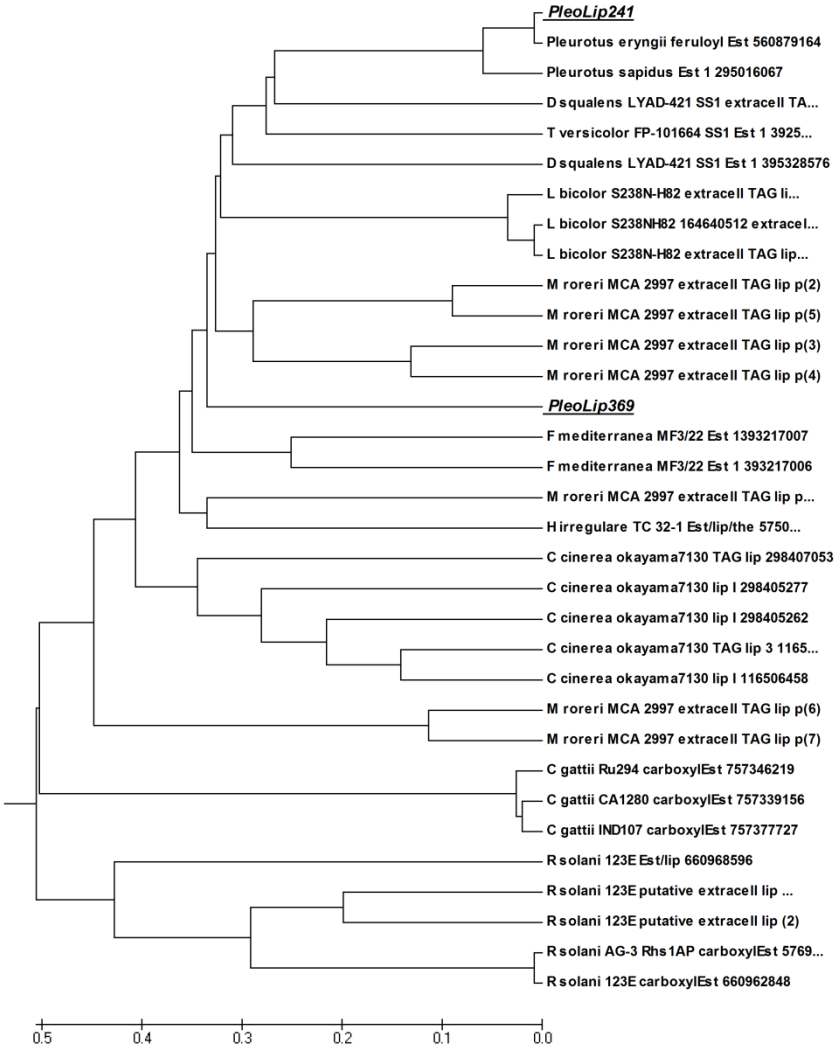

Figure 3: Neighbour Joining tree of lipase amino-acid sequences. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pair wise deletion option). Phylogenetic analyses were conducted in MEGA4. *P. ostreatus* lipases are highlighted.

Comparison of the aminoacid sequence of PleoLip241 with similar sequences from different organisms indicated a percentage identities of 97% with a feruloyl esterase from *Pleurotus eryngii*. The high percentage of similarity of PleoLip241 with a feruloyl esterase, indicate the possibility of PleoLip241 to work as a bifunctional enzyme.
It is known that proteins with similar aminoacid sequences have a tendency to adopt similar 3D structures, so it became possible to predict the 3D structure of the putative lipase PleoLip241. An acetylcholinesterase (c4bdtA) showing the highest scoring homology (100% of confidence for 92% of the PleoLip 241 sequence) has been used as template to model the 3D structure of the studied lipase through The Phyre 2 server (Kelley and Sternberg, 2009). Based on the 3D structure the location of catalytic triade was deduced: S 207, E 338, H454.

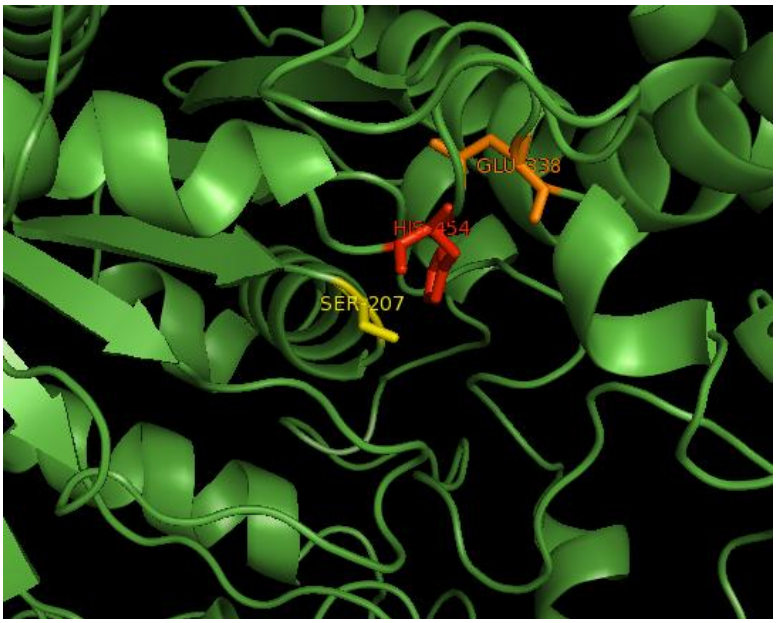

Figure 4: 3D model obtained by Phyre2 close up on the aminoacid of the catalytic triade, shown as stick. The picture has been elaborated with Pymol (DeLano, 2002).

Recombinant expression

The *P. pastoris* codon-optimised synthetic Pleolip241 gene, coding for 536 amino acid protein with a six Histidie residue at C-terminus, has a 47% G/C content, close to G /C content showed in *P. pastoris* (43%). The synthetic *PleoLip 241* was cloned into pJGGαKR under the control of the constitutive GAR promoter. The Pleolip241 gene was directionally cloned and fused to the coding sequence of the α-factor secretion signal, allowing thus secretion of the gene product. The cloning strategy involved the addition of a His tag to the C-terminus to facilitate purification steps. Transformation of electrocompetent *P. pastoris* cells with pJGGαKR/PleoLip241 allowed the selection of 5 out of 18 transformants. These clones were checked for lipase activity expression in small scale shake flasks cultivation and the selected clone was scaled up in 1L flask. Lipase activity production reached about 700 U/l after 6-days growth at 28°C (Figure 5), increasing the activity of more than 20 times, in comparison with production from the native strain.

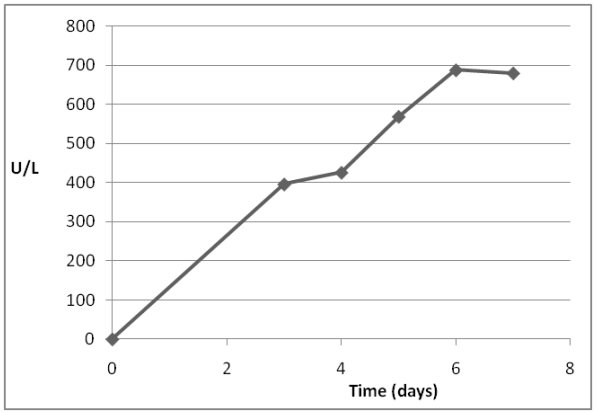

Figure 5: Time course of extracellular lipase production of the selected transformants. Experiments have been conducted in triplicate. Standard deviation <15%.

Conclusions

P. ostreatus has been widely studied for its ability to produce a wide range of enzyme, but no study reports lipase production by this fungus. This is the first study about production of extracellular lipases from *P. ostreatus*. Five putative extracellular lipases have been induced in different growth conditions. A phylogenetic analysis of two of the five lipases, expressed in two differen conditions, has been performed leading to the selection of PleoLip241 to be heterologously expressed. PleoLip241 was cloned and expressed in *P. pastoris*. The recombinant laccase was successfully secreted by yeast cells in an active form.

Matherial and methods

Fungal strains

The basidiomycetes fungus used in this study was a strain of the white rot fungus *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC MYA-2306) from ATCC, the Global Bioresource Centre.

Growth conditions

All pre-culture was prepared as follow: 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 flashs of 3'' at maximum rpm) and were inoculated, in a 1:10 ratio in PDY broth added with olive oil, glucose, Olive Mill Wastewater and glycerol, at different concentrations.

Chromogenic agar plates and detection of lipase activity

Chromogenic substrate plates were prepared according to Singh and coworkers (Singh *et al*., 2006), using 1% olive oil as substrate. Different amount of substrate were spotted onto a small disc of filter paper and placed on top of the chromogenic substrate plate. The plate were incubated a room temperature. The commercial Lipase (Sigma-Aldrich) was used as positive control.

Lipase assay

Lipase activity was determined spectrophotometrically 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 the supernatant previously obtained from the broth culture centifugation and 20 µl of 10mM the *p*-nitrophenyl decanoate (ε_{405}= 3,390 $M^{-1}cm^{-1}$). One unit of lipase activity was defined as International Units. All measurements were carried out in triplicate.

Semi-denaturating SDS–PAGE zymography

Polyacrylamide gel electrophoresis (PAGE) was performed at an 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 to the gel.

Gene identification

Active protein were excised from the gel and washed first with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubating the bands 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 bands 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 300–2400*m*/*z*), followed by MS/MS scans (mass range 100–2000*m*/*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).

Phylogenetic analysis

Phylogenetic analysis was carried out on PleoLip 241 and PleoLip 369. Sequences were aligned with ClustalW using default settings for multiple sequence alignments. Based on this alignment, a Neighbour Joining tree was constructed using MEGA 4.0 software (Tamura *et al.*, 2007).

Gene synthesis, and plasmid and strain 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 gene were digested with *BsaI* and then ligated. The ligation mixture was 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^{-1} of ampicillin antibiotic

The pJGGαKR /PleoLip241 vector was linearized with *BsiW*I, 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 in BMGY(13 gL^{-1} yeast nitrogen base with ammonium sulfate without amminoacids; 10 gL^{-1} yeast extract; 20 gL^{-1} peptone; 100 mM potassium phosphate, pH 6.0; $4x10^{-4}$ gL^{-1} biotin; 1% glycerol) liquid culture. The BMGY culture were incubated at 28°C under shaking condition. Lipase-producing transformants were identified by enzymatic assay.

Cultivation of Pichia pastoris

Recombinant cells from solid culture of pJGGαKR/PleoLip 241 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. Cells were grown on a rotary shaker (250 rpm) at 28°C.

Acknowledgements

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Chapter 3:

A laccase from the cold Psuedoalteromonas haloplanktis heterolougously expressed in E. coli and its application for dye synthesis

Abstract

The ability to oxidise different phenolic substrates as dye precusors resulting in the formation of different coloured polymeric molecules is being exploited for developing “green” enzymatic dyeing processes. Laccases can catalyse oxidative and coupling reactions in which coloured product are formed. In this study a bacterial laccase has been heterologously expressed in *E. coli* and partially purified by heat-treatment, resulting active in the soluble intracellular fraction. This new laccase has been found to be significantly active against the dye precursors 2,5 DABSA and resorcinol (1,3-hydroxyphenol).

Introduction

Dyes are widely used in textile, leather, plastic, pharmaceutical and cosmetic industries.
The use of biocatalytic processes to synthesize colorants could be an efficient way for dyeing at mild conditions, in terms of chemicals, pH and temperature. Since several groups described the ability and efficiency of laccases to catalyse the formation of coloured compounds, these enzymes now represent an attractive route towards dye synthesis (Shaw and Freeman, 2004; Bruyneel *et al.*, 2008) Laccases catalyse oxidation of colourless phenolic and aromatic compounds resulting in dimeric, oligomeric or polymeric colourful compounds. Laccases can catalyse three types of reactions in which coloured product are formed: *i)* direct oxidation of phenolic derivates, *ii)* mediated oxidation of phenolic and non-phenolic derivates and *iii)* coupling of reactive radicals (Polak *et al.*, 2012). In previous works a permanent colouration of cotton fabrics based on the oxidative coupling of catechol (1,2-hydroxyphenol) and the colourless 2,5-diaminobenzenesulfonic acid (2,5-DABSA) using a laccase has been demonstrated (Calafell *et al.*, 2007; Hadzhiyska *et al.* 2006, Fu *et al*., 2012) (Figure 1).

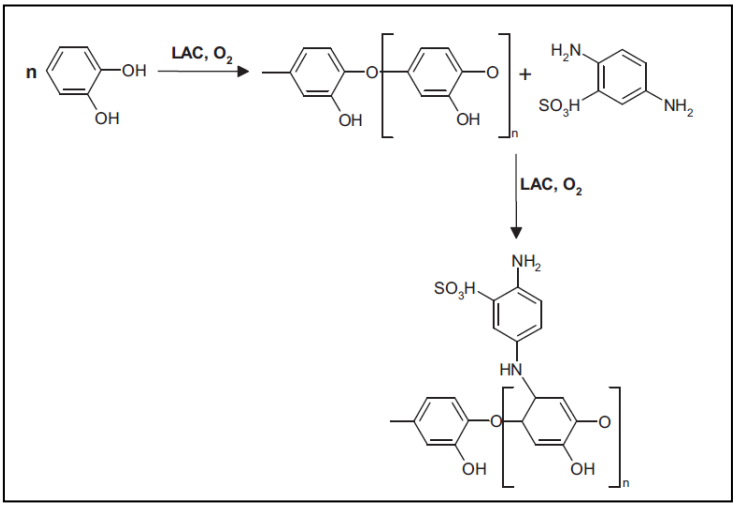

Figure 1: Reaction pathway for oxidative cross-coupling of 2,5-DABSA-cathecol mediated by laccase

In this study a bacterial laccase from the artic bacterium *P.haloplanktis* has been recombinantly expressed in *E.coli.* The oxidative potential of the recombinant laccase towards 2,5-DABSA and resorcinol (3-hydroxyphenol), has been evaluated.

Results

Laccase recombinant expression and production

An *in silico* analysis of the genome of *P. haloplanktis,*revealed the presence of a DNA sequence coding for a putative laccase-like protein. This annotated laccase coding gene was synthesized and heterologously expressed in *E.coli.* Two variants of the protein were expressed: with and without T7 tag at N-terminus.

Protein production was optimized as a function of growth medium composition, inducer concentration, time of induction, temperature after induction, and copper concentration for both variants.

When the recombinant strain was grown in TB and induced for 18 h with 0.5 mM IPTG at 20°C in the presence of 2mM $CuSO_4$, a predominant band corresponding to the expected size (58 kDa) was detected by SDS-PAGE in the cellular extracts expressing recombinant protein without tag (fig2). A comparable overexpression was not obtained when the protein was produced fused to the T7 tag (Figure 2). Even if it is known that the presence of a tag may provoke negative effects on the tertiary structure or biological activity of the fused protein (Rosano and Ceccarelli, 2014), it is yet unknown what is the effect of the tag on the fused protein expression.

The heterologously expressed Laccart is active against syringaldazyne, but it displays no activity against ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), a substrate generally oxidized by laccases. These results indicate that, when cloned without tag, the recombinant laccase is efficiently overexpressed in a soluble and active form.

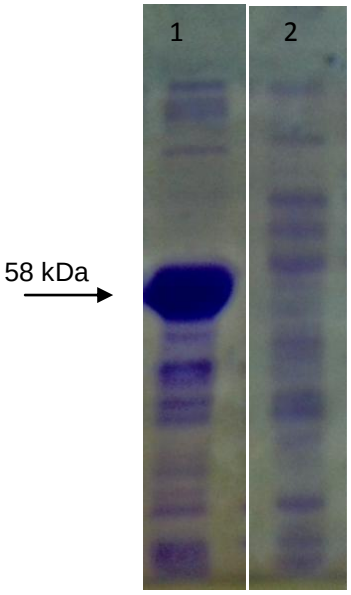

Figure 2: SDS-PAGE analysis of espresse Laccart in *E.coli* at 20°C.Lane 1: pet21a+/Laccart, intracellular soluble fraction Lane 2 pet21a+/T7Laccart, intracellular soluble fraction.

Several purification protocols of the soluble intracellular Laccart have been adopted, obtaining, in most of the cases, low purification yelds.

Thus, an alternative strategy of heat-treatment was used to purify Laccart. After an incubation at 60°C for 90 min,100% of the initial laccase activity was recovered with an increased specific activity. This treatment results in denaturation of most of *E. coli* proteins and is generally used to enrich heat resistant proteins. The heat-treatment has also been described for other bacterial laccases expressed in *E. coli* (Koschorreck *et al.*, 2008, Reiss *et al.*, 2011, Rosconi *et al.*, 2005). This treatment also indicate an unaspected heat-stability of the artic laccase at 60°C.

Laccart application

Laccases catalyse conversion of catechol to a highly reactive quinone specie which undergoes homo-molecular C-O-C coupling, and then non-enzymatic C-N coupling with 2,5-DABSA takes place through 1,4 nucleophilic addition of the latter to the quinoid rings (Calafell *et al.,* 2007).

The potentiality of the bacterial laccase Laccart, in the production of dyes, has been investigated. In particular the oxidative capability against resorcinol, a cheaper cathecol isomer. It is interesting to observe that the artic laccase studied in this work displays an oxidative capacity against 1,3 resorcinol comparable with that of other fungal and commercial laccases, and 10 times higher towards 2,5 DABSA (Table 1). It is reported that especially fungal laccases show a high oxidative potential, resulting interesting in the *de novo* synthesis of dyes (Bruyneel *et al.*, 2009; Polak *et al.*, 2012). This results let us to consider Laccart as a promising enzyme for developing eco-friendly dye synthesis processes.

Table 1: Oxidative ability of Laccart, POXA1b (Giardina *et al.*, 1999) and a commercial laccase against 2,5 DABSA and resorcinol

Enzyme	2,5 DABSA ΔAbs_{458}	RESORCINOL ΔAbs_{488}
Fungal Laccase POXA1b	2.39±0.16	0.24±0.04
Commercial Laccase	2.30±0.06	0.37±0.07
Laccart	**24.48±2.55**	**0.3±0.02**

Conclusions

We found that laccase from the Artic bacteria *P. haloplanktis* can be properly expressed in *E.coli*. This enzyme is stable at 60°C for 90 minutes. Moreover, this study demonstrates the feasibility of this laccase for dye synthesis.

Matherial and methods

Gene design and insert preparation

The *Laccart* gene was designed to optimise its codon usage for the expression in *E.coli* and synthesised by Geneart Lifetechnology (Germany). Six more ammminoacids, recognized by thrombin, were added to N-terminus. Two different strategies were used to clone Laccart gene in the expression vector: *i) SacI*/*Hind*III digestion to express a fusion protein with T7 tag *ii) NdeI*/*XhoI* digestion to express the protein without any tag (Figure 3). Laccart gene was ligated with plasmid pet21a+ digested with the same enzymes. The ligation mixture was 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 μgmL^{-1} of ampicillin antibiotic.

The pet21a+/Laccart vector was transformed into cells of E.coli competent BL21 (DE3) fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS strain. Positive clones were selected on LB agar plates supplemented with 100 $\mu g\ mL^{-1}$ of ampicillin antibiotic.

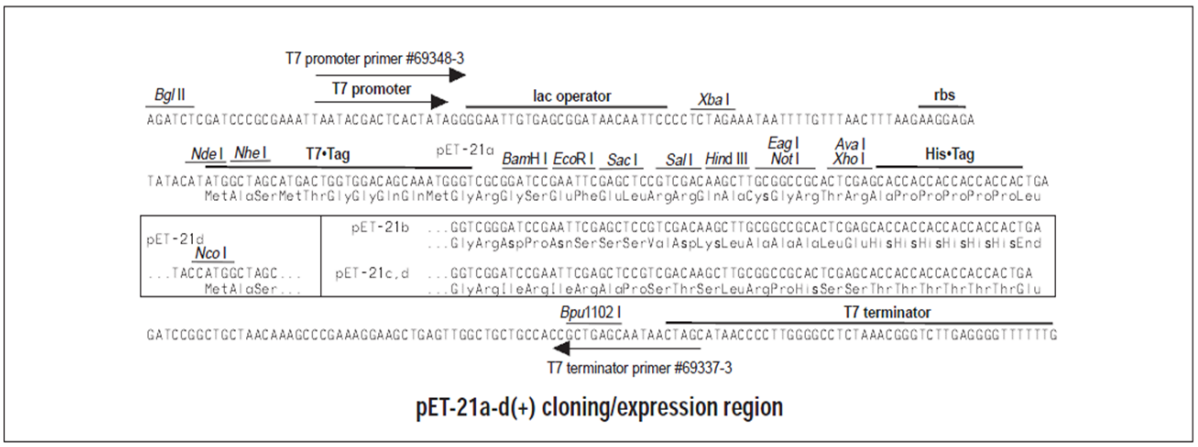

Figure 3: Cloning strategy

Expression of Laccart

Escherichia coli BL21(DE3) transformant containing plasmid pET21a+/Laccart was grown in LB and TB broth plus ampicillin (100 µg/mL), at 37°C. When the culture growth reached 0.4-0.8 OD_{600}/mL in LB, or 1.2-1.6 OD_{600}/mL in TB, the production was induced by adding IPTG (final concentration 0; 0.1; 0.3; 0.5; 0.7; 1 mM) and $CuSO_4$ (final concentration 200µM, 1 and 2 mM). Three different temperature were applied after induction (20°, 28° and 37°C). After 18 hours induction cells were harvested by centrifugation (6,000 rpm, 10 min, 4°C). The cell sediment was suspended in 50mM potassium Phosphate buffer pH 7+$CuSO_4$ 200µM and 0.1mM PMSF (phenylmethanesulfonylfluoride). Cells were disrupted in a French pressure cell (at 19,000 p.s.i.) followed by centrifugation (10,000 rpm, 20 min, 4 °C) to remove cell debris and membranes.

Chromatgrafic purification

The resulting soluble extract was loaded onto: *i)* an ion exchange DEAE FF column (bed volume 1 ml) equilibrated with Tris-HCl (20 mM, pH 7) + 0,1 mM $CuSO_4$. Elution was carried out with a linear step NaCl gradient (0–1M) in the same buffer. *ii)* an hydrophobic interaction Phenyl HP column equilibrated with 50 mM potassium phosphate pH 7 + 1M $(NH_4)_2SO_4$. Elution was carried out with a linear step $(NH_4)_2SO_4$. gradient (1-0 M). *iii)* an ionic exchange MonoQ column equilibrated with 50 mM potassium phosphate pH 7. Elution was carried out increasing the molarity of potassium phosphate pH 7 (50-500mM).

Heat-treatment

The soluble extract from *E. coli* was incubated at 60°C for 3h. Each 30 min an aliquots was assayed for laccase activity.

SDS-PAGE analysis

Bacterial cell suspension was mixed with the same volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The mixture was boiled for 5 min, and the samples were electrophoresed on 12% SDS-PAGE (Laemli 1970, . Proteins were then stained with Coomassie blue R250.

Laccase activity assay

The activity was measured by monitoring the oxidation of syringaldazine. The reaction mixture contained aliquots of the crude enzyme and in 0.1 M sodium phosphate buffer (pH 6.5) and 100 µM syringaldazine (ε_{525} = 65,000 $M^{-1}cm^{-1}$) in a total volume of 1 mL. The oxidation was monitored spettrofometrically at 525 nm. One unit of enzyme was defined as International Units.

Resorcinol oxidation

Laccase (0.5 U) were added to 1mL of 250mM resorcinol dissolved in Potassium Phosphate 50 mM pH7, incubated 10 min at 60°C. The oxidation was monitored spectrophotometrically at 488nm.

2,5 DABSA oxidation

Laccase (0.5 U) were added to 1mL of 25mM 2,5 DABSA dissolved in Potassium Phosphate 50 mM pH7, incubated 5 min at 60°C. The oxidation was monitored spectrophotometrically at 458nm.

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Chapter 4:

Metagenomic DNA from surface Mediterranean Sea water: a source of biocatalysts

Abstract

Marine ecosystems represent a large and yet under explored reservoir of industrially relevant bio-systems. The application of molecular genomics to marine microbes (both cultivable and uncultivable) has a substantial impact on the search for novel industrial enzymes. The aim of this study was to isolate new lipases and laccases from commercial harbour of Naples sea water (Mediterranean Sea), exploiting a metagenomic approach. Lipases and laccases are useful enzymes for a variety of applications. A metagenomic library of about 30,000 fosmid clones was constructed from DNA extracted from surface sea water. High throughput plate screening on 1% tributyrin agar resulted in the identification of 40 positive putative lipase coding clones, with an hit rate of 0.05%. The functional-screening on syringaldazyne, ABTS and Remazol Brilliant Blue R agar plate, and the sequence-based metagenomic did not lead to the identification of any clone putatively coding for laccases.

Introduction

Microbial marine ecosystem is typified by a great reservoir of diversity (Felczykowska *et al.*, 2012). Microbial inhabitants of these ecosystems are exposed and adapted to survive under harsh environmental conditions, such as high salinity, high hydrostatic pressure, reduction or absence of light. As a consequence enzymes produced by marine microorganisms often show features that make them suitable for many industrial processes (Trincone *et al.*, 2011; Dionisi *et al.,* 2012). However more than 99% of microorganisms within marine habitat are not readily cultivable by using pure-culture based approach (Amann *et al.*, 1995), thus their enzymes are not accessible. Metagenomic based approaches provide a solution to this problem by facilitating the identification of novel enzymes. As a fact, metagenomic based approach allows to gain insights into the genomes of uncultivable microorganisms. Metagenomic describes the functional and sequence based analysis of collective microbial genomes contained in an environmental sample. Screening of genes, based on a selected function or on sequence identity, have led to the identification of numerous distinct enzymes from metagenome libraries of various natural ans extreme environments such as soil, water, sediment, and extreme environments (Handelsman, 2004; Lorenz and Eck, 2005; Schmeisser *et al.,* 2007). In the present study, the screening of metagenomic DNA from superficial sea water samples was used to investigate the presence of two classes of potentially novel biocatalysts: laccases and lipolytic enzyme.

To date, almost all studied laccases have been isolated by a culture-based approach, even if a gene encoding a multicopper oxidase with laccase activity has been identified trough an activity based functional screening from a metagenomic library from mangrove soil (Ye *et al.*, 2010) and a a new bacterial laccase gene has been obtained from a marine microbial metagenome of the South China Sea by using a sequence screening strategy (Fang *et al.*, 2011).

Actually, many lipase producing microorganisms have been isolated from different habitats, such as oil contaminated environment and wastes. Moreover, many genes encoding metagenomic lipases have also been identified in metagenomic libraries constructed from soils, pond and lake water, solfataric field, artic and antartic region, marine sponge (Berlemont *et al.*, 2011, Selvin *et al.*, 2012, Nacke *et al.*, 2011, Khan *et al.*, 2013, Jeon *et al.*, 2009).

Results

Metagenomic library

To explore the untapped esterases/lipases and laccases in intertidial marine environment, metagenomic approach was applied to the samples from superficial sea water collected in a contaminated area in the commercial harbour of Naples (Mediterranean sea) (Figure1). Contaminated sea water was chosen, as source of environmental DNA because it should lead to a high number of salt-tolerant enzymatic active clones in the metagenomic library.

A metagenomic library consisting of 30,000 fosmid clones was constructed transforming *E. coli*. Each clones contained an insert of approximately 40Kb, thus total amount of genetic information covered in this library is approximately 1200 Mb.

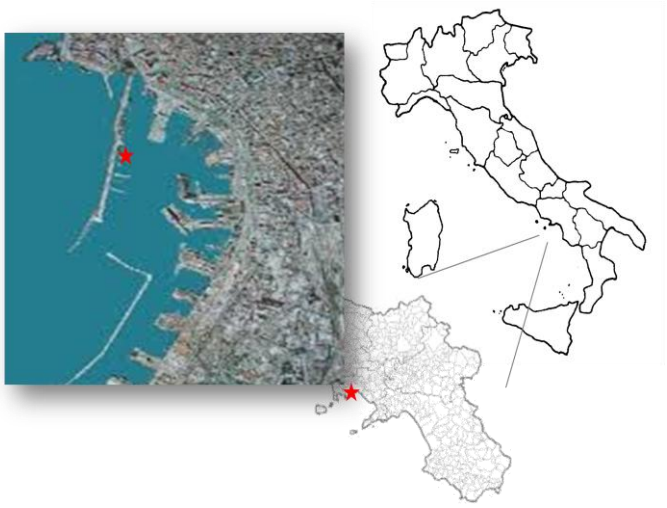

Figure1: Water sampling site in the commercial harbor of Naples, Naples, Italy.

Lipolytic enzymes

To isolate lipase coding genes from the metagenomic library, a function-based method was adopted. This strategy gives aid to discover novel full-length lipolityc genes. The functional screening to detect the fosmid clones exhibiting lipolytic activity was performed on agar plates containing 1% tributyrin (TBN). The hydrolysis of tributyrin causes the formation of an halo. This functional screening resulted in the initial identification of 40 positive clones. The candidate clones were re-streaked on 1% trybutyrin agar plates to confirm their hydrolytic capacity (Figure2). Three out of the 40 producing clones demonstrated significantly higher activity compared with the others and were selected for further characterisation. In order to identify the putative lipase gene(s) the fosmids were extracted from each clone and subsequently subjected to sequence analysis (sequencing in progress). In this study the hit rate for lipolytic clones marine metagenomic library is 0.05%. This value is in accordance with values reported in other studies. As a fact, when the enrichment step prior to DNA extraction are not applied, hit rate of lipolytic clones of metagenomic libraries generally ranges between 0.001 and 0.081% (Liaw *et al.*, 2010).

Figure 2: Identification of lipolytic clones that formed a hydrolysis halo in the tributyrin plate assay.

Laccases

An high throughput function-driven screening method to detect the fosmid clones exhibiting laccase activity was performed on agar plates containing syringaldazine (SNG) or ABTS or Remazol brilliant Blue R, as substrate. These functional screenings did not lead to the identification of laccase activities. The absence of laccase activity might be due to the absence of potential laccase genes in the library. Recombinant proteins can aggregate in misfolded and thus biologically inactive form (Villaverde and Carrio 2003) in dependence to the growth rates, culture temperature, and doses of gene expression inductor (Sorensen and Mortensen 2005). Moreover, bacterial laccases are generally intracellular. To overcome the inefficiency of screening to detect intracellular activity the clones cellular membrane was permeabilized by adding chlorophorm, and Tween 80 to plates after 24h of cellular growth. Neither in this case any activity was detected (Figure 3), whereas a positive control displays an activity halo.

To be sure that the absence of activity was due to the absence of laccase coding genes, a sequence-based strategy was also applied. The screening for laccase DNA fragments of laccase genes, was performed by amplifying by PCR based on conserved signature sequences in bacterial laccases (Ausec *et al.*, 2011; Fang *et al.*, 2013). These experiments confirm the absence of laccase coding genes in the treated metagenomic DNA sample. The presence in literature of only few new laccases isolated from marine bacteria and the enormous diversity of bacterial genes for laccase-like enzymes, as revealed by bionformatic analysis (Ausec *et al.*, 2011) demonstrate that the identification of this enzyme is quite difficult.

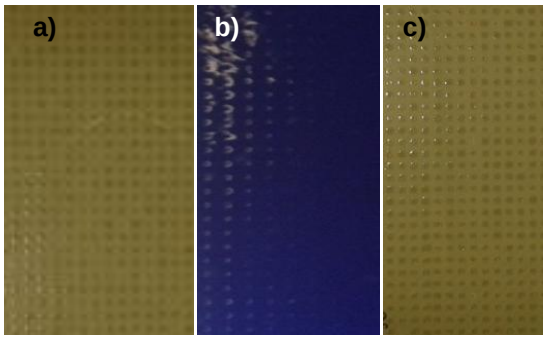

Figure 3: Metagenomic screening for laccase activity a) ABTS agar plate b) Remazol Brilliant Blue c) SNG agar plate

Conclusions

Lipases and laccases are versatile industrial enzymes. There is yet an interest in searching for new biocatalysts with unique properties with respect to specificity, stability, temperature and pH dependence. In the present study, a metagenomic library was constructed for the screening of lipase and laccase coding genes in the surface sea water metagenome. The study led to the identification of new lipase activities that could represent potential novel enzymes.

Materials and methods

Seawater sampling and Metagenomic Dna extraction

Seawater sample was aseptically collected from the harbour of Naples, Italy at the end of June 2014. The sampling method is the following: (*i*) collection of 30L of surface seawater in sterile plastic bottles, (*ii*) prefiltering through Watman paper disks, (*iii*) filtering through a 0.22 µm Sterivex cartridge (Millipore). One filter was used for 2L of water according to manufacturing specific. The metagenomic DNA was extracted according to Neufeld and co-workers (Neufeld *et al.*, 2007) and stored at -80°C. DNA was analysed by gel electrophoresis and quantified using a spectrophotometer (Nanor.drop ND-100 (NanoDrop, Wilmington)).

Metagenomic Clone Library Construction

The metagenomic DNA extracted from seawater sample (mDNA) was fractionated by pulse-field gel electrophoresis. The mDNA was mixed with loading dye, warmed at 55°C and loaded on a 1% agarose gel which was then separated by pulse field gel electrophoreses for 16h with initial switching time of 1'', final switching time of 25'', a gradient of 6 V/cm and an included angle of 120°. 1 Kb plus (Thermo Fisher Sicentific, Whatman, MA USA) and Lambda Ladder PFG Marker (NEB, Ipswich, MA, USA) standard marker ladders were also loaded on the gel. A gel slice corresponding to about 40 Kbp was excised from the gel according to Bradly 2007.

DNA was electroeluted in TBE buffer from the gel by electrophoresis for 3h at 80V in 14,000 MWCO Biodesign Dialysis Tubing (Fisher Scientific). The electroeluted DNA was concentrated in VivaSpin20 5,000 MWCO spin column. DNA was analysed on 1% agarose gel and quantified by Nanodrop.

Preparation of insert

The purified and concentrated DNA was blunt-ended by T4 DNA polymerase and T4 polynucleotide kinase treatments. 2-15 µg of DNA were added to 1xTANGO buffer (Thermo Scientific), 15 U of T4 DNA polymerase (2µL 2mM) dNTPs and sdH_2O. The reaction was incubate at 11°C for 30 min and the enzyme was inactivated at 70°C for 10 min. To the reaction were added ATP (50µM) and T4 polynucletide kinase, 20 U (Thermo Scientific). The reaction was incubate at 37°C for 30 min and the enzyme was inativated at 70°C for 10 min. The DNA was precipitated using sodium acetate (0.1v) and isopropanol (0.7v). The DNA pellet was washed with 70% ethanol and suspended in TE buffer.

Preparation of vector

The cloning vector pCCERI-1-Fos, a modified version of the pCC-1Fos vector (epicentre Biotechnology) was used. The vector was digested to completion with BstZ171, in a 3h incubation at 37°C using NEB buffer 4 (New England Biolabs).

The digested DNA was dephosphorylated adding the alkaline phosphatase to the reaction and continuing the incubation for 30 min. The phosphatase was inctivated by incubating at 70°C for 15min. And the BstZ171 was inactivated by phenol chloroform extraction.

The DNA was precipitateed by sodium acetate, isopropanol and dissolved in 20 µL of TE buffer. The DNA was checked by 1% gel electrophoresis and quantified by Nanodrop.

Ligation, Phage Packaging and Transfection

The blunt-ended DNA was ligated to the vector in a 10ul reaction comprising 0.5 µg of vector and 0.25µg of insert DNA 1X Ligase buffer and T4 ligase (Fermentans). The reaction mixture was incubated at 4°C overnight. The ligation reaction was checked on a 1% agarose gel.

The packaging reaction was performed using MaxPlaxTM Lambda Packaging Extracts and following the manufacturer's instruction.

Before plating the library, the titre of the phage particles (packaged CopyControl Fosmid clones),was estimated. Serial dilution of the packaged phage particles in Phage Dilution Buffer (PDB), were made. 10 µl of each above dilution, and 10 µl of the undiluted phage were individually added to 100 µl of the prepared EPI300 host cells previously grown in LB broth supplemented with 10mM $MgSO_4$ 0,2%

maltose and 50µg/mL Kanamycin, at an optical density between 0.8-1. The mixture was incubated 1 h at 37°C. The infected EPI300 cells were spread on an LB plate + 12.5 µg/mL chloramphenicol + 50 µg/mL Kanamycin and incubate at 37°C overnight to select for the CopyControl Fosmid clones. After calculating the titre of the packaged phage particles, the reaction was scaled up to obtain as many as clones as possible.
The generated clones were picked, arrayed and replicated using a QPix 2 xt robotic system (Genetix, Hampshire, UK).

Lipase activity screening
For activity screening, the transformed cells were plated onto Luria-Bertani (LB) agar plates containing 1% $_{(v/v)}$ emulsified tributyrin as substrate.
Laccase activity screening
Laccase activity screening on the metagenomic library was conducted on three different substrate (1mM ABTS, 1mM syringaldazine 0.04 % Remazol Brilliant Blue as substrate) added to LB agar plates containing 250µM $CuCl_2$ and incubated at 37°C.
Laccase sequence-based screening
Target laccase gene fragments were amplified by PCR with primers Cu1AF (5′-ACM WCB GTY CAY TGG CAY GG-3′) (Kellner *et al.*, 2008)/ Cu4R (5′-TGC TCV AGB AKR TGG CAG TG-3′) (Ausec *et al*., 2011) LacK 155F (5′-GGCTACGGCYTCGAGAAG-3′)/ LacK120F (5′-CCCATGTCGGAGTGGCTC-3′) LacI11F (5’-CCAGTGGTGGTACCACGACCA-3’)/Lac192r (5’-ATCATGCCCATGTCCTCGTG-3’) (Fernandes *et al.*, 2014) with the following protocol: an initial denaturation at 94 °C for 3 min, 30 cycles of 30 s at 94°C, 30 s at 48°C, 1 min at 72°C, ending with 5 min at 72°C. 50-µl reactions contained 5 µl of each primer (20 µM), 1 µl of dNTP (10 µM), 6 µl of BSA (10 mg/ml), 6 µl of $MgCl_2$ (25 mM), 500 ng of DNA template,5 µl of PCR buffer and 1 U of Taq DNA polymerase.

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Chapter 5:

Phylogenetic analysis of Microbial Communities in sea water from two nearby sites of the same Geographical Location in the Mediterranean Sea

Abstract

Marine Surface MicroLayer (SML) is considered to be an extreme environment, being characterised by a high concentrations of inorganic and organic compounds and intense solar radiation temperature fluctuations and salinity changes. Thanks to these features it is really interesting to study the biodiversity of SML marine habitat. In the present study the biogeography of Sea SML in two different sites in the same Geographical location (Naples commercial harbour, Mediterranean Sea) were explored. Analysis of the two microbiota revealed that the two sites share a set of aboundant OTUs, despite that some taxonomic group are exclusively present in one of two sites.

Introduction

Thanks to the advancement in sequencing technologies, it can be hypothesized that all microorganisms from an habitat can be analysed bypassing the cultivation steps. An high throughput microbial identification based on 16S rDNA next-generation sequencing of the bacterial metagenomic DNA is a powerful instrument to investigate the biodiversity of microbial communities (Fabrice and Didier, 2009). This kind of approach needs the availability of bioinformatic tools able to analyse the huge amount of data obtained by sequencing and of a robust universal reference taxonomy (Tringe and Hugenholtz, 2008). QIIME (Quantitative Insights Into Microbial Ecology) is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data (Caporaso *et al.*, 2010). QIIME is designed to take users from raw sequencing data generated on the Illumina or other platforms through publication quality graphics and statistics. This includes demultiplexing and quality filtering, OTU picking, taxonomic assignment, phylogenetic reconstruction, and diversity analyses and visualizations.
The marine environment represent a resource in search of biological diversity. The marine environment contains several habitats such as the sea SML. Even though a lot of advance have been reached in biogeography in diverse marine habitats, sea SML remains, until now, poorly characterized. The sea SML is an environment with unique properties, proposed to support a high microbial density and a specific community (the neuston) (Ward and Bora, 2006). The surface microlayer is characterised by a high concentration of inorganic and organic nutrients, intense solar radiation in the ultraviolet and visible spectra, high concentrations of heavy metals, temperature fluctuations and salinity changes (Agoguè *et al.*, 2005). Therefore, the surface microlayer has often been considered as an extreme environment for microorganisms that may contain unusual species and taxa. In order to increase the understanding of the microbial communities associated with that marine habitat, the commercial harbor of Naples was chosen as geographical location for the sampling. Naples, a city of the southern coast of Italy, has one of the most important commercial harbors of the Mediterranean Sea. It covers an area of 1,366,000 m^2, the water surface has an area of 2,792,550 m^2. The opening of interoceanic maritime routes and the environmental pollution is supposed to entail the development of novel species in the bay (Flagella *et al.*, 2006).

Results

Water sampling

Water sampling was performed in June 2014. 10L water samples were collected in triplicate from two nearby locations in the Mediterranean Sea (Figure1). The metagenomic DNA extracted was subjected to deep-sequencing to describe the microbiomes structures and relationships. In this kind of experiment sequencing errors can lead to overestimations of microbial diversity and the 16S rRNA region targeted for amplification can also influence the analysis through primer biases. Amplicon sequence reads which cover both a variable region and a hypervariable region of the target gene have been recommended. For that reason we targeted the V5–V6 region of the 16S rRNA gene. The primers here used have previously been used to successfully classify bacterial and archaeal pyrosequencing reads. To further minimise any possible biases, triplicate PCR reactions were performed for each sample. During the sampling in the two sites (C and U) the temperature were respectively 24 and 22 °C, and pH values were 7.2 and 7.4.

Figure1: Water sampling sites in the commercial harbor of Naples, Naples, Italy.

Total pyrosequencing reads and OTUs

A total of 200,000 individual 16S rDNA sequence reads were obtained by pyrosequencing. Errors in the dataset were analysed and corrected using Acacia (Bragg *et al.*, 2012). The sequence length of all quality checked sequences was superimposed between 100 and 290 bp. After denoising and quality filtering, a total of 104,543 sequences were obtained. These data were processed using QIIME (Quantitative Insights Into Microbial Ecology) Pipeline. Operational taxonomic units (OTUs) were selected at 97% of similarity and chimera sequences were removed using Chimeras slayer following alignment. The total of OTUs obtained from each sample (C and U) are reported in the Table 1.

Table1: Analysis of 16S rRNA pyrosequencing reads from the seawater samples collected in C and U sites. Chao1 species richness and Shannon diversity indices were calculated at 95% sequence identity.

Sample	Sequences	N° OTUs (97%)	Archeal Phyla	Bacterial Phyla	Chao Index	Shannon Index
C1	9282	154	1	12	1972	8.01
C2	20823	232	1	12	4880	9.09
C3	17587	188	1	15	2311	8.11
U1	19855	258	2	21	5680	9.52
U2	29468	309	2	19	3921	8.35
U3	7523	174	2	14	2152	7.97

Rarefaction curves, based on OTUs at 97% similarity, approach but do not reach a plateau, indicating that the sequencing depth used was not enough to assess the community full diversity (Figure 2). Nevertheless, in complex communities the full sequencing depth is rarely if ever obtained. Full sequencing depth is only obtained from much simpler microbiomes than seawater. Shannon diversity indices calculated for all three samples were reflective of the relative diversities observed (Table 1). Chao1 species estimators suggest that about 4% of the seawater communities have been sampled in this study (Table 1).

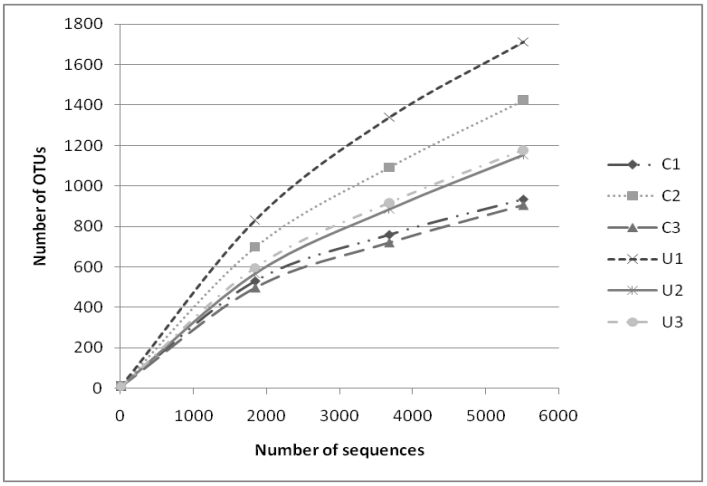

Figure 2. Normalized Rarefaction Curves. Rarefaction curves for seawater and sponge derived pyrosequencing reads from the V5–V6 region of 16SrRNA genes from Bacteria and Archaea. The x-axis refers to the number of sequencing reads sampled in a random sampling process.

The similarity among the different communities was obtained by UniFrac analysis (Lozupone and Knight, 2005), a β-diversity metric (Figure 3). Although the U2 community (one of the triplicate from U site) clusters with the C communities, the C communities also form their own cluster and are closer to each other than they are to the U communities, as expected, being the communities (U and C) from two different sites.

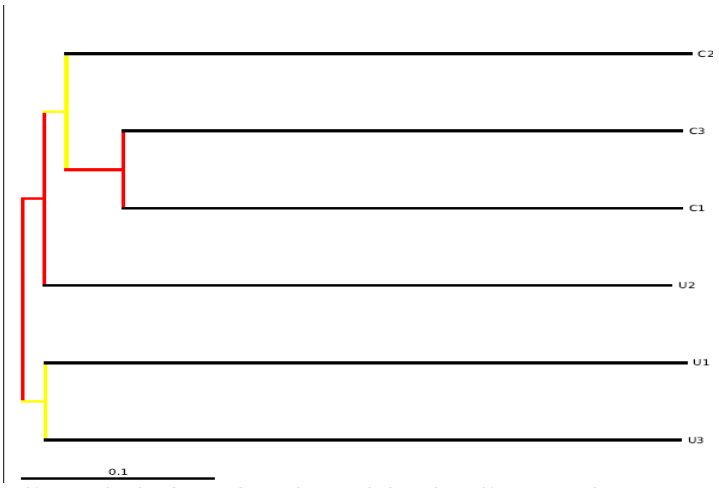

Figure 3: Jackknife analysis, based on the weighted Unifrac metric, generated by Qiime (www.qiime.org), describing statistical support for clustering of the bacterial communities. Red nodes represent jackknife support of 75-100% and yellow nodes stand for jackknife support of 50-75%

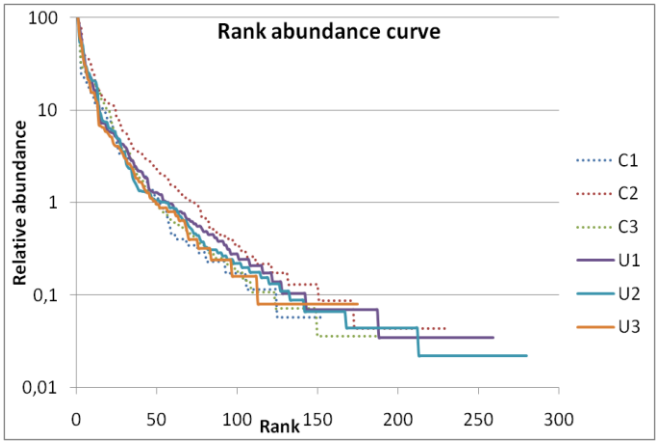

Figure 4: Rank-Abundance curves. Rank-Abundance curves for seawater samples derived pyrosequencing reads from the V5–V6 region of 16S rRNA genes from Bacteria and *Archaea*. OTUs are based on 97% sequence similarities.

Rank-abundance curves indicated that a majority of the reads belonged to rare organisms represented by only a few sequence reads, whereas all samples contained relatively low proportions of highly abundant Bacteria or *Archaea* (Figure 4).

Taxa analysis

All seawater derived sequence reads were classified in the **domains** *Archaea* or *Bacteria.* The abundance of *Archaea* in both sites is less than 0.2%. This value is really lower than other previously reported *Archaeal* aboundances in other seawater samples, (Karner *et al.*, 2001; Jackson *et al.*, 2011; Lloyd *et al.*, 2013). At the **phylum level**, two archaeal phyla (*Euryarchaeota* and *Thaumarchaeota*) and twenty-five bacterial phyla were noted in sea water datasets (Figure 5). Among Archaea the *Thaumarchaeota* were exclusively found in the sample from U site. Study of pelagic *Thaumarchaeota* has revealed high levels of spatial variability, which has been suggested to be associated with the dissolved oxygen (DO) concentration, the presence of organic substrates, salinity and sulfide concentration (Bale *et al.*, 2013).

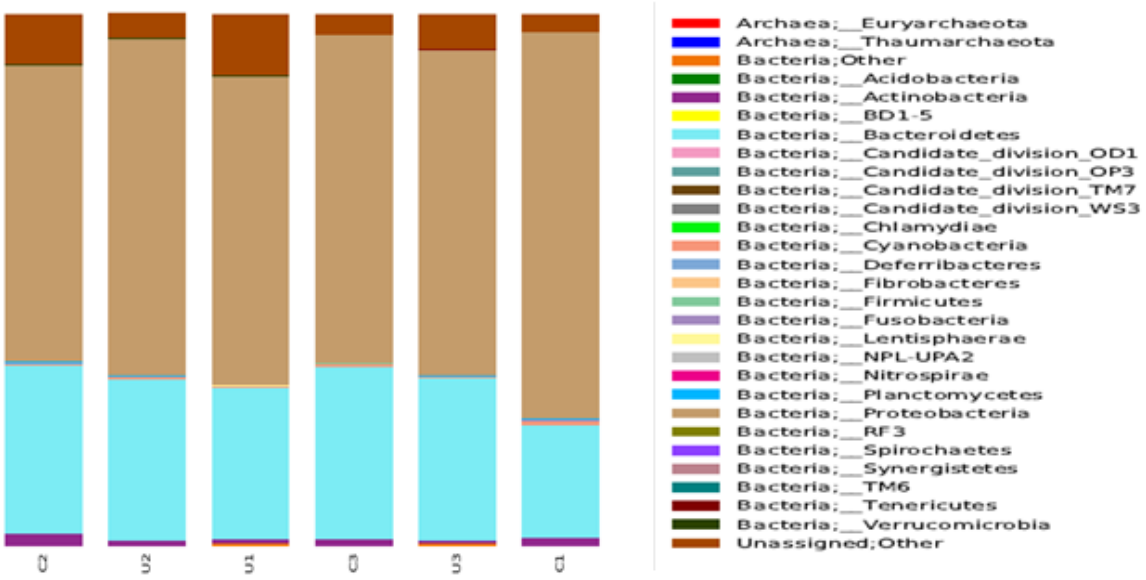

Figure 5: Relative abundance of 16S tag by phylum from seawater

In both sites the bacterial phyla with a relative aboundance higher than 0.5 % are *Proteobacteria*, *Bacteroidetes*, *Actinobaceria* and *Cyanobacteria,* representing more than the 90% of total reads. The relative amounts of *Proteobacteria* and *Bacteroidi* are comparable in the two sites, while *Actinobaceria* and *Cyanobacteria* are twice as abundant in the C site (Figure 6). In marine environment *Actinomycetes* are reported to contribute to the break-down and recycling of organic compounds (Goodfellow and Haynes 1984). In addition, they play a significant role in mineralization of organic matter, immobilization of mineral nutrients, fixation of nitrogen, improvement of physical parameters and environmental protection (Das *et al.*, 2006). *Cyanobacteria* distribution was related to the N:P ratio, and they were scarce when the atomic ratio of total inorganic N / total P exceeded 50 (de Hoyos *et al.*, 2004).

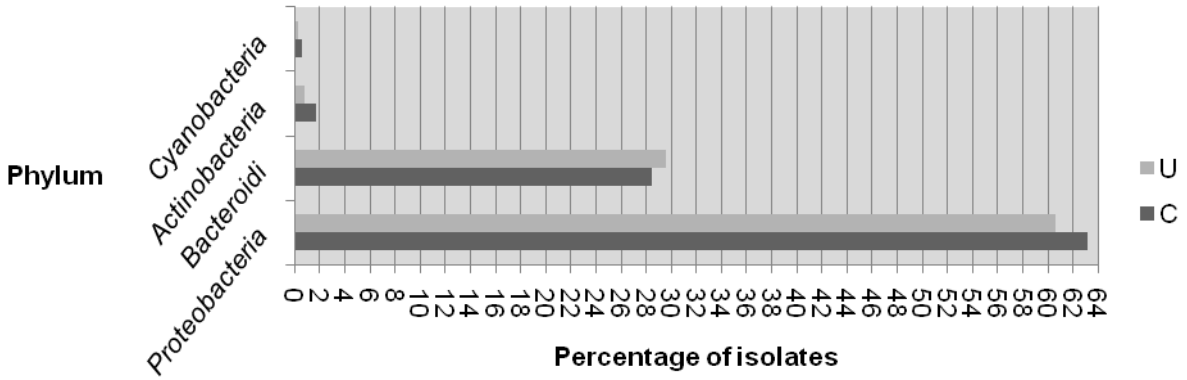

Figure 6. Relative number of isolates by percentage from the C and U seawater samples

Among the phylum ***Proteobacteria***, that represent the 62% of phyla, *Alphaproteobacteria* constituted the most dominant group (34.2%), followed by *Gammaproteobacteria* (25.5%), *Betaproteobacteria* (1.4%) and *Deltaproteobacteria* (0.2%). In the class *Alphaproteobacteria,* the most aboundant genus is *Roseobacter clades* that are widespread and abundant across diverse marine environments from coastal to open oceans and from sea ice to sea floor (Buchan *et al.*, 2009; Brinkhoff *et al.*, 2004; Giebel *et al.*, 2011). The *Gammaproteobacteria* include the high number of OTUs (106), distributed mainly in the *Alteromonadales* (29 OTUs), *Oceanospirillales* (30 OTUs) and *Thiotrichales* (12 OTUs) orders. 28 OTUs were detected in *Betaproteobacteria* and 18 in *Deltaproteobacteria.*
76 OTUs in the dataset belong to ***Bacteroidetes*** phylia, mainly represented by four classes: *Flavobacteia*, *Sphingobacteria*, *Bacteroidia*, *Cytophagia*. The most diffused genera in this phyla are: Owenweeksia, *Formosa*, NS3a and NS5 *Marine group* and *Winogradskyella*. ***Actinobacteria*** and ***Cyanobacteria*** constitute the 1.3% and 0.5% of phyla with11 and 5 OTUs respectively.

Conclusions

Most of biogeography study have been proposed for sample from different geographical locations, different habitats of marine environment or from the same geographical location but collected in different time. This is one of the first studies aimed at studying the microbiomes in SML of two site in the same geographical location. Nevertheless it was unexpected to find huge diversity in the two microbiome, experimental results (in particular UniFrac analysis) show very clearly that the samples are different.

Material and methods

PCR Amplicon Library preparation for Pyrosequencing

The 16S rDNA was amplified by PCR for multiplexed pyrosequencing using barcoded primers. The universal forward primer U789F (5' -TAGATACCCSSGTAGTCC-3') and the reverse primer U1068R (5'-CTGACGRCRGCCATGC-3') for amplification of bacteria and archaea, were adapted for pyrosequencing by addition of 10 nucleotide barcode. Each 50 uL of PCR reaction contained 0.5µL 50µM of each primer, 1µl of DNA Template 0.5µl FailSafe PCR Enzyme Mix (1.25 Units) 25µl of the FailSafe Master Mix B and sdH_2O. PCRs were performed in the following conditions: initial denaturation at 94°C for 5 min; 26 cycles at 94°C for 30s, 53.5°C for 30s and 72°C for 45s; and a final extension at 72°C for 6 min. To reduce PCR bias 3 individual reactions were performed per template and equimolar amounts of PCR products from each of the three reactions were pooled for pyrosequencing. PCR products were purified using Qiagen PCR purification Kit (Qiagen Ltd., UK). A mixture of PCR products was prepared by mixing 200 ng of the purified 16S amplicons from each seawater sample and then pyrosequenced on GS FLX Titanium Platform (454 Life science).

Pyrosequencing data analysis

Adapter sequences were removed from all pyrosequencing reads. Datasets were denoised using Acacia (Brag *et al.*, 2012) and sequences were subsequently analysed using a custom script in the Quantitative Insights Into Microbial Ecology (QIIME: http:// qiime.org) software package (Caporaso *et al.*, 2010).Sequencing reads were assigned to samples according to their barcodes. Reads with incorrect barcodes, incorrect primer sequences, average quality scores of 25 or less, homopolymers of 6 or more and read lengths of 200 bp were removed from further analysis. OTUs were picked using the default setting (97% similarity) using UCLUST. Representative sequences from each OTU were aligned using PyNAST (Caporaso *et al.*, 2010). Chimeric reads were removed using Chimera Slayer (Haas *et al.*, 2011). Taxonomic assignments were assigned by comparison to the Greengenes database (http://greengenes.lbl.gov) (De santis *et al.*, 2006). Rarefaction curves and diversity metrics (alpha diversity: Chao1 and Shannon indices, beta diversity: UniFrac) were generated at 97% sequence identities.

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Guarino L., Lettera V., Sannia G., *Pleurotus ostreatus* laccases: New media for maximum production at minimum cost (2014) Journal of Biotechnology, S71-185

RESEARCH ACTIVITY IN FOREIGN LABORATORY

From July 4th to November 12th my research activity was carried out in Prof. Alan Dobson's laboratory at University College of Cork, Environmental Research Institute, Cork, Ireland